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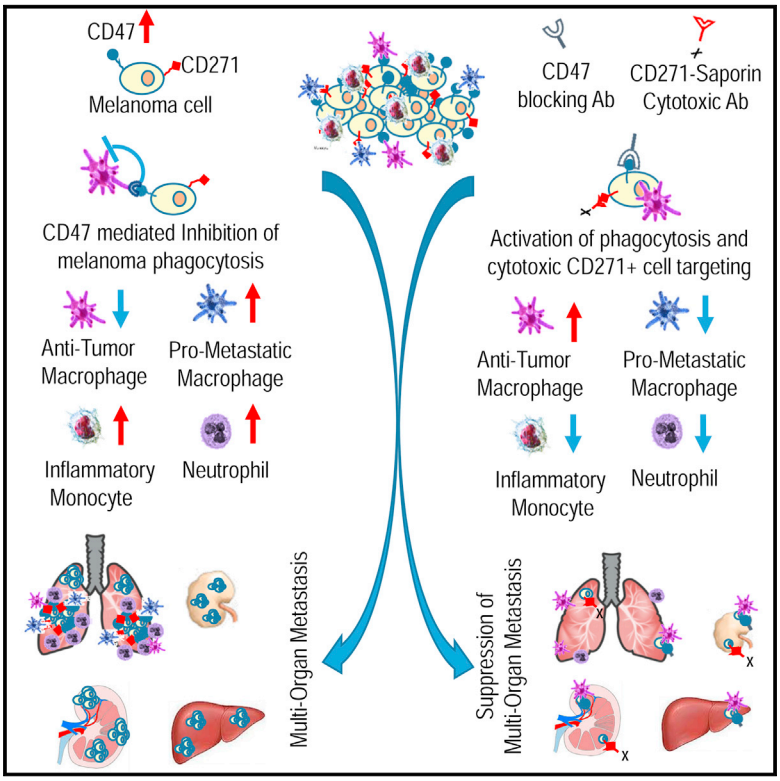
Antibody Therapy Targeting CD47 and CD271 Effectively Suppresses Melanoma Metastasis in Patient-Derived Xenografts

Graphical Abstract



Highlights

- Increased CD47 expression correlates with tumor metastasis in melanoma patients
- CD47 blockade activates mouse macrophage-induced phagocytosis and inhibits metastasis
- Targeting CD271^{+} melanoma cells augments the anti-metastatic effect of CD47b mAb
- Metastasis suppression is mediated by remodeling of the tissue immune microenvironment

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In Brief

Ngo et al. find that metastatic progression in melanoma is associated with overexpression of an anti-phagocytic signal, CD47. Blockade of CD47 and activation of innate immunity via macrophage-induced phagocytosis effectively suppress melanoma metastasis in patient-derived xenografts. Coupled with targeting of CD271^{+} melanoma cells, this regimen produces the most potent therapeutic response.

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Antibody Therapy Targeting CD47 and CD271 Effectively Suppresses Melanoma Metastasis in Patient-Derived Xenografts

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SUMMARY

The high rate of metastasis and recurrence among melanoma patients indicates the existence of cells within melanoma that have the ability to both initiate metastatic programs and bypass immune recognition. Here, we identify CD47 as a regulator of melanoma tumor metastasis and immune evasion. Protein and gene expression analysis of clinical melanoma samples reveals that CD47, an anti-phagocytic signal, correlates with melanoma metastasis. Antibody-mediated blockade of CD47 coupled with targeting of CD271$^+$ melanoma cells strongly inhibits tumor metastasis in patient-derived xenografts. This therapeutic effect is mediated by drastic changes in the tumor and metastatic site immune microenvironments, both of whichwhich exhibit greatly increased density of differentiated macrophages and significantly fewer inflammatory monocytes, pro-metastatic macrophages (CCR2$^+$/VEGFR1$^+$), and neutrophils, all of which are associated with disease progression. Thus, antibody therapy that activates the innate immune response in combination with selective targeting of CD271$^+$ melanoma cells represents a powerful therapeutic approach against metastatic melanoma.

INTRODUCTION

Melanoma, the most lethal type of skin cancer, has high metastatic potential and arises as cells of melanocytic lineage undergo tumorigenic transformation. Although surgical removal and treatment of primary tumors prior to metastasis have a favorable 5-year survival rate for 90% of patients, this figure drops sharply to 65% for those diagnosed with regional metastatic disease and to 15% for those diagnosed with distant metastases (American Cancer Society, 2015). Despite being one of the most immunogenic tumors, the success rate of immune-therapeutic regimens in achieving long-term, complete responses in metastatic melanoma patients remains low (Zikich et al., 2013). This might be due to the fact that the antigens selected for these approaches do not cover the full spectrum of melanoma cells present in a tumor, including the most dangerous, melanoma-initiating subset, defined by cluster of differentiation (CD)271 expression (Boiko et al., 2010; Civenni et al., 2011). In addition, interaction between tumor cells and the immune microenvironment has been shown to play a pivotal role in disease progression as well as the ability of the disease to regress in the presence of activated immune components (Biswas and Mantovani, 2010; Murdoch et al., 2008). Importantly, primary melanoma cells that fail to be detected and removed by the innate immune system—macrophages in particular—are highly likely to metastasize to visceral organs and tissues, resulting in incurable disease.

Macrophages comprise a major component of the innate immune system and act as professional phagocytic cells throughout the body. Most adult tissue-resident macrophages differentiate from bone marrow-derived monocytes, with an exception of microglia and subsets of epidermal Langerhans cells, which are derived from the yolk sac and fetal liver, respectively (Hoeffel et al., 2012; Sheng et al., 2015). When a tissue undergoes remodeling due to injury or inflammation (also hallmarks of tumor growth), Ly6C^{hi}/C-C chemokine receptor (CCR)2$^+$ monocytes are rapidly recruited to these sites, where they extravasate and differentiate into macrophages and certain types of dendritic cells (Ginhoux and Jung, 2014; Serbina and Pamer, 2006). In cancer patients, macrophages elicit their activity by binding to and phagocytosing malignant cells, contributing to reduced tumor growth (Biswas and Mantovani, 2010). Moreover, macrophages are often found at high concentrations in the afferent sinuses of lymph nodes and the surrounding blood vessels, indicating that they constitute a major barrier to the formation of metastases. Conversely, besides their anti-cancer function, macrophages found at tumor sites can also play a pro-tumorigenic/metastatic role upon adopting a polarized phenotype, characterized by the expression of CCR2 and

vascular endothelial growth factor receptor 1 (VEGFR1) (Qian et al., 2011; Ren et al., 2012). The presence of $CCR2^+$/$VEGFR1^+$ macrophages at metastatic organs creates a favorable microenvironment for arriving circulating tumor cells and could augment tumor colonization (Qian et al., 2011). Precise functional delineation of macrophages has been hampered by their high degree of heterogeneity and plasticity that underlies the broad spectrum of macrophage involvement in very diverse processes such as defense against pathogenic cells, maintenance of tissue integrity, and immunomodulation. Expression of genes that include Mrc1 (CD206), Fizz1, Ym1, Mgl1 (CD301a), Arg1, Nos2, and Il10 has commonly been used to classify macrophage populations into the M1 and M2 (M2a–M2c) subgroups (Qian and Pollard, 2010; Roszer, 2015). However, in vivo classification of macrophages into distinct M1/M2 subtypes based on established patterns of gene expression needs to be approached with caution, and depends in each case on the homeostatic and pathological conditions of the living tissue from which the macrophages are being sampled (Franklin et al., 2014; Martinez and Gordon, 2014). In addition to macrophages, the microenvironment of the metastatic site can also be affected by the presence of neutrophils, which are known to respond to inflammation associated with cancer cell growth (Cools-Lartigue et al., 2013; Fridlender and Albelda, 2012). Clinical studies indicate that elevated frequencies of neutrophils at metastatic sites indicate an adverse prognosis for patients with gastric and lung cancers (Sarraf et al., 2009; Walsh et al., 2005). Based on these findings, we hypothesized that a therapeutic approach that activates the pro-phagocytic response and shifts the balance between anti- and pro-tumorigenic macrophages and neutrophils would determine the eventual outcome of progressing metastatic disease.

Tumors of hematopoietic and epithelial origin have recently been found to avoid macrophage phagocytosis by overexpressing CD47, which is known as a "don't eat me" signal (Jaiswal et al., 2009; Mawby et al., 1994; Willingham et al., 2012). CD47 is a transmembrane integrin-associated protein whose ligands include thrombospondin 1 (TSP1) and signal-regulatory protein alpha (SIRPα). Cells expressing CD47 interact with macrophages via SIRPα (Mawby et al., 1994; Vernon-Wilson et al., 2000); CD47 binding to the macrophage SIRPα receptor leads to the tyrosine phosphorylation of its cytoplasmic tail and subsequent activation of Src homology-2 (SH2)-domain-containing protein tyrosine phosphatases SHP1 and SHP2. Activity of these enzymes results in cytoplasmic membrane modifications that inhibit macrophage-mediated phagocytosis of target cells (Chao et al., 2012; Okazawa et al., 2005; Vernon-Wilson et al., 2000). Recent studies found that masking CD47 with anti-CD47 monoclonal antibodies on the surface of hematopoietic and epithelial cancer cells leads to stimulation of phagocytosis in vitro and decreased tumor burden in vivo (Jaiswal et al., 2009; Majeti et al., 2009; Willingham et al., 2012). In addition to SIRPα, CD47 has also been shown to interact with TSP1 (Isenberg et al., 2006). Disruption of this signaling network leads to increased radioprotection of normal tissue cells and delays tumor growth, including that of melanoma, in syngeneic mouse models of cancer (Maxhimer et al., 2009). On the other hand, researchers have shown that ligation of CD47 on the cell surface of breast cancer cell lines or promyelocytic leukemia cells by soluble TSP1 induced their direct killing via caspase-independent apoptosis (Johansson et al., 2004; Manna and Frazier, 2004; Mateo et al., 1999; Saumet et al., 2005). These seemingly opposite effects of CD47-TSP1 interaction on tumor fate are likely due to the differences in cancer cell types as well as systemic tissue levels of TSP1 in vivo.

Our group and others previously demonstrated that melanoma cells expressing the neural crest stem cell marker CD271 are capable of initiating tumor growth in vivo and, importantly, also give rise to melanoma cells capable of metastasis (Boiko et al., 2010; Civenni et al., 2011; Redmer et al., 2014). CD271 is a low-affinity nerve growth factor receptor (NGFR/p75) whose main ligands are neurotrophins, a family of protein growth factors that stimulate survival and migration of neuronal and neural crest-derived cell lineages (Marchetti et al., 1993). Subsequently, overexpression of CD271 was found to positively regulate the invasive properties of melanoma, enhancing its metastatic potential (Guo et al., 2014; Marchetti et al., 2004). Our experiments established that upon transplantation into the skin of NOD-*scid* IL2Rgamma^{null} mice (NSG) mice, $CD271^+$ melanoma cells isolated from clinical samples can give rise to metastatic tumors that spread to the lymph nodes, lungs, liver, and kidneys, mimicking human disease (Boiko et al., 2010). Evasion of immune surveillance during primary tumor growth, dissemination, and homing to visceral tissues is a main prerequisite for metastasis. Thus, we hypothesized that melanoma tumor cells, especially metastatic subsets, rely on CD47 expression for phagocytosis blockade and successful colonization of distant organs. We further thought to determine the feasibility of targeting both the CD47 and CD271 antigens to determine the therapeutic effects of a pro-phagocytic response, combined with the elimination of $CD271^+$ tumor cells, on melanoma metastasis.

Here we find that CD47 is expressed on all clinical melanoma samples, including first-passage xenografts, and that the frequency of $CD47^+$ melanoma cells is significantly higher in tumors isolated from metastatic sites compared to tumors from primary sites. We next demonstrate that antibodies that block the interaction of CD47 with SIRPα increase phagocytosis of melanoma cells in vitro. We further establish that the combination of CD47 blocking with the targeting of $CD271^+$ cells using CD271-saporin antibody greatly reduces metastases of xenotransplanted clinical samples of human melanoma in vivo. Significantly, we show that the therapeutic effects of these antibodies are mediated by remodeling the immune microenvironment of the primary tumor and metastatic sites. As a result of CD47 blockade, macrophages were dramatically increased over undifferentiated monocytes at primary tumor sites, while at the same time they harbored lower frequencies of pro-metastatic $CCR2^+$/$VEGFR1^+$ subsets. Moreover, myeloid cells infiltrating the tumor in control mice were found to express higher levels of Mrc1, Fizz1, Mgl1, Ym1, and Il10—characteristic of immune cells that positively regulate tumor growth and dissemination. Mice treated with CD47 blocking monoclonal antibody (mAb) also exhibited an increase in differentiated macrophages at pulmonary sites of metastasis. In contrast, lungs of control mice were dominated by neutrophils, previously shown to positively regulate the homing of metastatic cells (Cools-Lartigue et al., 2013; Fridlender and Albelda, 2012).

Taken together, these results provide direct evidence that targeting melanoma immune-evasive properties and $CD271^+$

melanoma cells represents a very effective regimen against aggressive metastatic disease.

RESULTS

CD47 Expression Is Upregulated in Metastases Compared to Primary Tumors in Melanoma Patients

CD47 is overexpressed on the cell surface of hematopoietic malignancies, protecting them from phagocytosis by macrophages as the disease is spread through the blood and lymphatic systems (Jaiswal et al., 2009). In this study, we sought to first examine whether CD47 plays a similar role in melanoma tumors. Multiple surgical samples representing primary tumors and metastases were collected from melanoma patients immediately after surgery and processed into viable single-cell suspensions as previously described (Boiko, 2013; Boiko et al., 2010). Patient cells were then incubated with fluorescently labeled anti-CD47 mAb (along with mAbs to gate out non-tumor cells) and analyzed for the presence of cell-surface CD47 by fluorescence-activated cell sorting (FACS). Alternatively, clinical tumor samples that were too small to isolate sufficient numbers of cells were implanted into NSG mice, after which first-passage xenografts were used to generate viable single-cell suspensions for FACS analysis. From these experiments, we found that $CD47^+$ melanoma cells comprised 30%–99% of the entire tumor cell population (Figure 1A, left) among various patients. Importantly, the proportion of melanoma cells that were $CD47^+$ in metastatic lesions was significantly higher (mean 87%) than the proportion of melanoma cells that were $CD47^+$ in primary tumors (mean 61%, $p < 0.05$) (Figure 1A, right). To further investigate whether CD47 expression increases as melanoma progresses from primary to metastatic form, we analyzed a previously reported cohort of melanoma patients (Xu et al., 2008) ($n = 85$) containing clinical information linked to the gene expression profile for every tumor. This analysis revealed that CD47 expression is significantly elevated in metastatic lesions compared to primary tumors ($p = 8.4e^{-5}$) (Figure 1B, left). We next analyzed cutaneous melanoma cases ($n = 310$) collected as part of The Cancer Genome Atlas (TCGA) project. Based on pathological data, we were able to classify most melanoma lesions into four main categories: primary tumor, regional cutaneous metastasis, regional lymph node metastasis, and distant metastasis (DM). Comparison of CD47 expression between these categories revealed a significant increase in CD47 mRNA between the primary tumor site and both regional cutaneous ($p = 0.043$) and lymph node ($p = 0.002$) metastasis (Figure 1B, right). At the same time, CD47 expression in a much smaller group of melanoma samples isolated from DM sites did not differ significantly from samples isolated from primary sites (Figure 1B, right). Overall, our examination of two large melanoma patient cohorts (430 total samples), with the exception of DM samples ($n = 38$), revealed a significant positive correlation between overexpression of CD47 and metastatic melanoma.

Treatment of Melanoma Cells with Anti-CD47 Antibodies In Vitro Enables Macrophage-Mediated Phagocytosis

CD47 expressed on the surface of target cells can interact with the SIRPα receptor on macrophages, leading to the inhibition of macrophage-mediated phagocytosis (Chao et al., 2012; Okazawa et al., 2005). To determine whether CD47 present on metastatic melanoma cells plays a protective role against macrophages, we analyzed the ability of tumor cells to resist phagocytosis in the presence and absence of CD47 blocking antibody. To conduct these experiments, we fluorescently labeled metastatic melanoma cells (M213) with carboxyfluorescein succinimidyl ester (CFSE) dye and co-incubated them with activated NSG mouse bone marrow-derived macrophages in the presence of IgG1 isotype control or blocking CD47 mAb (B6H12). This antibody was previously shown to bind to the CD47 antigen, thereby inhibiting the interaction between CD47 and SIRPα and enabling phagocytosis (Jaiswal et al., 2009). Using in vitro assays measuring the macrophage phagocytic index, we revealed that blocking CD47 antibody promotes efficient phagocytosis of melanoma cells by macrophages compared to matched IgG controls (Figure 2A). To demonstrate the effects of CD47 blockade on macrophage-induced phagocytosis of melanoma in a broader context, we examined additional patient-derived cells representing metastatic variants of the disease (M727 and M1626). In these experiments, we used GFP-expressing lentiviral vector and FACS to derive homogeneously labeled GFP populations from each tumor sample (M213, M727, and M1626). GFP-labeled melanoma cells were then co-incubated with activated macrophages in the presence of a humanized CD47 blocking mAb (hCD47b) (Hu5F9-G4; Lonza) or control IgG as described above. Next, we labeled macrophages with macrophage-specific fluorescently conjugated antibody (F4/80) and used FACS to measure phagocytic efficiency by determining the frequency of double-positive (GFP^+, $F4/80^+$) cells. Our results convincingly demonstrate that CD47 blocking antibody induced efficient phagocytosis of metastatic melanoma (Figures 2B–2D). These findings also demonstrated interspecies cross-reactivity between mouse macrophages and human melanoma cells and paved the way for in vivo studies using a melanoma xenograft model. It is important to note that the CD47 blocking antibody did not cause tumor cell death prior to phagocytosis.

Generation of Patient-Derived Xenograft Metastatic Models of Melanoma

Upon demonstrating that CD47 was abundant on human melanoma tumors and that blocking CD47 was sufficient to induce phagocytosis in vitro, we sought to examine the therapeutic potential of anti-CD47 antibodies in vivo using a mouse xenograft model of metastatic melanoma. Although many melanoma cell lines and their metastatic derivatives have been established in vitro and shown to have highly invasive and aggressive phenotypes, the extent to which these phenotypes reflect original disease characteristics as opposed to changes acquired during the course of adaptation to tissue-culture conditions is unclear. Thus, we decided to establish mouse xenograft models of metastatic melanoma that recapitulate the progression of human disease using freshly obtained surgical samples. Through this process, we identified a human melanoma sample (M213) that, upon intradermal injection into mouse skin, forms primary tumors at injection sites that later metastasize to the lymph nodes and visceral organs, typically the lungs and liver (Figure S1A).

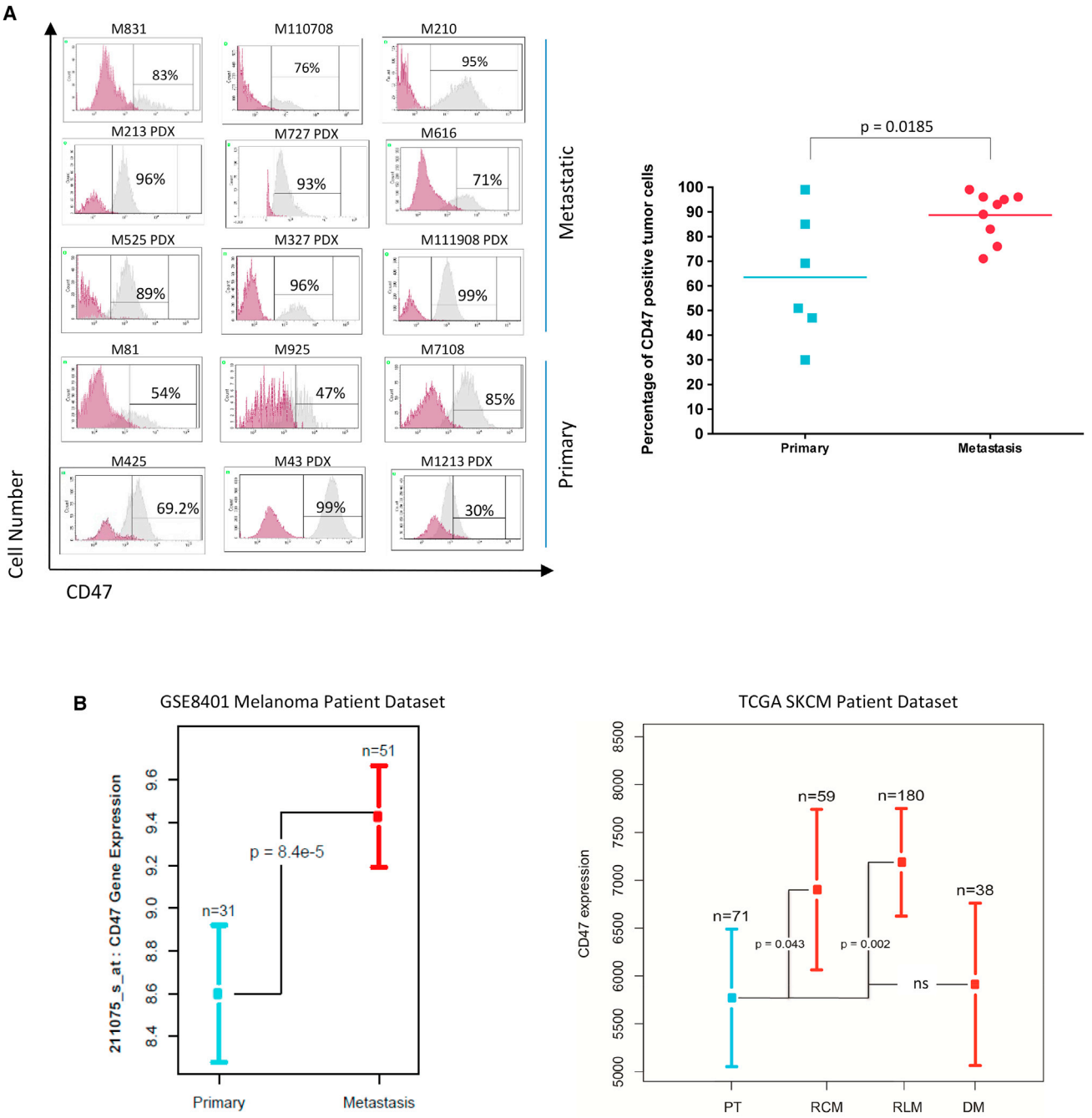


Figure 1. CD47 Is Upregulated in Human Melanoma Metastases

(A) Frequency of CD47$^+$ melanoma cells in patient tumors excised from metastatic locations compared to primary lesions as determined by FACS. Gating strategy for CD47$^+$ melanoma cells (gray histograms) was set up in accordance with the isotype controls (red histograms) conjugated to the same fluorochrome as CD47 mAb. Horizontal bars illustrate mean values for each group.

(B) Relative CD47 mRNA expression was analyzed for previously reported cohorts of melanoma patients GSE8401 (n = 85) and TCGA_SKCM (n = 310) who were subdivided into primary and metastasis categories based on clinical information. The TCGA_SKCM cohort contains primary tumor (PT), regional cutaneous metastasis (RCM), regional lymph node metastasis (RLM), and distant metastasis (DM) subgroups. ns, not significant.

Consistent with our earlier findings in human patient samples, cell-surface CD47 levels increase significantly for melanoma cells that form pulmonary metastasis in mice (Figure S1B). Next, we established a second, independent metastatic xenograft model from patient M727. Intradermal injection of M727 into mouse skin resulted in the formation of primary tumors

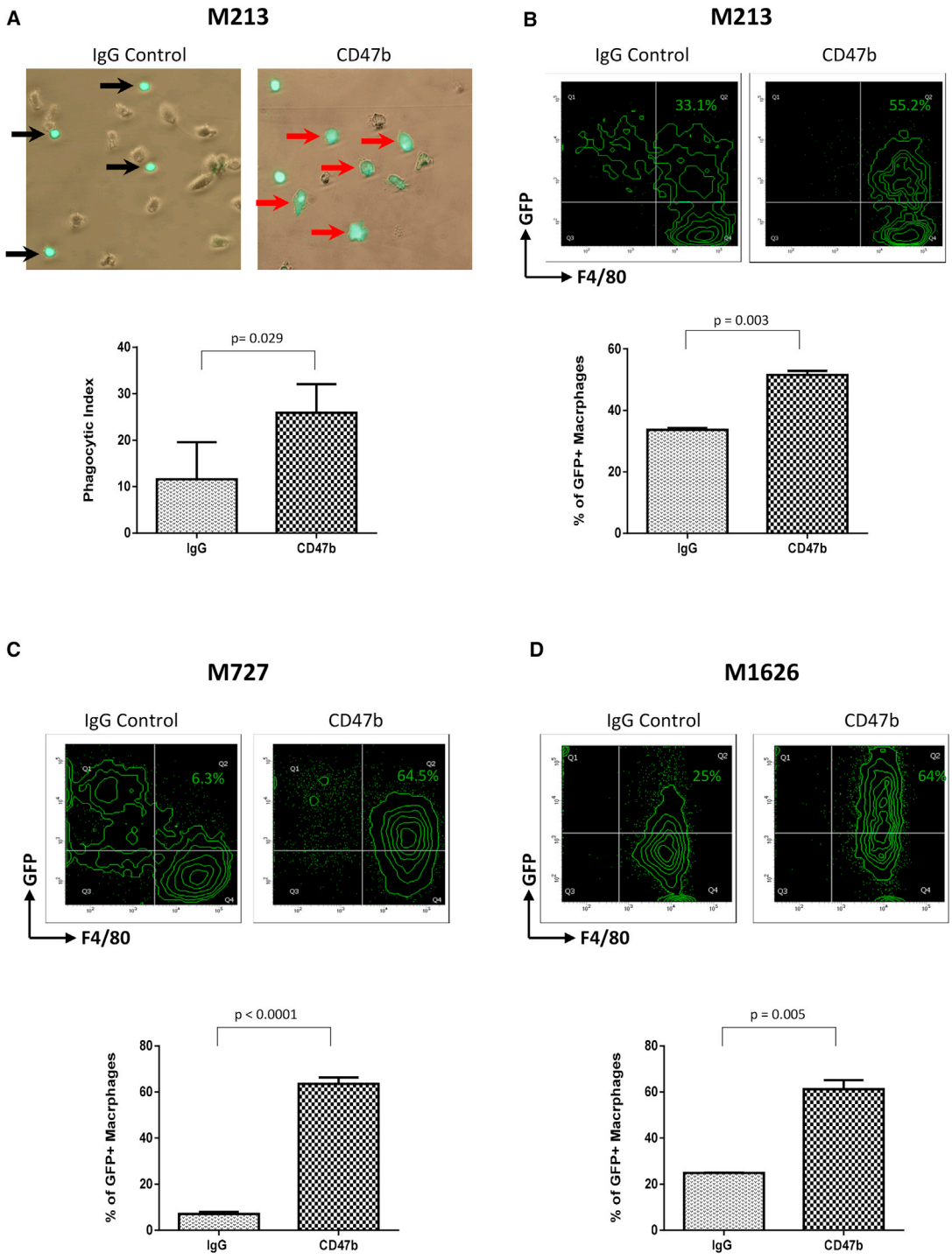
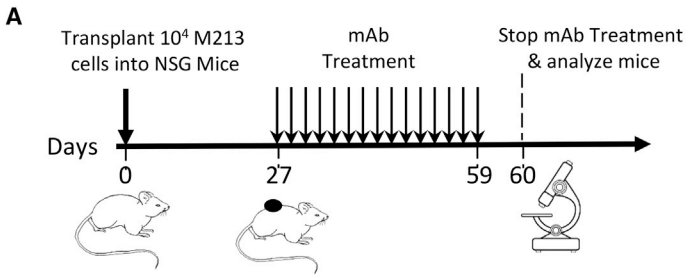


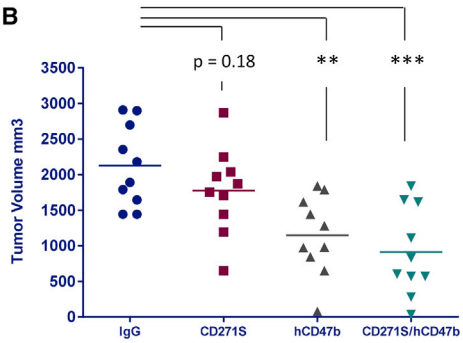
Figure 2. Blockade of CD47 on the Cell Surface of Melanoma Cells by mCD47b and hCD47b mAbs Induces Their Effective Phagocytosis by Activated Macrophages

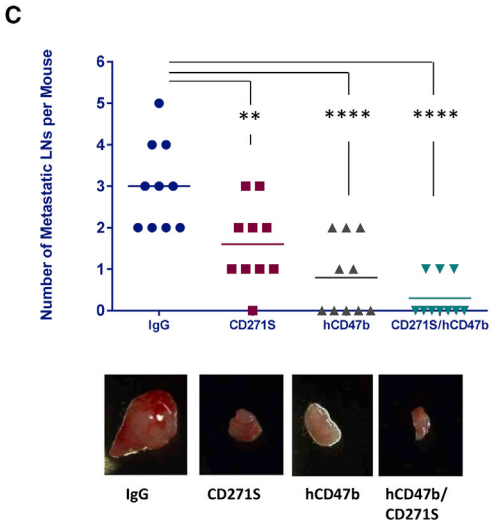
(A) Phagocytic index of M213 cells that were labeled with CFSE dye and incubated with NSG mouse bone marrow-derived macrophages in the presence of mCD47b (B6H12; Bio X Cell) blocking mAb or matching IgG control. Representative images of co-cultured melanoma cells and NSG mouse bone marrow-derived macrophages following antibody treatment are shown. Black arrows indicate melanoma cells that had not been phagocytosed by macrophages; red arrows point to phagocytosed tumor cells.

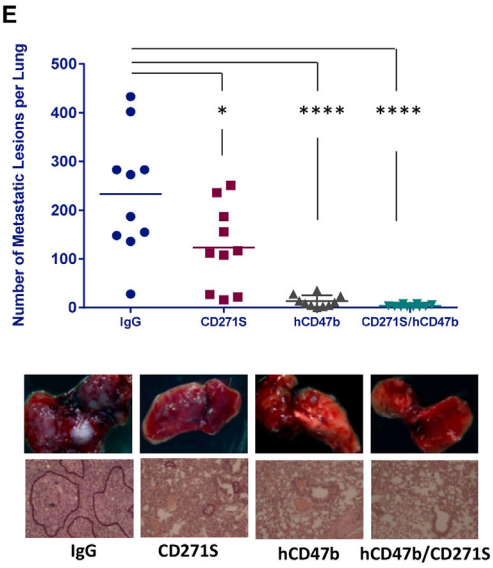
(B–D) Phagocytosis of GFP-labeled M213, M727, and M1626 patient-derived melanoma cells that were co-incubated with bone marrow-derived NSG macrophages in the presence of hCD47b (Hu5F9-G4; Lonza) blocking mAb or IgG control.

Data are shown as means with SDs.



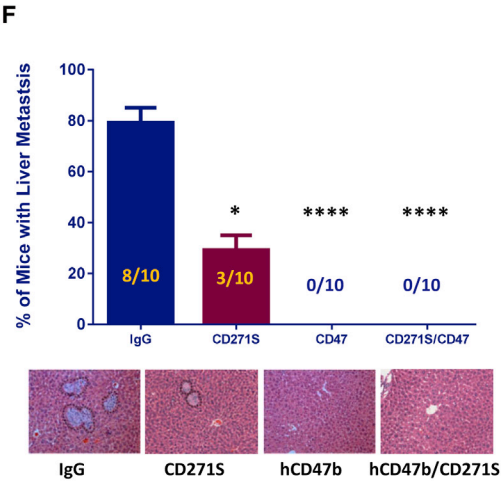






D

Treatment	Lymph Node: Left Side Axillary	Left Side Inguinal	Right Side Axillary	Right Side Inguinal
IgG	N	big	N	big
IgG	N	medium	big	medium
IgG	medium	N	big	N
IgG	N	N	big	medium
IgG	big	big	big +small	big
IgG	N	N	big +small	big
CD271.S	N	N	medium	N
CD271.S	N	N	N	N
CD271.S	N	N	medium	N
CD271.S	N	N	medium	N
CD271.S	medium	N	2 medium	
CD271.S	medium	N	medium	N
CD47	small	N	medium	N
CD47	Big	N	N	small
CD47	N	N	N	N
CD47	N	N	N	N
CD47	N	N	N	N
CD47	N	N	small	N
CD47/CD271.S	N	N	N	N
CD47/CD271.S	N	N	N	N
CD47/CD271.S	N	N	small	N
CD47/CD271.S	N	N	small	N
CD47/CD271.S	N	N	N	N
CD47/CD271.S	N	N	N	N



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that later metastasize to the lungs and kidneys, without affecting lymph node compartments. Establishing these models enabled us to assess the effects of antibody treatment on different types of melanoma metastasis and stages of disease progression.

Blocking CD47 and Cytotoxic CD271 Antibodies Inhibit Melanoma Metastasis In Vivo

We previously demonstrated that in human melanomas, cells that have increased tumor-initiating capacity and also give rise to metastatic subpopulations express CD271 (Boiko et al., 2010). Because the process of metastasis requires evasion of the innate immune system, we investigated how direct targeting of $CD271^+$ melanoma cells and blockade of CD47 antigen would affect tumor progression, using the metastatic xenograft models described above. In these experiments, we utilized hCD47b mAb (Willingham et al., 2012) and a CD271-saporin mAb (CD271S), which is capable of selectively killing $CD271^+$ cells by delivering an inhibitor of ribosomal assembly (saporin) to cells expressing the CD271 receptor on their surface (Fine et al., 1997) (Figure S2).

The general outline of the experiment is shown in Figure 3A: 10,000 M213 cells were introduced intradermally into NSG mice via unilateral injection of the single-cell suspension in Matrigel into the right flank of the back of the animal. Melanoma cells were given 4 weeks to grow and form tumors, a time frame that we previously determined to correspond to the onset of tumor metastasis. Animals were randomly divided into four antibody treatment groups (each group n = 6 for experiment 1 and n = 4 for replicate experiment 2): IgG control, hCD47b, CD271S, and a combination of hCD47b and CD271S. Antibody administration was performed once every 2 days for 32 days, after which all animals were sacrificed and examined for primary and metastatic melanoma growth. Upon gross pathological examination, metastasis was detected in multiple organs, including lymph nodes, lungs, and liver, in control (IgG)-treated mice. Strikingly, administration of hCD47b, CD271S, or both hCD47b and CD271S significantly reduced the presence of metastatic lesions in all the organs mentioned above and, in some animals, generated a complete response, resulting in the absence of detectable metastatic growth in lymph nodes and visceral sites (Figures 3C–3F). At the same time, mAb therapy had only a partial effect on established primary tumors, decreasing tumor volume by a mean of 16.5% ($p = 0.18$) after CD271S administration, 46% ($p = 0.001$) after hCD47b administration, and 57% ($p < 0.001$) after combined hCD47b and CD271S administration (Figure 3B). Notably, when the cellular composition of treated tumors was analyzed, we discovered that administration of hCD47b alone or in combination with CD271S not only reduced tumor size but also significantly decreased the proportion of human melanoma cells within tumors (Figure S3), which also contained cells of inflammatory, stromal, vascular, and blood lineages, among others.

During melanoma pathogenesis, the onset of metastatic disease is often associated with lymph node (LN) metastasis, most affecting LNs closest in proximity to the primary tumor site. Despite the fact that the primary tumors grew on the upper right side of the back of all treated mice, in the control (IgG)-treated group large metastatic lymph nodes were detected on both lateral sides in most of the animals, many of which had large metastatic growth at the axillary and inguinal LN locations (Figure 3D) in the same animal. This indicated rapid spread of metastasis, characteristic of melanoma pathogenesis. In sharp contrast, mice treated with CD271S displayed significant reduction in the size and number of metastatic LNs (Figure 3C), which appeared primarily on the right side of the body at the axillary location (Figure 3D). More pronounced results were observed in hCD47b-treated mice: metastatic LNs were absent on average in 50% (3/6 and 2/4) of examined animals (Figure 3C). Combined hCD47b and CD271S treatment yielded the most striking effect: on average, 70% of animals (4/6 and 3/4) had no detectable LN metastases. Moreover, in the animals that were affected, LN metastasis was restricted to a single small metastatic LN on the right axillary site (Figure 3D). Very importantly, examination of visceral organs revealed that lung metastases were significantly reduced after CD271S mAb administration (Figure 3E), whereas administration of hCD47b reduced them even further and a combination of both mAb treatments in the same animals virtually eliminated metastatic disease from their lungs (Figure 3E). In drastic contrast, 90% of control, IgG-treated mice developed numerous (>150) large pulmonary metastases. As melanoma progresses to more advanced stages, metastasis often affects multiple organ systems, representing a major challenge for existing therapies, which eventually fail due to widespread disease. In our xenograft model of human metastasis, M213 cells also metastasize to the liver, in addition

Figure 3. Administration of Antibodies Blocking Tumor CD47 and Targeting $CD271^+$ Melanoma Cells Results in Elimination of Regional and Distant Melanoma Metastases

Plotted data points for tumor volume and metastatic lesion/organ count represent a combination of two replicate experiments (experiment 1 n = 6 and experiment 2 n = 4 for each treatment group).

(A) General outline of the experiment. Antibodies were injected once every 2 days at the following doses: IgG control, 100 μg; hCD47b, 100 μg; CD271S, 1 μg; and hCD47b + CD271S, 100 μg + 1 μg, respectively.

(B) Primary tumor volumes at the end of each indicated treatment.

(C) Analysis of LNs from all treatment groups reveals dramatic reduction in metastatic LN burden and size after treatment with the indicated mAbs in comparison to control IgG.

(D) Representative experiment (experiment 1 n = 6) analysis of metastatic LN location and their distribution throughout the body of mice treated with the indicated mAbs. N, none.

(E and F) Pathological examination of lungs (E) and livers (F) from all treatment groups of mice reveals significant reduction in metastatic lesions in each organ after the indicated mAb administration. Representative areas of H&E-stained tissues containing metastatic foci are shown at 4× magnification and traced by brown lines.

Horizontal bars illustrate mean values for each group. p values were calculated using unpaired parametric Student's t test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$).

to the LNs and lungs. Examination of control, IgG-treated mice revealed liver metastases on average in 80% of animals (5/6 and 3/4), ranging from 3 to 13 lesions (Figure 3F). Treatment of mice with CD271S significantly reduced liver metastasis: on average, only 30% of animals (2/6 and 1/4) had detectable metastatic lesions. Even more strikingly, administration of hCD47b alone or in combination with CD271S completely prevented liver metastasis in 100% of animals, per detailed pathological examination (Figure 3F). Overall, we observed progressive diminishment of metastatic burden (particularly that of LNs) that was correlated with the reduction of primary tumor volumes. Further experiments will be required to determine the extent to which tumor volume and dissemination of metastatic cells are correlated, and whether any paracrine factors secreted by primary tumor cells support the homing and survival of their metastatic counterparts.

To evaluate the effect of the treatment antibodies on the early onset of metastases, we transplanted 100,000 M213 cells intradermally into NSG mice as described above and began treating them 14 days after cell implantation (Figure S4A). After 10 days of treatment, all mice were sacrificed and their tissues were examined for metastatic lesions. As in the experiments described above, we observed greatly reduced lung metastases and the complete absence of metastatic LNs in both mouse CD47 blocking mAb (mCD47b)- and CD271S-treated animals (Figure S4B). A decrease in metastases was accompanied by partial reduction of tumor volume, which, although significant in the case of mCD47b-treated tumors (34%, $p = 0.012$), was not significant for CD271S-treated tumors (13.5%, $p = 0.20$) (Figure S4C), indicating that primary tumor size reduction is not the sole mechanism through which mAb action suppresses the development of metastases.

In order to validate our findings in an independent metastatic melanoma model, we utilized M727 patient-derived cells. As indicated above, upon intradermal xenotransplantation into NSG mice, these cells form skin melanomas that later metastasize to the lungs and kidneys, without affecting the animals' LNs. This allowed us to test the effectiveness of blocking CD47 and targeting $CD271^+$ cells in a melanoma model characterized by a different mechanism of metastasis (metastasis of primary melanoma cells through the blood vasculature but not the lymphatic system). The general outline of the experiment is shown in Figure 4A; melanoma (M727)-bearing mice were treated with the indicated antibodies for 39 days, after which all animals were sacrificed and examined for metastatic melanoma growth. Upon pathological examination, we detected that lungs from control-treated mice contained grossly deformed tissues that consisted of large metastatic lesions fused together, essentially replacing epithelial lung structures with tumor cells, in 100% of cases (Figure 4B). We calculated the metastatic area index (MAI) in these animals by taking the ratio of tumor area to total lung area in the examined cross-section. Treatment of mice with CD271S resulted in, on average, a 2-fold decrease in MAI, with the worst-affected animal having a 0.23 MAI (23% of lung area colonized by metastatic tissue) compared to 0.43 MAI for the worst-affected control animal (43% of lung area colonized by melanoma). Administration of hCD47b alone or in combination with CD271S resulted in nearly complete elimination of lung metastasis in 100% of treated animals, with only a few micro-metastases (mets) existing throughout the entire lung area per histological examination (Figure 4B). Furthermore, analysis of kidneys revealed large metastatic lesions in 100% of control animals, ranging from 3 to 18 macro-mets per animal. Targeting $CD271^+$ melanoma cells with CD271S significantly reduced the number of kidney mets, ranging from 0 to 3 micro-mets per animal (Figure 4C). Importantly, administration of hCD47b alone or in combination with CD271S achieved the best response, almost completely eliminating kidney metastasis, per histopathological analysis (Figure 4C).

In summary, these results provide strong evidence that melanoma metastasis can be effectively treated and suppressed by blocking CD47 antigen with CD47b mAb on tumor cells, and that this therapeutic effect is augmented by specifically targeting $CD271^+$ melanoma cells with CD271S mAb.

CD47 Treatment Increases Macrophage Density and Changes the Balance of Pro-Metastatic and Anti-Tumor Myeloid Cells

Tumor immune microenvironment has been shown to play a pivotal role in carcinogenesis by either inhibiting or promoting tumor growth, depending on its cellular composition (Qian and Pollard, 2010). To investigate how the immune cell composition of a tumor changes following mAb treatment in melanoma-bearing NSG mice, we resected primary tumors at the end of each treatment and performed rigorous immunohistochemical (IHC) and FACS analysis of myeloid cell populations. IHC staining of tumors from each treatment group using the macrophage-specific mAb F4/80 indicated that CD47 blockade induces a massive increase of differentiated macrophages within the tumor (Figure 5A). We next performed flow cytometric analysis of treated tumors following their resection and isolation into single-cell suspensions to determine the types of immune cells infiltrating the tumor. Remarkably, we discovered that in control (IgG)- or CD271S-treated tumors, up to 85% of the myeloid lineage comprised inflammatory monocytes (Lin^-, $CD45^+$, $Ly6C^{hi}$, $CCR2^+$, $CD11b^{lo}$, $CD11c^-$, $F4/80^-$) and only 10% comprised differentiated macrophages (Lin^-, $CD45^+$, $CD11b^+$, $F4/80^+$, $Ly6C^{lo\text{-}hi}$, $Ly6G^-$). In sharp contrast, tumors treated with hCD47b, whether alone or in combination with CD271S, contained significantly fewer inflammatory monocytes and many more differentiated macrophages (45% and 42%, respectively) (Figure 5B).

Recent studies of epithelial malignancies suggest that macrophages, characterized by the expression of VEGFR1 and CCR2, aide tumor cells in migrating from their primary site and seeding metastatic growth at distant organs (Qian et al., 2011; Ren et al., 2012). We therefore analyzed the frequency of these pro-metastatic macrophages in tumors from each treatment group. Flow cytometric analysis of treated tumors revealed that combined hCD47b and CD271S mAb administration resulted in an almost 2-fold reduction in the frequency of pro-metastatic macrophages (Lin^-, $CD11b^+$, $F4/80^+$, $Ly6C^{lo\text{-}hi}$, $Ly6G^-$, $VEGFR1^+/CCR2^+$) compared to control (IgG)-treated mice: 6% and 11.5%, respectively (Figure 6A). Recent evidence indicates that $VEGFR1^+/CCR2^+$ macrophages not only influence the tumorigenic properties of primary tumor cells but are also

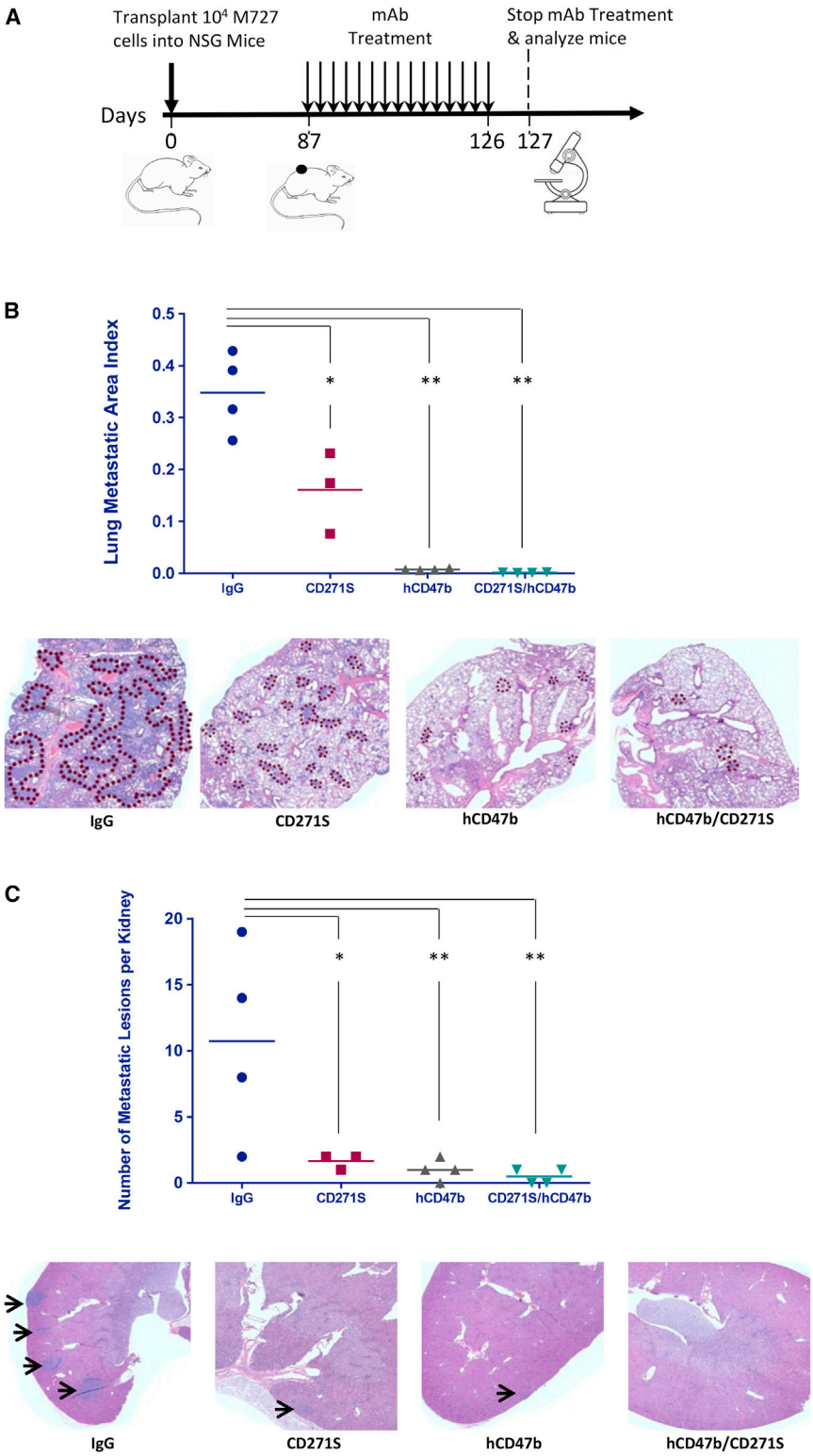


Figure 4. Antibody Therapy of Blocking CD47 and Targeting CD271⁺ Melanoma Cells Significantly Reduces Lung and Kidney Metastases

(A) General outline of the experiment. Mice bearing M727 melanomas were randomized into four treatment groups (n = 4, except CD271S n = 3) and treated with the indicated mAbs for 39 days at the following doses: IgG control, 100 μg; hCD47b, 100 μg; CD271S, 1 μg; and hCD47b + CD271S, 100 μg + 1 μg, respectively. At the end of the treatment, organs from all treated mice were removed and examined for the presence of metastatic melanoma foci.

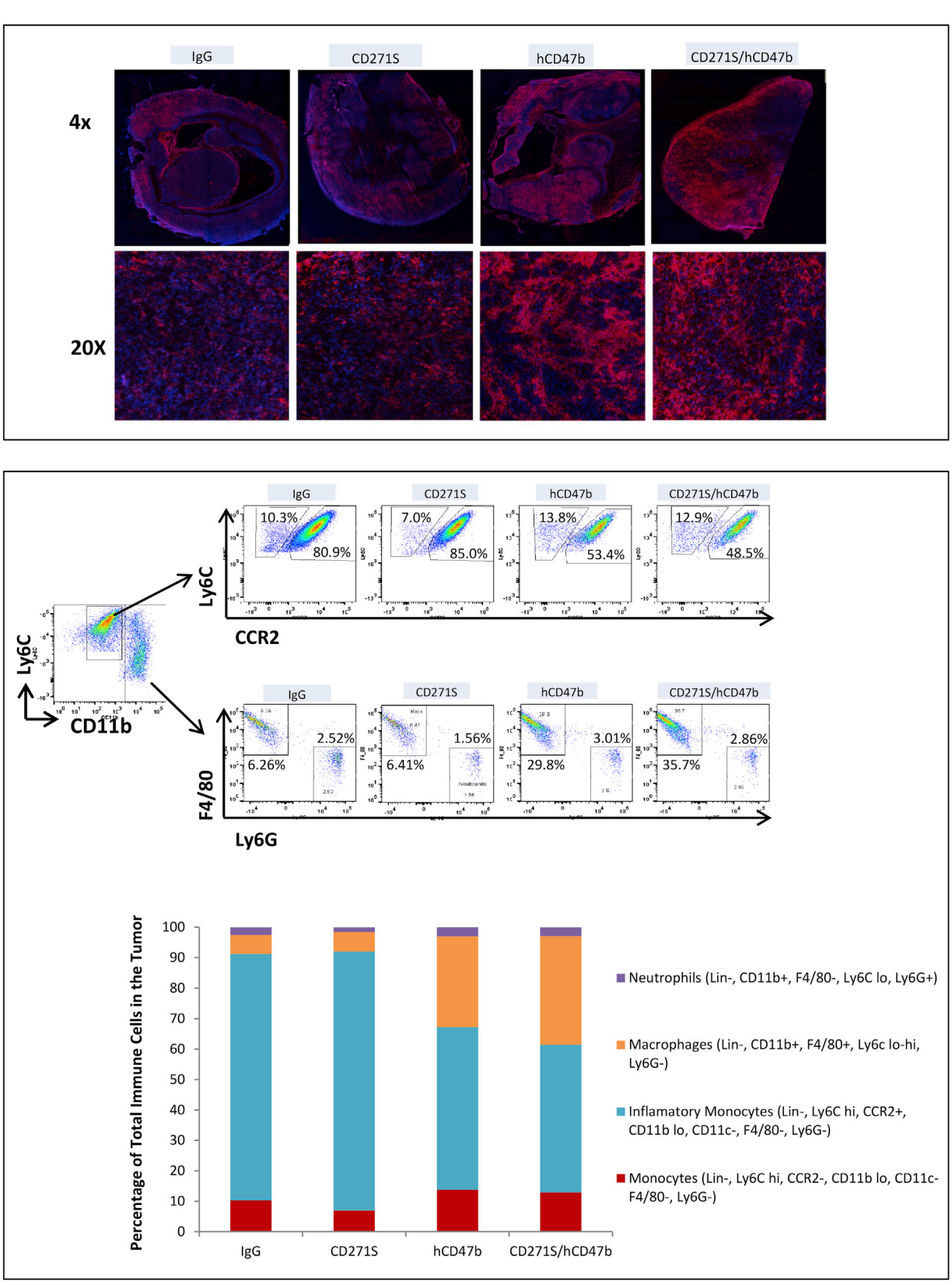
(B and C) Pathological analysis of lungs (B) and kidneys (C) from each treatment group reveals significant reduction of metastases in these organs. Representative areas of each H&E-stained tissue are shown at 20× magnification, and metastatic lesions are traced by brown dotted line or indicated by black arrows. Horizontal bars illustrate mean values for each group.

*p < 0.05, **p < 0.005, ***p < 0.0005.

recruited to pulmonary sites and positively regulate extravasation and initiation of metastatic growth (Ren et al., 2012). In our experiments, these macrophages were present in lungs from all treatment groups; however, we found that combined hCD47b and CD271S treatment (resulting in the most significant decrease of pulmonary metastatic burden) induced a 7-fold reduction in the frequency of pro-metastatic macrophages compared to control treated group (0.27% and 2%, respectively) (Figure 6B).

We next characterized the expression of specific genes commonly upregulated in myeloid cells found at tumor sites and associated with disease progression (Franklin et al., 2014). Using real-time PCR, we found that tumors treated with hCD47b alone or in combination with CD271S contained myeloid cells that expressed significantly lower levels of Mrc1, Ym1, Mgl1, Fizz1, and Il10 (Figure S5). Unexpectedly, we found no difference in Arg1 and Il12 expression and lower levels of Nos2 in the same cells as compared to control-treated mice. Interestingly, a previous report indicated that macrophages whose SIRPα had been activated by CD47 antigen binding display increased NO production (Alblas et al., 2005). Our results directly support this notion demonstrating that blockade of CD47 with mAbs decreases expression of Nos2 mRNA, which encodes the key enzyme in NO synthesis, iNOS (Figure S5).

Because CD47 is expressed in virtually all hematopoietic cell lineages, we tested hCD47b mAb binding to mouse myeloid cell subsets to rule out the possibility that the aforementioned changes in the immune cell profiles of treated mice are caused by antibody binding.



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We co-incubated freshly isolated mouse macrophages and neutrophils, as well as human melanoma (M727) cells, with hCD47b mAb, which was then detected by fluorescently conjugated secondary antibodies (IgG4-FITC [fluorescein isothiocyanate]). Our results validate that hCD47 mAb is specific to human CD47 only and lacks specific binding activity against the mouse cell populations tested (Figure S6).

Therapeutic Antibody Treatment Reduces Neutrophil Infiltration at Metastatic Sites

Similar to pro-metastatic macrophages, neutrophils also play a significant role in the colonization of visceral organs during metastasis when recruited to these sites. There are multiple mechanisms by which neutrophils act to promote the homing of circulating metastatic cells (CMCs), including contact-dependent tethering of CMCs to end-organ endothelium, formation of neutrophil extracellular traps (NETs), and secretion of soluble factors that create a favorable milieu for arriving metastatic tumor cells (Cools-Lartigue et al., 2013; Fridlender and Albelda, 2012). Mounting evidence points to a direct correlation between poor patient prognosis and high frequencies of neutrophils at metastatic sites (Sarraf et al., 2009; Walsh et al., 2005). In our experiments described above, we observed significant changes in some of the myeloid cell lineages that infiltrate tumors during mAb treatments (Figure 5). To more fully understand the therapeutic effects of antibody treatment on melanoma metastasis, we investigated the visceral organs of experimental mice for the presence of neutrophils and differentiated macrophages. Very importantly, we discovered that at pulmonary sites of melanoma metastasis in control (IgG)-treated mice, up to 75% of the myeloid cell lineage comprised differentiated neutrophils/granulocytes (Lin^-, $CD11b^+$, $F4/80^-$ $Ly6C^{lo}$, $Ly6G^+$) (Figure 7A; Figure S7), whereas only 8% comprised differentiated macrophages. In sharp contrast, when mice were treated with hCD47b alone or in combination with CD271S, the proportion of neutrophils/granulocytes decreased to 30%, whereas that of differentiated macrophages increased to 50% (Figure 7). These drastic changes in the myeloid cell microenvironment provide an additional mechanism by which to explain the potent therapeutic effect of hCD47b and combined hCD47b and CD271S mAb treatments against visceral metastasis.

DISCUSSION

In this study, we used surgical melanoma samples and gene expression datasets to confirm CD47 as a marker of metastatic melanoma development and demonstrate its role in disease progression. Blocking the CD47 antigen with hCD47b mAb, which activates macrophage-induced phagocytosis, considerably reduced melanoma metastasis in a mouse xenograft model of human disease. When this treatment is combined with mAbs targeting $CD271^+$ melanoma cells, which were previously determined to have tumor- and metastasis-initiating properties (Boiko et al., 2010; Civenni et al., 2011; Redmer et al., 2014), the therapeutic effect of CD47 blockade is enhanced even further. We also show that the anti-metastatic properties of CD47 blockade on tumor cells not only induce their phagocytosis in vitro and reduce their metastasis in vivo but also result in critical changes in the tumor site immune microenvironment by increasing the density of differentiated macrophages and decreasing the presence of inflammatory monocytes, pro-metastatic macrophages, and neutrophils (at pulmonary metastases), all three of which are associated with disease progression. Because hCD47b mAb treatment also results in partial reduction of primary tumor volume, which may affect its ability to metastasize, further research is required to delineate the anti-metastatic properties of hCD47b mAb treatment, independent of its effects on primary tumor growth. In a model of metastatic leiomyosarcoma, which is also susceptible to CD47 blockade, surgical excision of primary tumors during the first week of treatment still resulted in the significant reduction of metastases at visceral sites (Edris et al., 2012). Combined with the data presented in this study, it indicates the strong potential of CD47 treatment as a neoadjuvant therapy for melanoma patients whose primary cutaneous tumors had been previously removed.

Integrin-associated protein CD47 plays a "self-signal" role by binding to the SIRPα receptor on macrophages and preventing their phagocytic function (Majeti et al., 2009; Okazawa et al., 2005; Vernon-Wilson et al., 2000). Overexpression of CD47 on the cell surface of normal hematopoietic stem cells (HSCs) has been shown to play a protective role when these cells travel through the bloodstream during mobilization from the bone marrow to various tissue sites (Jaiswal et al., 2009). Metastasis, the primary cause of death for many cancers, including melanoma, occurs by active migration mechanisms that involve tumor cell intravasation, survival in the bloodstream, extravasation, and the ability to proliferate at distant sites. Macrophages, a major part of innate immune surveillance, react to the presence of metastasizing cells at primary or visceral locations by binding to and phagocytosing these cells to prevent the spread of disease. Our analysis of CD47 expression in primary and metastatic tumor sites from melanoma patient databases and our in vivo CD47 blocking experiments provide evidence that melanoma cells that acquire a metastatic phenotype and colonize other organs employ the same anti-phagocytic protection mechanism as do mobilized HSCs.

Inhibition of CD47 using monoclonal antibodies was recently demonstrated to effectively eliminate hematologic and epithelial malignancies (Chao et al., 2010; Majeti et al., 2009; Willingham et al., 2012). However, for tumors of neural crest origin—in particular human melanoma—the relevance of this molecule is

Figure 5. Blockade of CD47 Antigen on the Surface of Tumor Cells Changes the Repertoire of Myeloid Cell Lineages at the Primary Tumor Site

(A) Immune fluorescence tumor analysis of infiltrated macrophages using mF4/80-A647 mAb. Red color indicates positive F/480 staining.

(B) The frequency of myeloid cell lineages at the primary tumor site in each treatment group was determined by isolating a tumor single-cell suspension and staining it with a panel of cell-surface markers specific for the following populations: inflammatory monocytes (Lin^-, $CD11b^{lo}$, $CD11c^-$, $F4/80^-$, $Ly6C^{hi}$, $Ly6G^-$, $CCR2^+$), macrophages (Lin^- $CD11b^+$ $F4/80^+$ $Ly6C^{lo\text{-}hi}$ $Ly6G^-$), and neutrophils (Lin^-, $CD11b^+$, $F4/80^-$ $Ly6C^{lo}$ $Ly6G^+$) that were then analyzed using a BD FACSAria instrument.

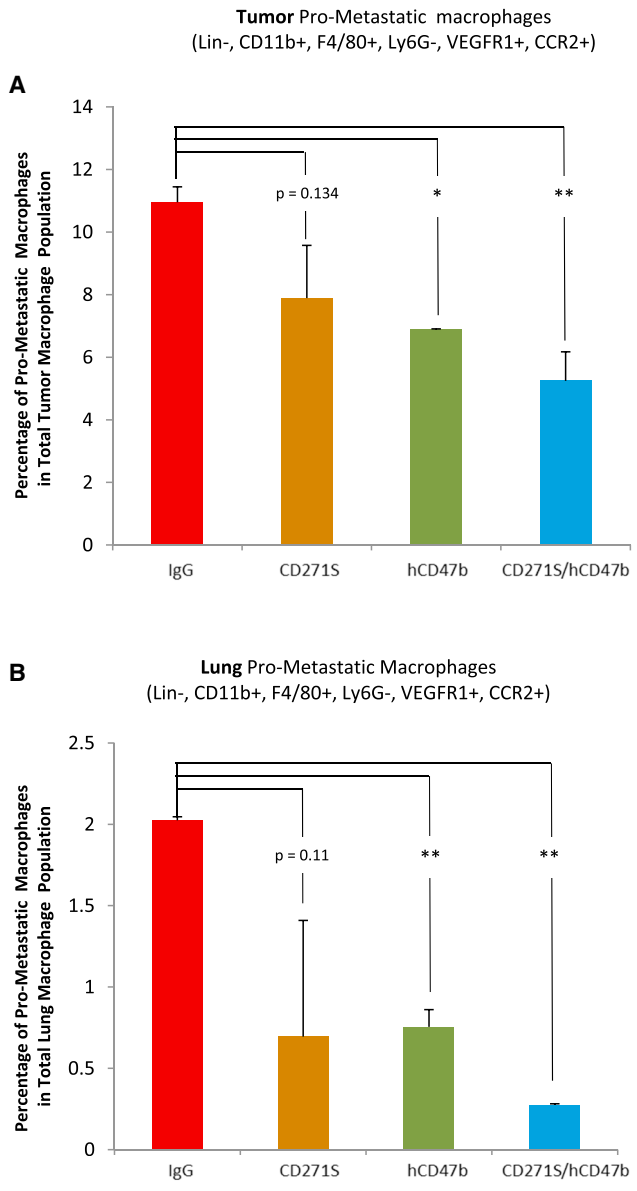

Figure 6. CD47 Blockade on Tumor Cells Decreases the Frequency of Pro-Metastatic Macrophages at Primary and Pulmonary Sites

M213 tumor-bearing mice were treated with the indicated mAbs and, at the end of the treatment, tumors and lungs were surgically removed and digested into a single-cell suspension. Cells were stained with a combination of cell-surface markers allowing quantitation of various macrophage populations. Frequency of pro-metastatic macrophages in the tumor (A) and lung (B) was determined as the percentage of Lin^{-} $CD11b^{+}$ $F4/80^{+}$ $Ly6C^{lo\text{-}hi}$ $Ly6G^{-}$, $CCR2^{+}$/$VEGFR1^{+}$ cells in the total macrophage population. Data are shown as means with SDs. p values were derived using unpaired parametric Student's t test (*$p < 0.05$, **$p < 0.005$).

only beginning to emerge. In a murine model of melanoma, B16F10, small interfering RNA (siRNA) targeting CD47 was shown to be effective against mouse tumors, suggesting that mRNA levels of CD47 are important for tumor homeostasis (Wang et al., 2013). Nonetheless, a significant gap exists between the B16F10 mouse melanoma model and the safe therapeutic delivery of siRNA into human patients. In the current study, we utilized a therapeutically developed and humanized mAb blocking CD47 and metastatic melanoma cells derived from clinical human samples to demonstrate that blockade of CD47 antigen by administering CD47b mAb considerably reduces metastasis in vivo. Significantly, these results confirmed our hypothesis that the CD47 antigen on metastatic melanoma cells plays a protective role against macrophage attack.

In most adult tissues, resident macrophages are derived from bone marrow monocytes. Classically differentiated macrophages are capable of eliciting anti-tumor responses, resulting in cell phagocytosis and antigen presentation. Accumulating evidence indicates that myeloid cell lineages, although helping the immune system to recognize and eliminate cancer cells, can also be subverted by tumors to play a supportive role for tumor progression and metastasis (Biswas and Mantovani, 2010; Murdoch et al., 2008). Both alternatively activated macrophages ($CCR2^{+}$/$VEGFR1^{+}$) and neutrophils have been shown to promote metastatic cell growth and seeding at distant organs (Cools-Lartigue et al., 2013; Qian et al., 2011; Ren et al., 2012). Alternative macrophage differentiation occurs in response to multiple external and internal factors and results in cells that aid tumor growth via cytokine secretion, stimulation of blood vessel growth, and matrix remodeling. Neutrophils (also derived from myeloid precursors) aid colonization of visceral organs by several independent mechanisms that involve formation of NETs and secretion of soluble factors that promote the adhesion of circulating tumor cells to an organ's endothelium (Cools-Lartigue et al., 2013; Fridlender and Albelda, 2012). Thus, the balance between different myeloid cell populations can be a critical determinant of eventual disease outcome and serve as a therapeutic target. In our current study, we demonstrate that blocking the CD47 antigen on the surface of tumor cells initiates a chain of events that changes the tumor immune microenvironment by significantly increasing the presence of anti-tumor macrophages and decreasing the presence of undifferentiated monocytes and pro-metastatic macrophages at primary tumor sites. Similar changes occur at the pulmonary sites of metastasis that harbor increased concentrations of anti-tumor macrophages and have a greatly decreased density of neutrophils. Because the hCD47b mAb is human specific and does not display binding activity against the murine CD47 antigen, observed changes in the type and number of myelomonocytic cells in treated melanomas at primary and metastatic locations must result from the biological effects of this antibody on the human tumor cells, and not the antibody-mediated killing of neutrophils and/or macrophages. Further studies will be required to elucidate the exact mechanisms by which CD47-induced phagocytosis of tumor cells affects macrophage polarization and monocytic lineage differentiation at the sites of actively proliferating disease.

During the last two decades, researchers have made numerous attempts to translate knowledge of immune system regulation into potent immune therapies in melanoma. These were mostly focused on potentiation of T cell responses and include administration of high-dose interleukin 2 and autologous cell transplantations that utilize patient-derived tumor-infiltrating lymphocytes or genetically engineered T cells against known melanoma tumor antigens such as TYR, MART-1, or

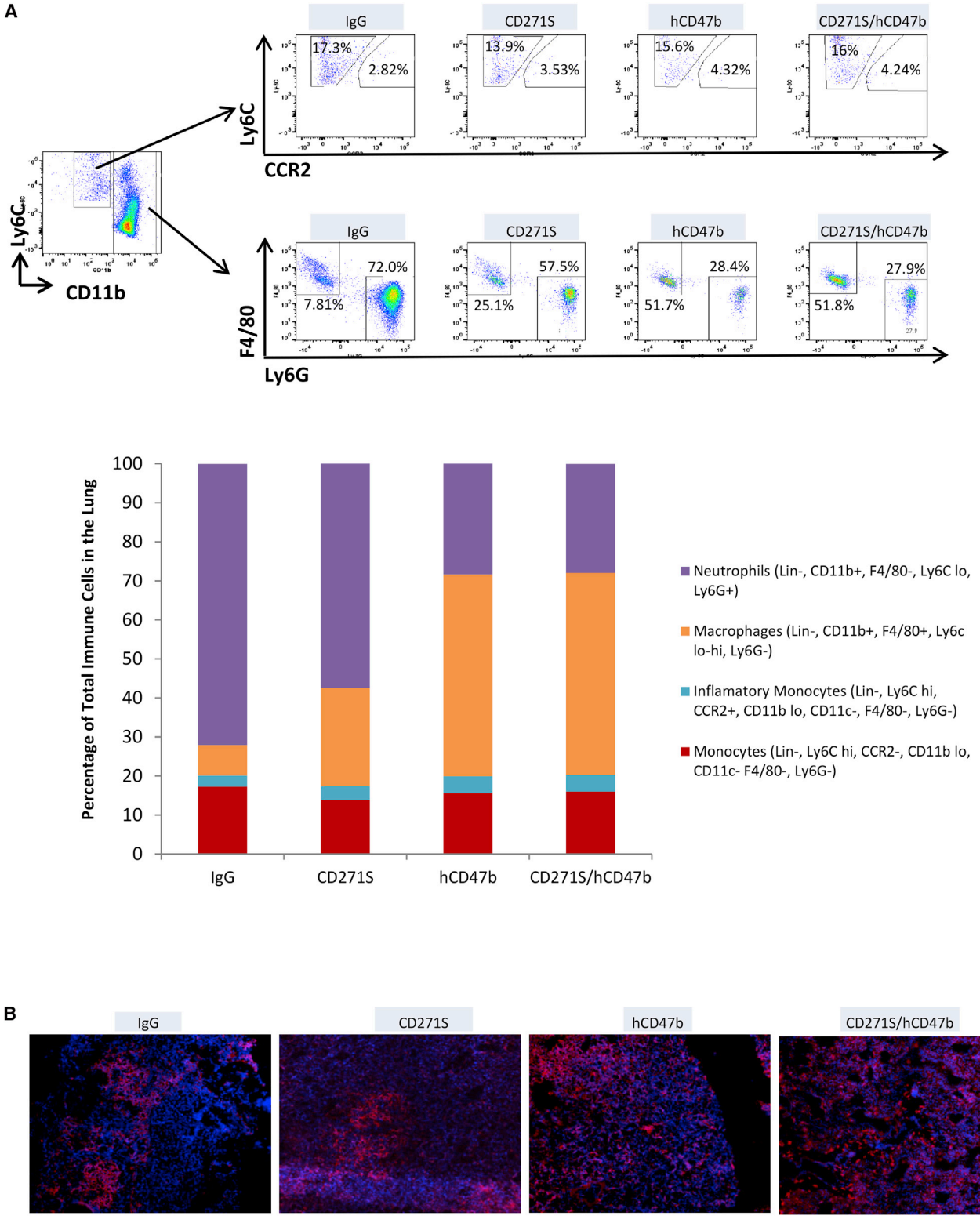

Figure 7. CD47 Blockade on Tumor Cells Reduces Neutrophil and Increases Differentiated Macrophage Densities at Pulmonary Sites of Metastasis

(A) M213 tumor-bearing mice were treated with the indicated mAbs, after which their lungs were surgically removed and digested into a single-cell suspension. Cells were stained with the combination of cell-surface markers specific for the following populations: inflammatory monocytes (Lin^{-}, CD11b^{lo}, CD11c^{-}, F4/80^{-},

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NY-ESO-1 (Cormier et al., 1997; Dudley et al., 2001; Morgan et al., 2006; O'Neil et al., 1993). More recent approaches also included application of monoclonal antibodies that target inhibitory immune checkpoints (CTLA-4) or anti-apoptotic pathways (PD-1/PDL1) to increase the cytotoxic anti-tumor response (Callahan et al., 2015; Hodi et al., 2010; Ribas et al., 2016). Recent clinical trial data show that ipilimumab and nivolumab are capable of inducing an objective response (OR) in 10%–43% of advanced melanoma patients, with the most effective regimen, based on concurrent administration of both antibodies, resulting in an OR rate of 61% (Postow et al., 2015; Robert et al., 2015). Although studies to determine disease-free survival rates in these patients are still ongoing, the latest clinically available data among ipilimumab-treated groups indicate that median overall survival was 9.5 months (95% confidence interval [CI], 9.0–10.0 months), with a 3-year survival rate of only 21% (95% CI, 20%–22%) (Schadendorf et al., 2015). This indicates that a significant proportion of patients still succumbs to the disease, most likely as a result of tumor adaptation that allows it to bypass recognition by activated T cells. Therefore, investigations continue to find regimens and cellular therapies that can be used in combination with T cell-activating approaches and thus significantly diminish the chances that resistant tumor clones emerge quickly in the patient. In this report, we provide biological evidence that blockade of CD47 activates strong anti-tumor prophagocytic response in vitro. A similar mechanism involving macrophage-induced phagocytosis of cancer cells may be responsible for the observed anti-metastatic effects of CD47b antibody in vivo. Thus, activation of innate immunity based on macrophage-induced phagocytosis can be harnessed as an effective therapeutic strategy against metastatic melanoma. Our findings also indicate that a treatment regimen aimed against multiple mechanisms of melanoma progression (blockade of CD47 antigen and targeting of the CD271^{+} melanoma-initiating cell subset) produces the most effective therapeutic response.

As clinical trials uncover the therapeutic effectiveness of antibodies targeting T cell checkpoint inhibitors, the data presented here on the antibodies that induce the anti-tumor macrophage response should lead to studies testing the synergy of activating both adaptive (the T cell response) and innate (macrophage-mediated phagocytosis) components of the immune system in advanced melanoma patients. Combining this strategy with the targeting of melanoma-initiating (CD271^{+}) cells may represent a synergistic therapy that more effectively activates the overall immune response against melanoma.

EXPERIMENTAL PROCEDURES

Human Samples

Human melanoma samples were obtained from consented patients at Stanford Hospital, per protocols approved by the Institutional Review Board of the Stanford University School of Medicine.

Flow Cytometry Analysis

For CD47 expression, surgical melanoma samples excised from primary and metastatic sites were dissociated into single-cell suspensions as previously described (Boiko, 2013; Boiko et al., 2010). Cell suspensions were stained with hCD47-phycoerythrin (PE) Ab and lineage (Lin) Abs: hCD45-Pacific blue (PB), hCD31-PB, and hCD235a-PB. For analysis of tumor- and lung-infiltrating cell populations, these tissues were digested into single-cell suspensions and stained with the following mAbs conjugated to the indicated fluorochromes: mCD45-PB, mCD11b-PeCy7, mCD11c-allophycocyanin (APC), mF4/80-APC, mLy6C-FITC, mLy6G-PE, mVEGFR1-APC, and mCCR2-A700. Flow cytometric analysis of stained cells was performed using a BD FACSAria instrument.

Analysis of CD47 mRNA Expression

Gene expression and clinical data were analyzed for previously described cohorts of melanoma patients: GSE8401 (n = 83) and TCGA_SKCM (n = 310). For GSE8401, Affymetrix probeset summaries were derived after normalizing the dataset with RMA (http://rmaexpress.bmbolstad.com/). Mean CD47 mRNA expression was computed based on probeset ID 211075_s_at using R software (https://www.r-project.org/). For melanoma TCGA, RNA-sequencing reads were processed with R_subread version 1.14.2 of the R package and summarized gene values were normalized to fragments per kilobase of transcript per million mapped reads (FPKM). Subsequently, CD47 gene expression was evaluated using "R Stats" R software version 3.2 in 310 subjects whose melanoma lesions were classified as primary tumor, regional cutaneous metastasis, regional lymph node metastasis, and distant metastasis.

Antibody Treatment of Xenograft Tumors

Mice bearing human melanoma tumors (M213 or M727) were randomized into four treatment groups: IgG (BD Pharmingen) control mAb, blocking anti-hCD47b (Hu5F9-G4; Lonza) monoclonal antibody, anti-CD271S (ME20.4-Sap; Advanced Targeting Systems) cytotoxic antibody, and combined blocking anti-hCD47b and anti-CD271S antibodies. M213, n = 6 for experiment 1 and n = 4 for replicate experiment 2 in each treatment group; M727, n = 4 in each treatment group, except CD271S n = 3. IgG control (100 μg) and CD47 antibodies (100 μg) were injected intraperitoneally (100 μl) once every 2 days. CD271S (1 μg) was injected directly into the center mass of the tumor (50 μl) once every 2 days. Antibody injections began 27 days (M213-injected mice) or 87 days (M727-injected mice) after initial tumor cell engraftment, and lasted for 32 and 39 days, respectively.

Quantitative RT-PCR

Total RNA was extracted using TRIzol reagent, and the mRNA was reverse transcribed into cDNAs using Verso cDNA synthesis kits (Life Technologies) followed by real-time PCR using LightCycler FastStart DNA Master SYBR Green I and a LightCycler 780 II (Roche) instrument. Mouse gene-specific primer sets (summarized in the table in Supplemental Experimental Procedures) were used to detect each mRNA. Expression values were normalized against B2m using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Data are shown as means with SDs.

Immunohistochemistry

To determine the levels of macrophage infiltration, fluorescence immunohistochemistry was performed on fresh-frozen, OCT-embedded tumor and lung tissue. Tissues were sectioned at 10 μm and stained with anti-F4/80 antibody. Slides were scanned and imaged using a fluorescence Nikon Ti-E inverted microscope.

Statistical Analysis

All data are presented as means ± SDs. Statistical analysis and graph preparation were performed in Prism 6 (GraphPad Software). p values were

Ly6C^{hi}, Ly6G^{-}, CCR2^{+}), macrophages (Lin^{-} CD11b^{+} F4/80^{+} Ly6C^{lo-hi} Ly6G^{-}), and neutrophils (Lin^{-}, CD11b^{+}, F4/80^{-} Ly6C^{lo} Ly6G^{+}) that were then analyzed using a BD FACSAria instrument.

(B) Immune fluorescence lung tissue analysis of infiltrated macrophages. At the end of each treatment, lungs were removed, cryosectioned at 10 μm, stained with anti-F4/80-A647 antibody, and imaged at 20× resolution. Red color indicates positive F/480 staining.

calculated using unpaired parametric Student's t test, assuming equal variances. Statistically significant difference is marked as *p < 0.05, **p < 0.005, ***p < 0.0001.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.07.004.

AUTHOR CONTRIBUTIONS

M.N. and A.D.B. designed the research. M.N., A.H., S.J.H., and A.D.B. performed the research. A.L. and D.S. conducted bioinformatics analysis. A.H.B., A.L., D.S., K.W., I.L.W., and A.D.B. contributed new reagents/analytic tools. M.N., A.H., A.H.B., and A.D.B. analyzed the data. M.N. and A.D.B. wrote the manuscript.

CONFLICTS OF INTEREST

A.D.B. declares patent applications pertaining to CD47-blocking therapies and CD271 targeting in melanoma assigned to Stanford University and equity and/or consulting with Forty Seven, Inc. K.W. declares patent applications pertaining to CD47-blocking therapies assigned to Stanford University and equity and/or consulting with Forty Seven, Inc. and Alexo Therapeutics. I.L.W. declares equity and consulting and serves as a director of Forty Seven, Inc. I.L.W. and A.D.B. have been issued U.S. patent 9,151,760, "Isolation and Use of Melanoma Cancer Stem Cells."

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