ABSTRACT

Anti-CD47 Targeting Therapy for the Treatment of Colorectal and Pancreas Cancers

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CD47 is a “don’t eat me” signal over expressed on cancer cells inhibiting their engulfment by phagocytes. A novel monoclonal antibody, Hu5F9-G4, blocking CD47’s interaction with its ligand SIRPα allowed for phagocytosis of colorectal and pancreas tumors by macrophages. In some models, treatment of xenotransplanted tumors in immunodeficient mice with anti-CD47 antibodies strongly reduced tumor growth. In other models, the combination of anti-CD47 and anti-EGFR antibodies regressed tumor burden, where neither single agent therapy was as effective. Even in *KRAS* mutated tumors, where use of anti-EGFR antibodies is not indicated, the combination of antibodies lead to major tumor reduction. Taken collectively, Hu5F9-G4 is an effective treatment in colorectal and pancreas cancers, and in the combination of Hu5F9-G4 with an anti-EGFR antibody is therapeutically effective in *KRAS* mutated tumors in mouse xenografts.

Cancer immunotherapy, including anti-CD47 blockade, tries to utilize the host’s own immune system to mount an immune response to cancer. To date, there have been a multitude of new therapies from cytokine treatments, to cell based vaccines, to antibody

therapies which all recruit immune cells that have been somehow silenced by the tumor microenvironment. The Weissman lab's own studies into the adaptive immune system suggest that upon phagocytosis, mediated by anti-CD47 antibodies, the subsequent T cell presentation preferentially recruits cytotoxic T cells. Here, we propose two additional model systems to further investigate the role CD47 plays in antigen presentation and downstream T cell recruitment to target cancer cells.

Recent advances with immunotherapy agents for the treatment of cancer has provided remarkable, and in some cases, curative results. Blockade of the CD47:SIRPα axis between tumor cells and innate immune cells increases tumor cell phagocytosis in both solid tumors and hematological malignancies. These phagocytic innate cells are also professional antigen presenting cells, providing a link from innate to adaptive anti-tumor immunity. Preliminary studies have demonstrated that APCs present antigens from phagocytosed tumor cells, causing T cell activation. Therefore, agents that block CD47:SIRP-α engagement are attractive therapeutic targets as a monotherapy or in combination with additional immune modulating agents for activating anti-tumor T cells in vivo.

Anti-CD47 Targeting Therapy for the Treatment of Colon and Pancreas Cancers

by

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ACT – adoptive cell therapy

ADCC – antibody dependent cell-mediated cytotoxicity

ADCP – antibody dependent cellular phagocytosis

AML – Acute Myeloid Leukemia

APC – antigen presenting cell

CRC – colorectal cancer

CTL – cytotoxic T lymphocyte

DC – Dendritic cell

EGFR – Epidermal Growth Factor Receptor

FACS – Fluorescent activated cell sorting

FBS – fetal bovine serum

FcγR –Fcγ receptors

HLA - Human leukocyte antigen

HSC – Hematopoietic stem cell

IP – Intraperitoneal

ITIM – Immunoreceptor tyrosine-based inhibitory motif

JAK – Janus kinase

Lin – Lineage

LSC – Leukemia stem cells

LTC-IC – Long-term culture- initiating cell assay

mAb – Monoclonal antibody

MCA – methylcholanthrene

MHC – major histocompatibility

MPP – Multipotent progenitor

NHL – Non-Hodgkin's lymphoma

NOD – Non-obese diabetic

NSG – NOD SCID Gamma

PDAC – Pancreatic ductal adenocarcinoma

PI3K - Phosphoinositide 3-kinase

PrCR – Programed cell removal

RBC – Red blood cell

SCID – Severe combined immunodeficiency

SHP - Src-homology domain containing phosphatase

SIRPα – Signal regulatory protein alpha

STAT – Signal transducer and activator of transcription

TAM – Tumor associated macrophage

TCR – T cell receptor

TH – T helper

TIL – Tumor associated lymphocyte

TKI – Tyrosine kinase inhibitors

WT – Wild-type

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CHAPTER ONE

Introduction

Identifying CD47 As a Target for Cancer Therapy

Identification of Hematopoietic Stem Cell

Hematopoiesis proceeds through an organized hierarchy initiated by hematopoietic stem cells that give rise to progressively more committed progenitors and eventually terminally differentiated blood cells. The prospective isolation of mouse hematopoietic stem cells (HSC) was accomplished through the use of monoclonal antibodies (mAbs) directed against cell surface markers in conjunction with fluorescent-activated cell sorting (FACS) and transplanting using congenic mouse strains. Application of these techniques to discrete populations within mouse bone marrow led to the additional identification of multipotent and lineage-committed progenitors, making it possible to model the mouse hematopoietic developmental hierarchy (Figure 1.1A). Through the use of multiple cell surface markers, mouse HSCs were identified at the single cell level, and using the current best available markers, approximately 1 out of every 2 single cell HSC transplants were successful (Spangrude et al. 1988; Kiel et al. 2005; Bryder et al. 2006).

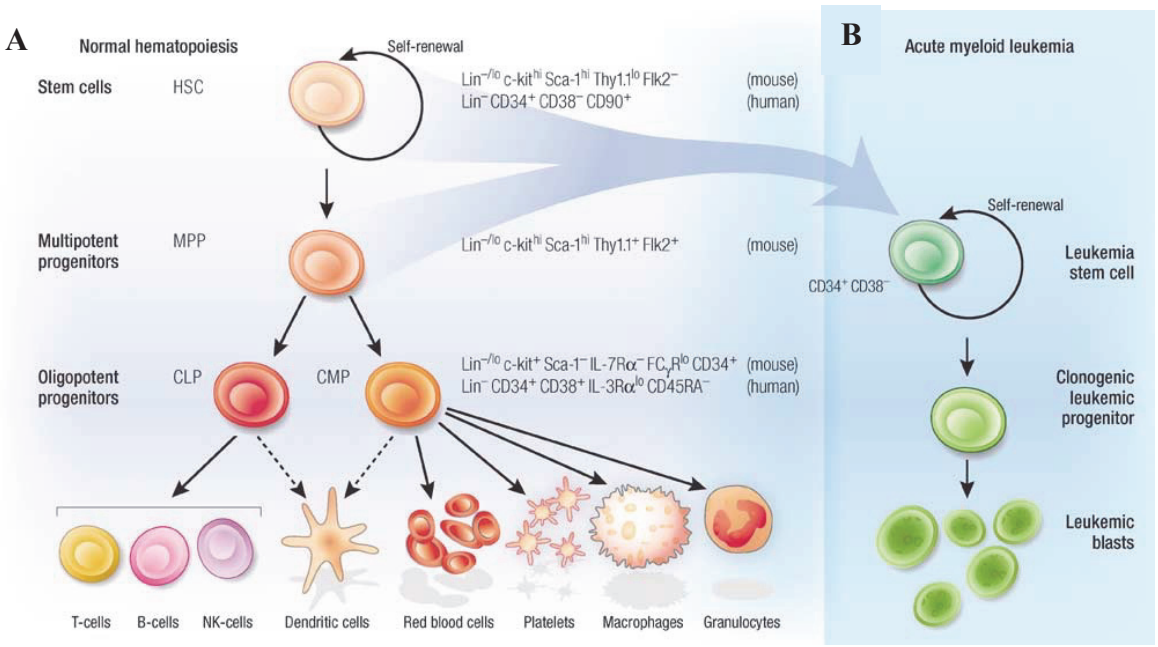

Figure 1.1. Hierarchical Model of Normal Hematopoiesis and Acute Myeloid Leukemia (A) Self-renewing HSCs give rise to multipotent progenitors that give rise to lineage-restricted progenitors and eventually to terminally differentiated blood cells. (B) AML is organized similarly by a self-renewing leukemic stem cell which gives rise to bulk leukemic blasts.
Cell surface immunophenotypes are indicated where shown. (Tan et al. 2006).

Human hematopoiesis also proceeds through an organized hierarchy initiated by hematopoietic stem cells that ultimately give rise to all the terminally differentiated cells of the blood. Both in vitro and in vivo experimental approaches have been utilized to identify human HSC (Kondo et al. 2003; Shizuru et al. 2004). The best in vitro assay of HSC function is the long-term culture-initiating cell assay (LTC-IC), which utilizes culture on bone marrow feeder cells to identify cells capable of producing hematopoietic cells for 6 weeks or longer (Sutherland et al. 1989). Using this technique, candidate human HSC were identified as negative for lineage markers (Lin-), positive for expression of CD34, and positive for expression of CD90 (Thy) (Baum et al. 1992; Craig et al. 1993; Murray et al. 1995). In separate reports, human HSC activity was localized to a Lin- CD34+CD38-/lo fraction using this LTC-IC assay (Terstappen et al. 1991; Murray et al. 1995; Hao et al. 1998; Miller et al. 1999). While in vitro assays provides important

information regarding lineage potential and possibly self-renewal, definitive demonstration of HSC function requires an in vivo assay. Dick and colleagues pioneered the xenotransplantation of normal human progenitors into immunodeficient mice, principally the NOD/SCID strain (McCune et al. 1988; Baum et al. 1992; Dick et al. 1997; Dick et al. 2001; Mazurier et al. 2003). By assaying for long-term multipotent human hematopoiesis in recipients and the ability to form secondary and tertiary transplants, several investigators demonstrated that human HSC reside in the Lin-CD34+CD38-/lo fraction of human progenitors (Bhatia et al. 1997; Guenechea et al. 2001; McKenzie et al. 2006), with the most enriched population residing in the lineage (Lin)-CD34+CD38loCD90+ subset (Baum et al. 1992; Uchida et al. 1999). Perhaps the best in vivo demonstration of HSC function comes from human clinical trials of autologous mobilized peripheral blood in clinical transplantation, where long-term engraftment was provided by transplantation of purified CD34+CD90+ cells (Negrin et al. 2000; Vose et al. 2001). These in vivo studies indicate that human HSC are contained in the Lin-CD34+CD38-CD90+ fraction of hematopoietic progenitors, and when taken from patients with metastatic cancers are cancer-free. Women with metastatic breast cancer who received myeloablative doses of combination chemotherapy were rescued either with cancer-free HSC or unpurified, cancer-contaminated mobilized blood; 33% of those receiving cancer free HSC were alive 18 years later, while only 7% of those who received mobilized blood (Müller et al. 2012).

Identification of the Leukemic Stem Cell in Acute Myeloid Leukemia

Human acute myeloid leukemia (AML) is a clonal malignancy characterized by the accumulation of immature myeloid cells defective in their maturation and function.

Much is now known about the biology of AML including: chromosomal translocations resulting in gene fusions, chromosomal and subchromosomal deletions of putative tumor suppressor genes, dysregulation of programmed cell death, single gene mutations correlating with leukemia, and derangements of the telomere/telomerase system (Gilliland et al. 2004). Recent experiments have added to our fundamental knowledge by demonstrating that AML is composed of a hierarchy of cells that is maintained by a small pool of leukemia stem cells (LSC) (Figure 1.1B) (Miyamoto et al. 2000; J.C.Y. Wang and Dick 2005; Tan et al. 2006). Stem cells in any biological process are defined by the properties of self-renewal, which is the ability to give rise to progeny identical to the parent through cell division, and multipotency, which is the ability to give rise to all the differentiated cells of that biological process (Reya et al. 2001). Most of the cells within AML do not possess these properties, which are restricted to the infrequent LSC that are able to maintain the leukemia.

Analogous to normal hematopoiesis, both *in vitro and* in vivo experimental approaches using FACS-purified subpopulations of leukemia cells were utilized to identify human AML LSC. Culture of AML subpopulations on bone marrow feeder cells identified long-term culture-initiating activity in the Lin-CD34+CD38- leukemia fraction of most samples assayed (Ailles et al. 1997). Dick and colleagues were the first to establish xenotransplantation models for human AML, and used this assay to identify long-term engrafting AML subpopulations, and proposed they were early HSC or progenitor stage cells (Lapidot et al. 1994; Levis et al. 2005). In published reports assaying a variety of clinical and biological subtypes of AML, LSC were found to be negative for expression of Lin-CD34+CD38- (Bonnet and Dick 1997; Levis et al. 2005;

J.C.Y. Wang and Dick 2005). In normal human hematopoietic development, this Lin-CD34+CD38- fraction is known to be enriched for hematopoietic stem cells (HSC), leading to a proposal that AML LSC arise from mutational transformation of HSC (Bonnet and Dick 1997; J.C.Y. Wang and Dick 2005). However, human HSC have been shown to be positive for expression of CD90, while AML LSC were found to lack expression of this marker (Blair et al. 1997). Miyamato and colleages showed that the HSC population, Lin-CD34+CD38loCD90+, had preleukemic cells with the leukemogenic translocation, and that cells in the multipotent progenitor (MPP) stage, Lin-CD34+CD38loCD90-, carrying the same translocation were the actual LSC at the MPP stage (Blair et al. 1997; Miyamoto et al. 2000; Majeti et al. 2007).

Differential Expression of HSC as compared to AML LSC

Cell surface molecules expressed on LSC are not only important for their isolation by FACS, but are also potential targets for therapeutic monoclonal antibodies. Others had investigated the expression of cell surface markers on LSC in comparison to HSC, however both CD123 and CD33 are well expressed on HSC as well (Jordan et al. 2000; Taussig et al. 2005). As a result, they were poor targets for therapeutic antibodies, evidence by the hematological toxicity observed in patients who are treated with CD33 targeting gemtuzumab ozogamicin (Mylotarg) (Larson et al. 2005). The Weissman lab FACS purified HSC and LSC based on

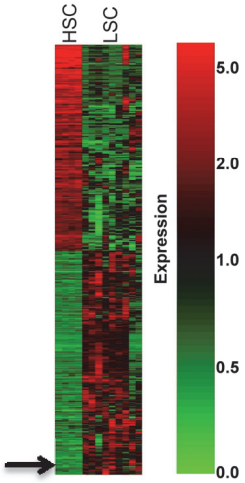

Figure 1.2. Gene Expression profiling of HSC versus AML LSC. As illustrated by the heat map, 4037 genes were differentially expressed in four HSC samples and nine LSC samples. The arrow gives the approximate location of CD47, the 62nd most over expressed gene on LSC relative to HSC (Majeti et al. 2009).

their cell surface profiles and ran the first genome-wide expression analysis of the two populations (Figure 1.2). Upon analysis of the gene expression data 4037 genes were differentially expressed, including 237 cell surface molecules (Majeti, Becker, et al. 2009). Among the cell surface molecules highly expressed on LSC but not HSC was the receptor CD47.

CD47 in Cancer

CD47 is a ubiquitously expressed transmembrane glycoprotein. It was originally identified in placenta and neutrophil granulocytes associated with $\alpha_b\beta_3$ integrins (Lindberg et al. 1993; Reinhold et al. 1995). Its ligands include integrins and thrombospondin, and has been implicated in both innate and adaptive immune responses, axonal development, and cell migration (Ticchioni et al. 1997; Oldenborg et al. 2000; Brown 2001; Miyashita et al. 2004; Grimbert et al. 2006; Tseng et al. 2013). Moreover, CD47 inhibits phagocytosis, providing a "don't eat me" signal through binding to its ligand signal regulatory protein alpha (SIRPα) (Figure 1.3A), which contains an ITIM motif able to recruit and activate the Src-homology 2 domain containing phosphatase (SHP)-1 and SHP-2 phosphatases, preventing myosin-IIA accumulation at the phagocytic synapse (Oldenborg et al. 2000; Miyashita et al. 2004; Tsai and Discher 2008). CD47 functions as a marker of self on normal cells, down regulating the surface protein when it is programed for cell removal, different than programed for cell death (Lagasse and Weissman 1994). We hypothesize that co-opting this mechanism is a key method by which cancers avoid immune clearance.

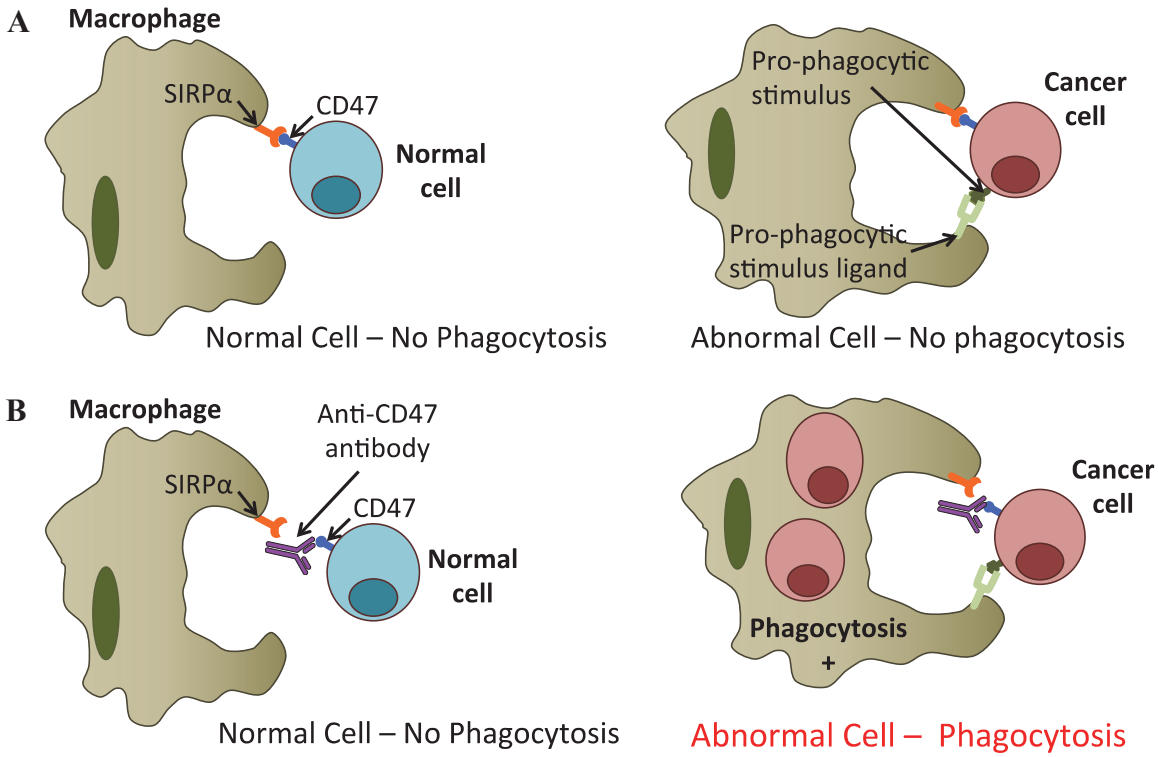

Figure 1.3. Macrophages phagocytose only abnormal cells with CD47 blockade. (A) Compared with normal cells (left), cancer cells (right) express increased levels of CD47 that contribute to pathogenesis by binding phagocyte SIRPα, which delivers a potent inhibitory "do not eat me" signal that predominates over a pro-phagocytic "eat me" stimulus. (B) A blocking monoclonal antibody directed against CD47 disrupts the dominant inhibitory SIRPα signal in phagocytes. This unmasks the pro-phagocytic stimulus on cancer cells resulting in their phagocytic elimination (right), but has no effect on normal cells, as they lack pro-phagocytic stimuli (left).

In the mid-80's, CD47 was identified as a cancer antigen in ovarian cancer (Poels et al. 1986). Subsequently, it has been identified on a multitude of cancer types including acute myloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, non-Hodgkin's lymphoma (NHL), glioblastoma, leiomyo sarcoma, bladder cancer, prostate cancer, breast cancer, and colon cancers among others (Jaiswal et al. 2009; Chao, Alizadeh, et al. 2010; Edris et al. 2012; Willingham et al. 2012). Moreover, the overexpression of CD47 is a poor prognostic marker in many liquid and solid tumors (Majeti, Chao, et al. 2009; Chao, Alizadeh, et al. 2011; Willingham et al. 2012).

The Weissman lab initially showed that blocking CD47 ability to interact with SIRPα, with an antibody, allowed for the phagocytes of AML *in vitro* as well as in vivo in primary patient xenograph models (Figure 1.3B), demonstrating the efficacy of anti-CD47 antibodies as a monotherapy (Majeti, Chao, et al. 2009). Further studies in hematopoietic malignancies, we demonstrate that an antibody against CD47 in combination with Rituximab, an anti-CD20 mAb, completely eradicated xenotransplanted NHL tumors in NOD SCID Gamma (NSG) immunodeficient mice (Chao, Alizadeh, et al. 2010). In addition to blockade of the "don't eat me" signal, the second antibody provided several potential complementary mechanisms of action including antibody dependent cellular phagocytosis (ADCP), complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (CDC) (Boross and Leusen 2012). The studies by Chao et al. were carried out in mice lacking full complement activity, and which had no NK cells, leaving ADCP as the functional mechanism in vivo. The combination therapy causes a complete remission in disease mice, which neither single agent could achieve (Chao, Alizadeh, et al. 2010). The lab then investigated if similar responses could be demonstrated in a host of solid tumor cancers as well. Using primary cell line xenotransplant models of ovarian, bladder, breast, head and neck, brain, and colorectal cancers (CRC), blocking mAbs are able to inhibit tumor growth as well as prevent the formation of metastases (Willingham et al. 2012). Critically unlike in the hematopoietic cancer setting, CD47 monoclonal antibodies (mAbs) have been unable to full eliminate disease in solid tumors as a monotherapy.

Epidermal Growth Factor Receptor Targeting Therapy

First identified by Cohen in 1962, Epidermal Growth Factor Receptor (EGFR) is a cell surface receptor tyrosine kinase in the ErbB family that is expressed at low levels on normal epithelial tissue and aberrantly on cancer cells (Cohen 1962). Overexpressing EGFR in tumors of the head and neck, breast, colorectal (CRC), non-small cell lung, glioma, and pancreas, among others, has been implicated in cancer proliferation (Yewale et al. 2013). Moreover, high expression of EGFR generally leads to a poorer prognosis (Mitsudomi and Yatabe 2010). EGF binding to EGFR leads to homodimerization or heterodimerization with other ErbB family members leading to receptor phosphorylation and activation of a multitude of downstream pathways including RAS, Janus kinase-signal transducer and activator of transcription (JAK-STAT), and Phosphoinositide 3-kinase (PI3K). Current EGFR therapies are primarily approved for the treatment of head and neck, lung, colorectal, and pancreas cancers. Two monoclonal antibodies, cetuximab (Erbitux) and panitumumab (Vectibix), are approved in the treatment of lung, colorectal, and head and neck cancers in patients that are either wild type for EGFR at the antibody binding site or wild type to downstream *KRAS* mutations in codons 12 and 13 (Figure 1.4), thereby inhibiting intracellular signaling pathways (Chong and

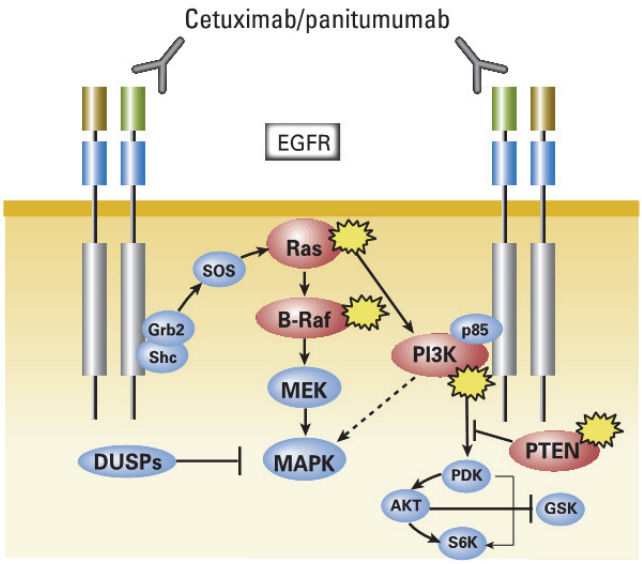

Figure 1.4. A representation of EGFR targeting antibodies and the downstream intracellular pathways. The red ovals correspond to location of known mutations in the pathway. (Normanno et al. 2006)

Jänne 2013). Additionally, four approved tyrosine kinase inhibitors (TKIs) acting upon the intracellular domain are used in patients as well. Especially with TKIs, patients that respond to EGFR targeting agents typically see a massive reduction in tumor burden, but eventually the subset of tumor cells that were resistant to the TKIs emerged, and the patients relapse rapidly and become refectory to additional therapy options (Yewale et al. 2013). Moreover, combination therapies with TKIs have been disappointing as well because of severe dose limiting toxicities (Wakelee et al. 2010).

Conclusion

Anti-CD47 therapy's efficacy requires both a blockade of the inhibitory CD47:SIRPα signaling as well as a second pro-phagocytic signal. This in turn enables antigen presenting cells (APCs) to engulf the cancer cells. Moreover, this mechanism can be enhanced with cancer targeting antibodies providing extra pro-phagocytic signaling in hematological malignancies. We investigate whether cancer targeting antibodies could have a similar effect in solid tumor malignancies, whereby a cancer targeting monoclonal antibody provides pro-phagocytic Fc which engages the Fc receptor on APCs, enhancing the effect of CD47 blockade.

Attribution

In the work previously published by McCracken et al, MNM and ACC contributed to the organization and communication of the review. MNM, ACC, and ILW contributed to the conceptual and intellectual formulation of the review.

CHAPTER TWO

The Treatment of Colorectal Cancer and Pancreatic Ductal Adenocarcinoma with Anti-CD47 Monoclonal Antibody Hu5F9-G4 and Anti-EGFR Antibodies

Abstract

CD47 is a "don't eat me" signal over expressed on cancer cells inhibiting their engulfment by phagocytes. A novel monoclonal antibody, Hu5F9-G4, blocking CD47's interaction with its ligand SIRPα allowed for phagocytosis of colorectal and pancreas tumors by macrophages. In some models, treatment of xenotransplanted tumors in immunodeficient mice with anti-CD47 antibodies strongly reduced tumor growth. In other models, the combination of anti-CD47 and anti-EGFR antibodies regressed tumor burden, where neither single agent therapy was as effective. Even in *KRAS* mutated tumors, where use of anti-EGFR antibodies is not indicated, the combination of antibodies lead to major tumor reduction and the inhibition of metastasis. Taken collectively, Hu5F9-G4 is an effective treatment in colorectal and pancreas cancers, and in the combination of Hu5F9-G4 with an anti-EGFR antibody is therapeutically effective in *KRAS* mutated tumors in mouse xenografts.

Introduction

Colorectal cancer is the third most common cancer in both men and women with incident rates that are declining significantly, -3.6%. Increased screen, primarily colonoscopies, has lead to removal of precancerous lesions as well as, early diagnose of localized or regional staged disease. This in turn has lead to increased 5-year survival for

patients with the diseases, +2.8% (Siegel et al. 2015). Surgery is the primary curative treatment in non-metastatic CRC, while chemotherapy has some effect as well. Most targeted therapies are against EGFR, expressed in 80% of CRC, and its downstream signaling molecules (Kuipers et al. 2015 Nov 5). Notably, both cetuximab and panitumumab are indicated for CRC patients, where the tumors are wild-type (WT) for mutations in codon 12 or 13 of *KRAS* (Normanno et al. 2006).

Pancreatic ductal adenocarcinoma (PDAC) is one of the least common types of cancer account for only 0.5% of all cancer incidents (Rahib et al. 2014). However, it has one of the most dismal 5-year survival rates, at 7%, making PDAC the fourth leading cause of cancer mortality (Siegel et al. 2015). Treatment options are also bleak, with surgical resection being the primary therapy. Moreover in the 10-20% of patients with respectable pancreatic cancer, the 5-year survival is only 20% (Raimondi et al. 2009). Other treatment options do not provide more than a couple months increase in median overall survival (Garrido-Laguna and Hidalgo 2015). Targeted therapies like antibodies are ineffective given that nearly 100% of PDAC tumors are positive for prognostically poor *KRAS* mutations (Bardeesy and DePinho 2002).

Current therapies in colorectal and pancreatic cancers are marginally effective at best. EGFR targeting therapies are only indicated for a subset of patients, however in combination with anti-CD47 the efficacy of treatment could be more broadly applied. Here we attempt to elucidate if combinational antibody therapy could overcome resistance to current single agent therapeutic options in both colorectal and pancreatic cancers.

Methods and Materials

Tumor Cell Lines and Luciferase-GFP$^+$ Lines

Pa01C, Pa02C, and Pa03C were a kind gift from the lab of Anirban Maitra at M.D. Anderson (Jones et al. 2008). Other lines were obtained from ATCC or DSMZ and cultured according to their protocols. GFP-luciferase+ lines were generated by transduction using a pCDH-CMV-MCS-EF1 puro HIV-based lentiviral vector (Systems Biosciences) engineered to express an eGFP-luciferase2 (pgl4) fusion protein (12). Stable lines were created by sorting for GFP expression on FACSAria II cell sorters (BD Biosciences). *KRAS* mutational status is listed in Table A.1.

Lentiviral Expression Vector for Luciferase-GFP

The vector was previously described in detail (Chao, Alizadeh, et al. 2010). In brief, the pCDH-CMV-MCS- EF1-puro HIV-based lentiviral vector (Systems Bioscience) construct contains a ubiquitin promoter driving the expression of a luciferase-eGFP fusion product. The luciferase gene is the Luc2 (pgl4) version (Promega). The eGFP portion derives from the pIRES2-eGFP plasmid (Becton Dickinson). Lentiviral production and concentration was accomplished using standard protocols.

Monoclonal Antibodies for In Vitro and In Vivo

Cetuximab (Erbitux) and Panitumumab (Vectibix) were obtained from the Stanford Health Care Pharmacy. Hu5F9-G4 was purified using standard IgG purification with procedures previously published (J. Liu et al. 2015). In vitro isotype control

antibodies were obtained from eBiosciences or BioXCell. Human IgG4 isotype was purchased from Eureka Therapeutics (Emeryville, Ca).

Cell Surface Expression Profiling

Cetuximab and Hu5F9-G4 were conjugated to Alexa Fluor 647 using standard conjugation methods according to manufacturer protocol (Life Technologies). Cells were incubated for 60 min on ice with 4 ug/ml of conjugated antibody, a known saturating dose, in FACS buffer (PBS with 2% FBS and 2 mM EDTA). Cell were washed and analyzed by flow cytometry.

Generation of Monocyte Derived Macrophages

For Human macrophages, Leukocyte Reduction System (LRS) chambers were obtained from the Stanford Blood Center from anonymous donors, and peripheral blood mononuclear cells were enriched by density gradient centrifugation over Ficoll-Paque Premium (GE Healthcare). Monocytes were purified on an autoMACS Pro Separator (Miltenyi) using anti-CD14 microbeads (Miltenyi) and differentiated to macrophages by culture for 7-10 days in RPMI+GlutaMax (Invitrogen) supplemented with 10% AB-Human Serum (Invitrogen) and 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen). NSG mouse macrophages were generated with bone marrow cells isolated NSG mice and differentiated in IMDM+GlutaMax supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, and 10 ng/mL murine M-CSF (Peprotech) for 7 days.

In Vitro Phagocytosis Assay

Phagocytosis assays were performed by co-culture of 50,000 macrophages with 100,000 GFP+ tumor cells for two hours in serum-free medium, then analyzed using an LSRFortessa or FACSCanto cell analyzer with high throughput sampler (BD Biosciences). Antibodies were used at a concentration of 10 ug/ml. Primary human macrophages were identified by flow cytometry using anti-CD14 or anti-CD206 antibodies. NSG macrophages were identified by anti-F4/80. All unlabeled target cells were labeled with 0.5 μM carboxyfluorescein succinimidyl ester (CFSE) according to manufatures protocols (Invitrogen). Dead cells were excluded from the analysis by staining with DAPI. Phagocytosis was evaluated as the percentage of GFP+ macrophages using FlowJo (Tree Star) and was normalized to the maximal response in a given experiment. All conditions were performed in three technical replicates and error bars show standard deviations between the replicates.

In Vivo Tumor Engraftment and Treatment

All tumor cells were suspending in growth media with 25% Matrigel Matrix, growth factor reduced (Corning) and engrafted subcutaneously on the flanks of NSG mice (Jackson Laboratories). NSG mice were treated by intraperitoneal (IP) injections with the following doses unless otherwise noted: PBS (100ul), Hu5F9-G4 (250 μg), cetuximab (500ug), panitumumab (500ug) for every other day treatments. For the weekly treatments of cetuximab and panitumumab 120ug was given IP.

Bioluminescent Imaging

Imaging was performed on an IVIS Spectrum (Caliper Life Science) and quantified using Living Image 4.0 or 5.0 software. D-Luciferin (firefly) potassium salt (Biosynth) solution was prepared by dissolving 1 g in 60 mLs PBS. Mice were injected intraperitoneally with luciferin solution (0.139 g luciferin per kilogram body weight) and imaged until peak radiance was achieved. Total flux (photons per second) values were obtained from the anatomic region of interest.

Results

Expression Levels of CD47 and EGFR in CRC and PDAC Cell Lines

By flow cytometery, we analyzed the surface expression levels of CD47 and EGFR using the same antibody clones as in downstream in vitro and in vivo assays. All CRC cell lines screened expressed CD47, though at varying levels (Figure 2.1). We also independently stained all cell lines for EGFR expression and found varying levels of cell surface expression. Notably, SW620 was negative for EGFR expression.

In Vitro phagocytosis of CRC by Mouse and Human Macrophages

We tested the ability of NSG bone marrow derived macrophages to phagocytose CRC cell lines. Each tumor line was incubated in the presence of human IgG4 isotype antibody, Hu5F9-G4, Cetuximab, or the combination of Hu5F9-G4 and Cetuximab. Isotype antibodies did not cause much phagocytosis. In all but HCT116, Hu5F9-G4 was able to induce phagocytosis of the CRC cells. In a few cases, SW480, SW48, and HT29, Cetuximab alone was able to induce phagocytosis by mouse macrophages as well. A noticeable increase in phagocytosis was seen in SW48 and HT29 in the context of both

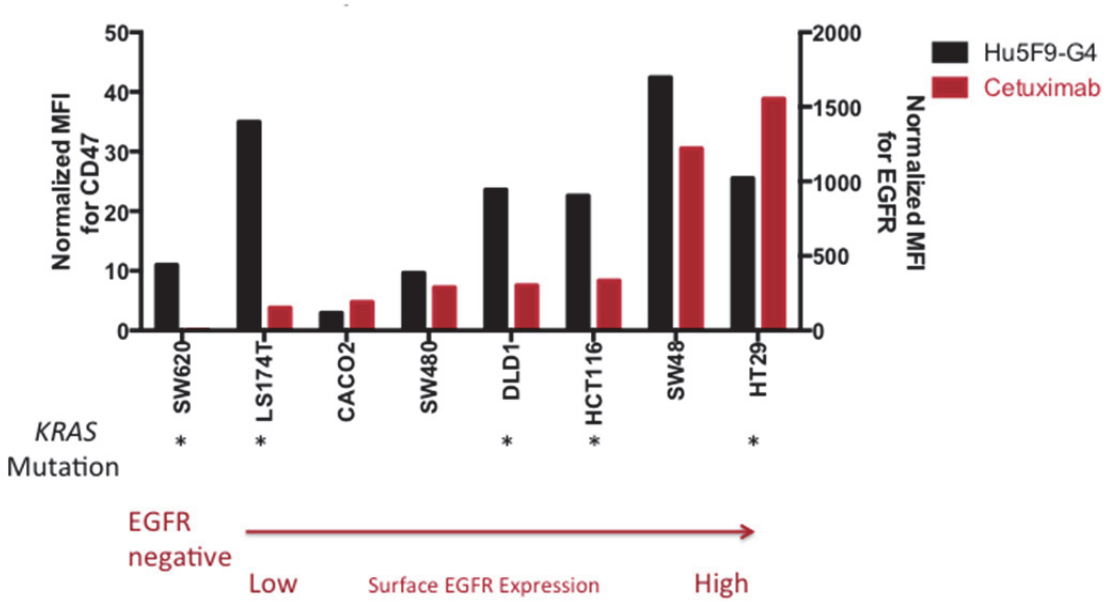

Figure 2.1. Cell Surface Expression of CD47 and EGFR on CRC lines as analyzed by FACS. Mean fluorescence intensity (MFI) was normalized to unstained cell line controls. The left Y-axis corresponds to relative CD47 expression levels, as measured by Hu5F9-G4 (black), while the right y-axis corresponds to EGFR expression levels, as measured by Cetuximab (red). A mutation in *KRAS* is indicated by the asterisk under a given cancer line. SW620 is negative for EGFR expression while, the other lines are ordered by ascending EGFR expression levels.

antibodies over single antibody conditions. Surprisingly, HCT116 could not be phagocytosed under any condition (Figure 2.2).

Next, we repeated the in vitro phagocytosis assays with human peripheral blood monocyte derived macrophages. Similar results were seen as compared to the in vitro phagocytosis assay performed with NSG macrophages. Isotype conditions did not cause much phagocytosis. Moreover, Hu5F9-G4 induced strong phagocytosis in the majority of CRC lines assayed. CACO-2 and HT29 also showed significant phagocytosis by cetuximab alone. With CACO-2 and DLD-1, significant phagocytosis was seen in the combination of both the anti-CD47 antibody and anti-EGFR antibody as compared to single antibody conditions. Moreover, Hu5F9-G4 induced strong phagocytosis in the majority of CRC lines assayed (Figure 2.3).

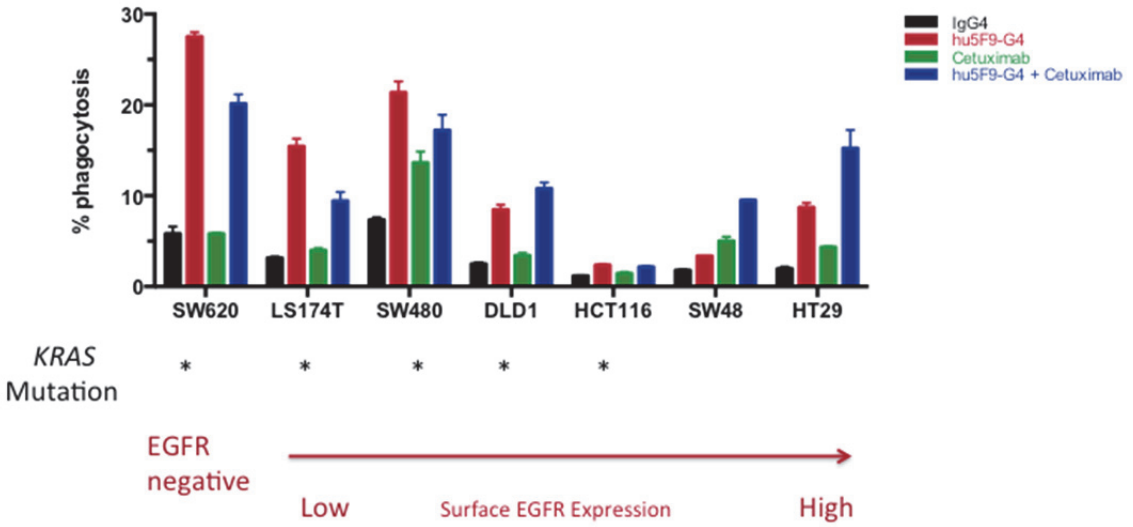

Figure 2.2. In vitro phagocytosis of CRC cell lines by NSG macrophages. Hu5F9-G4 blockade of CD47 enables phagocytosis and in some cases phagocytosis is enhanced with the addition of Cetuximab. CFSE labeled cell lines were co-incubated with NSG macrophages in the context of IgG4 isotype antibodies, Hu5F9-G4, Cetuximab or the combination of Hu5F9-G4 and Cetuximab. A mutation in *KRAS* is indicated by the asterisk under a given cancer line as reference, as well as the progressing levels of EGFR expression.

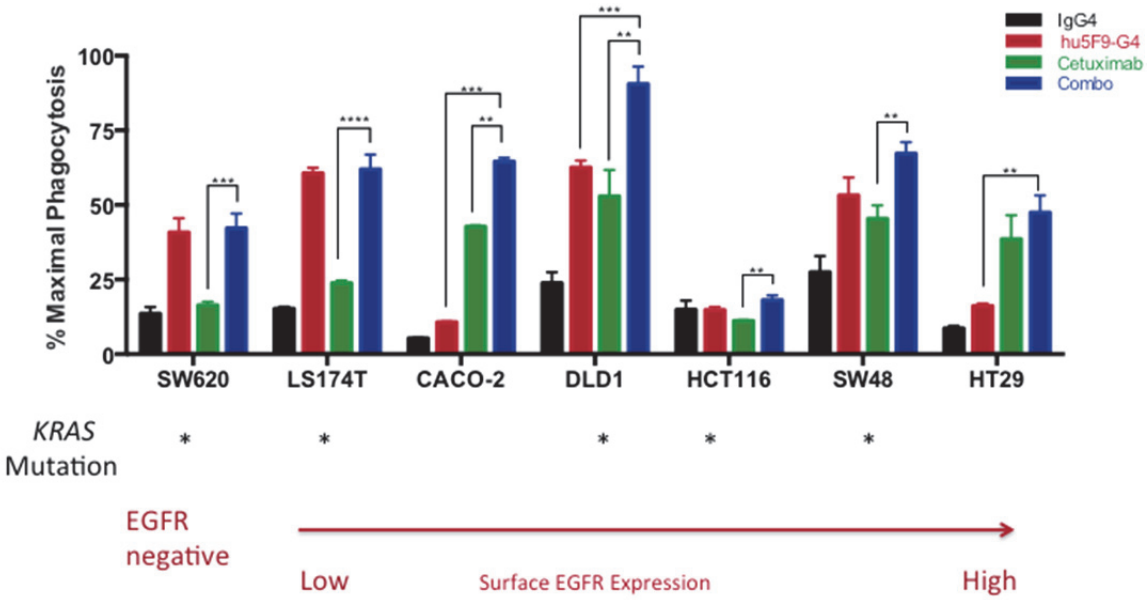

Figure 2.3. In vitro phagocytosis of CRC lines by human macrophages. Cell lines were labeled with CFSE and incubated with IgG4 isotype antibodies, anti-CD47 antibodies, anti-EGFR antibodies or the combination of anti-CD47 and anti-EGFR antibodies. A mutation in *KRAS* is indicated by the asterisk under a given cancer line as reference, as well as the progressing levels of EGFR expression.

Expression Levels of CD47 and EGFR in PDAC Cell Lines

The surface expression of CD47 and EGFR was detected by flow cytometry in PDAC cell lines using Hu5F9-G4 and cetuximab, respectively. CD47 and EGFR was detected ubiquitously, though at varying levels of expression. Also, all but BxPC3 have a mutation in *KRAS* (Figure 2.4).

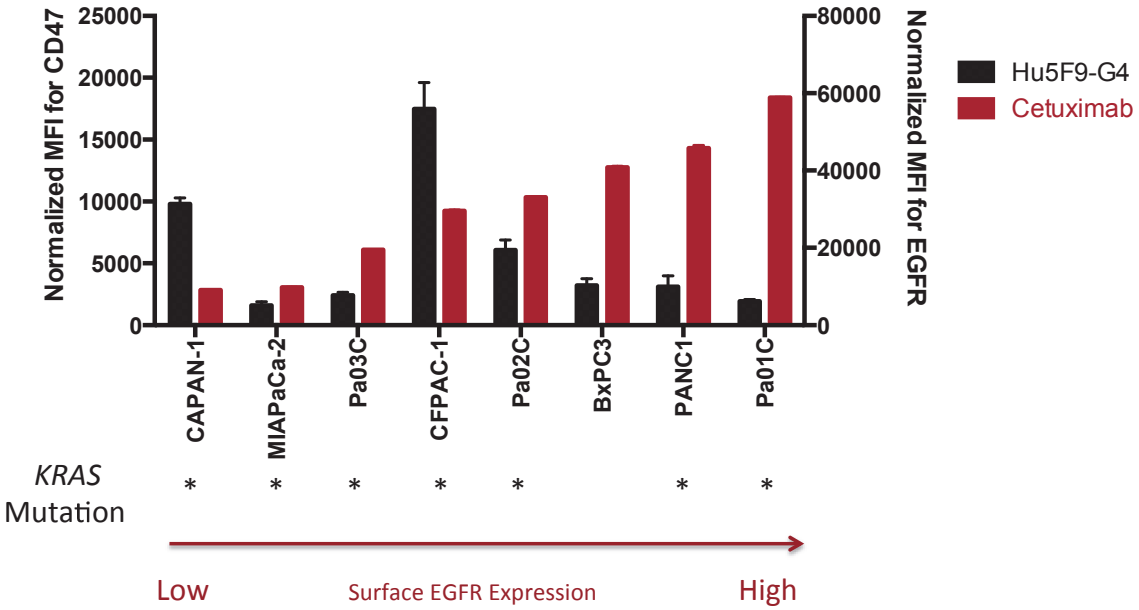

Figure 2.4. Cell Surface Expression of CD47 and EGFR on PDAC lines as analyzed by FACS. MFI was normalized to unstained cell line controls. The left Y-axis corresponds to relative CD47 expression levels, as measured by Hu5F9-G4 (black), while the right y-axis corresponds to EGFR expression levels, as measured by Cetuximab (red). A mutation in *KRAS* is indicated by the asterisk under a given cancer line. All cell lines stain for CD47 and EGFR, at varying expression levels.

In Vitro Phagocytosis of PDAC by Mouse and Human Macrophages

We wanted to test if blockade of CD47 by Hu5F9-G4 induced phagocytosis by mouse macrophages. Indeed Hu5F9-G4 enabled phagocytosis in all PDAC tumors except Pa03C. Cetuximab induced a greater amount of phagocytosis as compared to panitumumab, even though the both target EGFR. In the case of Pa01C and PANC-1,

combination antibody conditions caused greater phagocytosis than single antibody conditions (Figure 2.5).

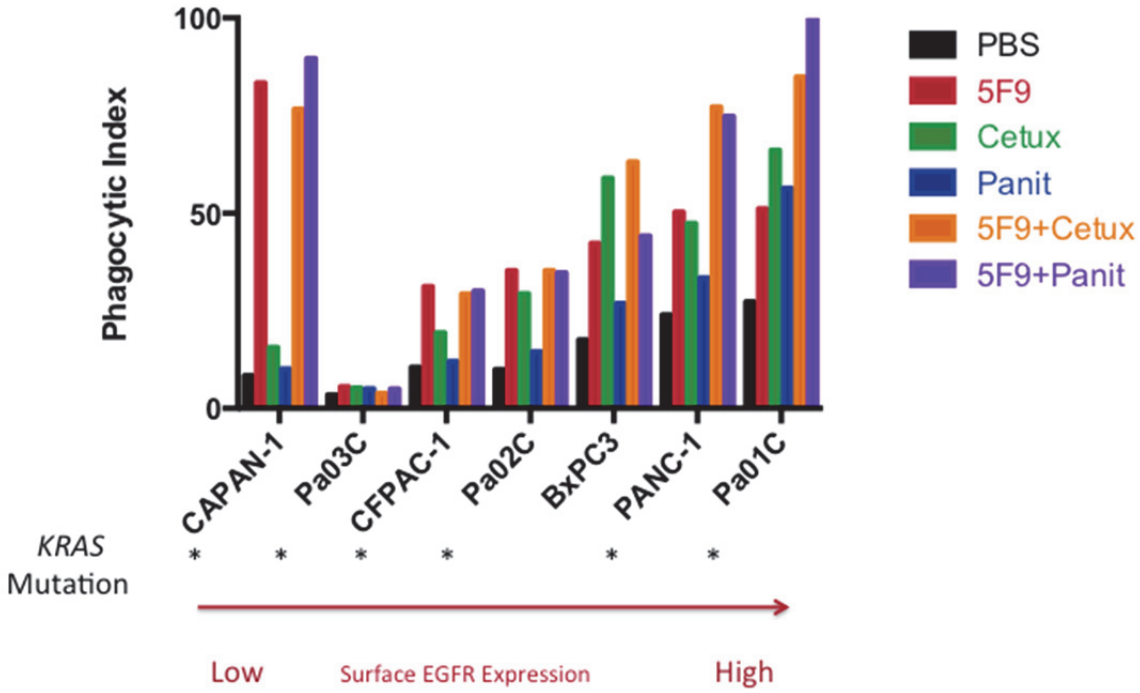

Figure 2.5. In vitro phagocytosis of PDAC cell lines by NSG derived macrophages. NSG bone marrow derived macrophages were co-cultured with CFSE labeled PDAC lines in the context of PBS, Hu5F9-G4, Cetuximab, Panitumumab, or the combination of Hu5F9-G4 with an anti-EGFR antibody. A mutation in *KRAS* is indicated by the asterisk under a given cancer line as reference, as well as the progressing levels of EGFR expression.

We also wanted to know if blockade by Hu5F9-G4 on PDAC lines would induce phagocytosis by human macrophages. In most cases, CD47 blockade enabled phagocytosis, except in the case of Pa03C. Cetuximab or pantiumumab alone also enabled phagocytosis of most of the cell lines at times to a greater degree than Hu5F9-G4. In a few cases like Pa02C, PANC-1, and Pa01C the combination of two antibodies synergized and gave increased phagocytosis of tumor cells greater than their single agent counterparts (Figure 2.6).

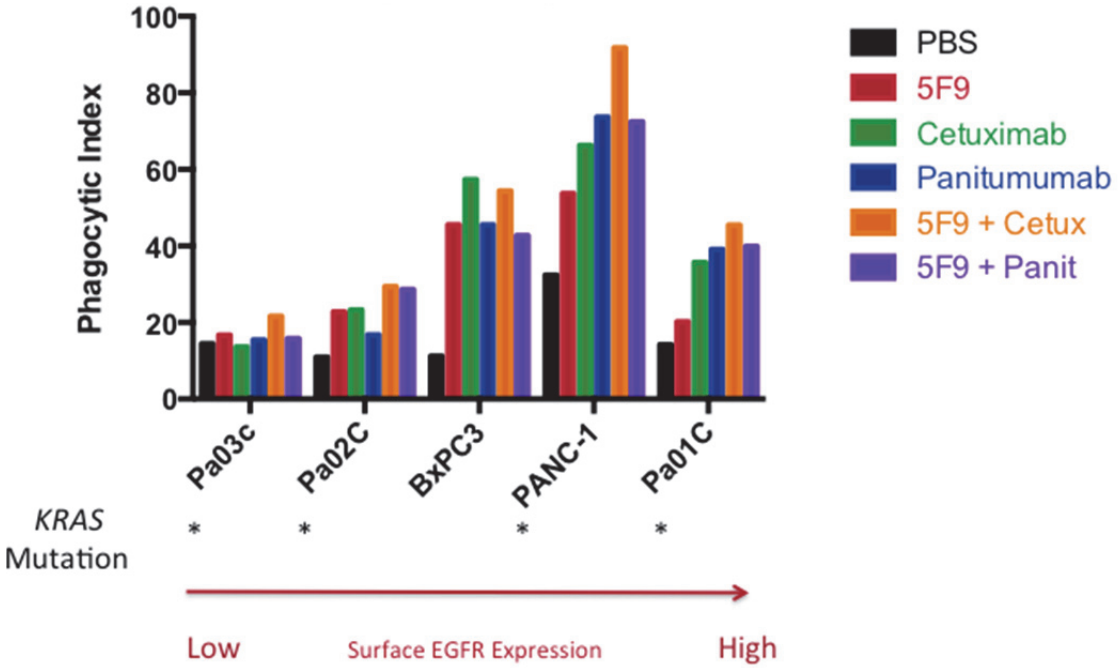

Figure 2.6. In vitro phagocytosis of PDAC lines by human peripheral blood derived macrophages. Cell lines were co-incubated with macrophages in with PBS, Hu5F9-G4, Cetuximab, Panitumumab, or the combination of Hu5F9-G4 with an anti-EGFR antibody. A mutation in *KRAS* is indicated by the asterisk under a given cancer line as reference, as well as the progressing levels of EGFR expression. Most lines responded to Hu5F9-G4 and Cetuximab. Only Pa02C, PANC-1, and Pa01C showed increased phagocytosis with the combination of antibodies.

Hu5F9-G4 as a Monotherapy and in Combination with an Anti-EGFR Antibody is Effective in PDAC in Xenotransplant Models

To better recapitulate disease pathology, we engrafted multiple PDAC tumor lines into mice. The tumor cells were injected subcutaniously onto the flanks of NSG mice, which lack T, B, and NK cells but maintain phagocytic cells (Ishikawa et al. 2005). All tumors were infected with a lentivirus containing a luciferase-green flourcent protein (GFP) fusion protein and the tumor growth was measure via bioluminicent imaging. The mice were given PBS, Hu5F9-G4, cetuximab, panitumumab, Hu5F9-G4 and cetuximab, or Hu5F0-G4 and panitumumab every other day for the duration of treatment.

In the model of PDAC wild-type for a *KRAS* mutation, we wanted to know if the combination of Hu5F9-G4 would have an increaesd effect on tumor regression. We engrafted 50, 000 BxPC3 cells in NSG mice and initiated treatment after one week (Figure 2.7B). Anti-CD47 antibody slowed tumor growth but did not increase survival noticably. As expectied with a *KRAS* WT tumor, BxPC3 responded well to both cetuximab and panitumumab treatment. The combination treatment also significantly reduced tumors but only marginally incrased survival over single agent anti-EGFR antibodies (Figure 2.7A).

In CAPANC-1 engrafted mice, Hu5F9-G4 alone dramatically reduces tumor burden as measured by luciferase imaging. A week after engrafting 20,000 cells, we initiated the combination treatment and monitored tumor growth for 20 weeks (Figure 12B). The mice treated with cetuximab slows the growth of the tumor but

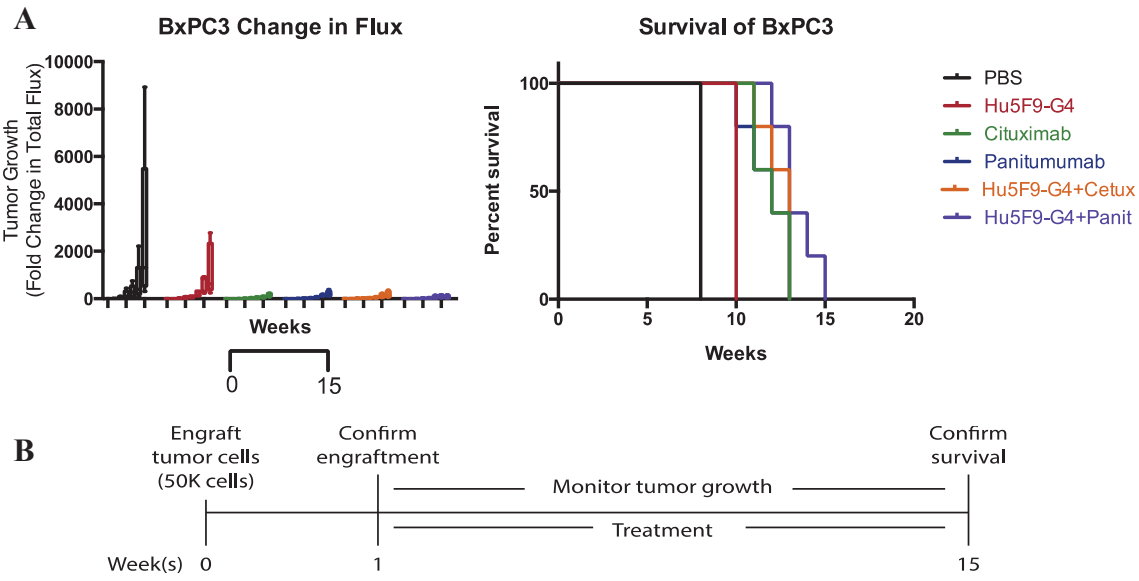

Figure 2.7. Combination treatment of BxPC3, in a *KRAS* wild-type PDAC tumor. (A) Tumor growth as measured by fold change over initiation of treatment at week 1 (left). Hu5F9-G4 was able to modestly slow tumor growth. Cetuximab and panitumumab were able to slow tumor growth significantly over PBS control treated mice. The combination of Hu5F9-G4 with an anti-EGFR antibody has a similar effect as the EGFR monotherapies, with marginally increased survival as seen by the Kaplan-Meier survival plot (right). (B) A timeline indicating 50,000 cells were engrafted as treatment was started one week later until all the mice reached maximum tumor burden at 15 weeks.

panitumumab does not, as compared to PBS control treated mice. Both combination therapies show similar decreases in tumor burdon, as seen in the anti-CD47 monotherapy (Figure 2.8A).

Pa02C engrafted mice, cetuximab, panitumumab, and Hu5F9-G4 with cetuximab treatment seem to have little effect as compared to PBS control mice. However, Hu5F9-G4 with panitumumab mice exhibit reduction in tumor load as measured by luciferase imaging (Figure 2.9A). In Pa03C engrafted mice, show no response to anti-CD47 antibodies. However, cetuximab, panitumumab, and Hu5F9-G4 with cetuximab slow tumor growth. We also observe that Hu5F9-G4 in combination with panutumumab shrinks the Pa03C to some extent as well (Figure 2.9C).

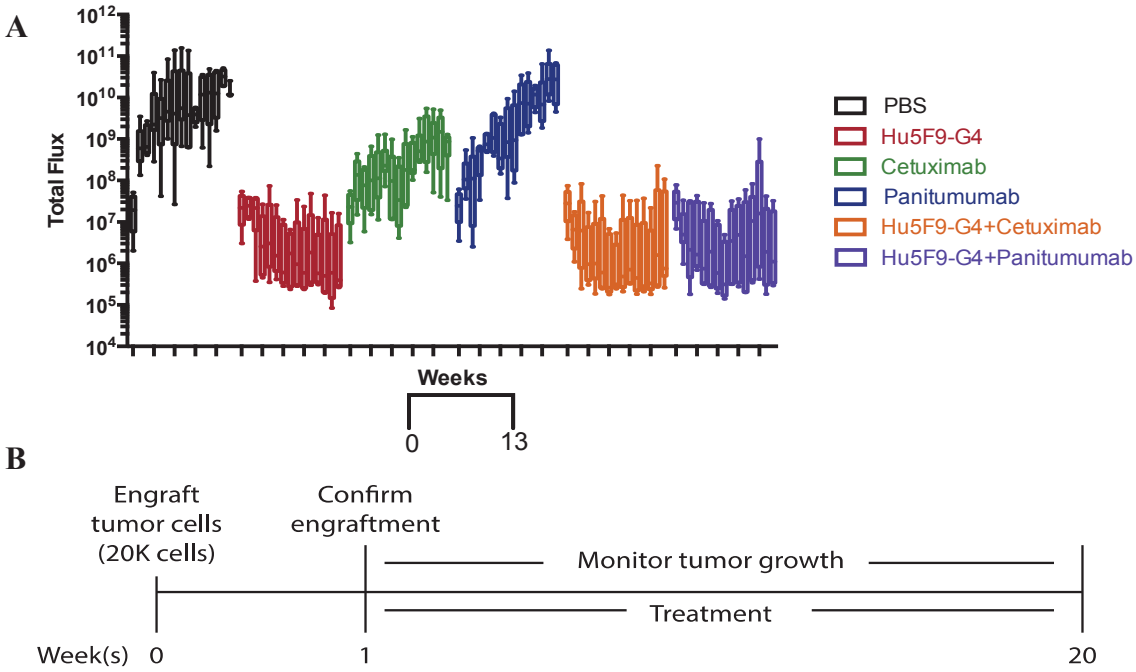

Figure 2.8. Combination treatment of CAPANC-1 with Hu5F9-G4 and anti-EGFR antibodies in xenotransplanted NSG mice. (A) Luciferase expressing CAPANC-1 cells are engrafted in NSG mice. After one week of engraftment, treatment was initated with PBS, Hu5F9-G4, Cetuximab, Panitumumab, Hu5F9-G4 and Cetuximab, or Hu5F9-G4 and Panitumumab. CAPAN-1 reduces tumor with anti-CD47 therapy. Total flux was graphed and is a surrogate for tumor growth. (B) A timeline indicating 50,000 cells were engrafted at treatment started one week later. The study was ended after 20 weeks.

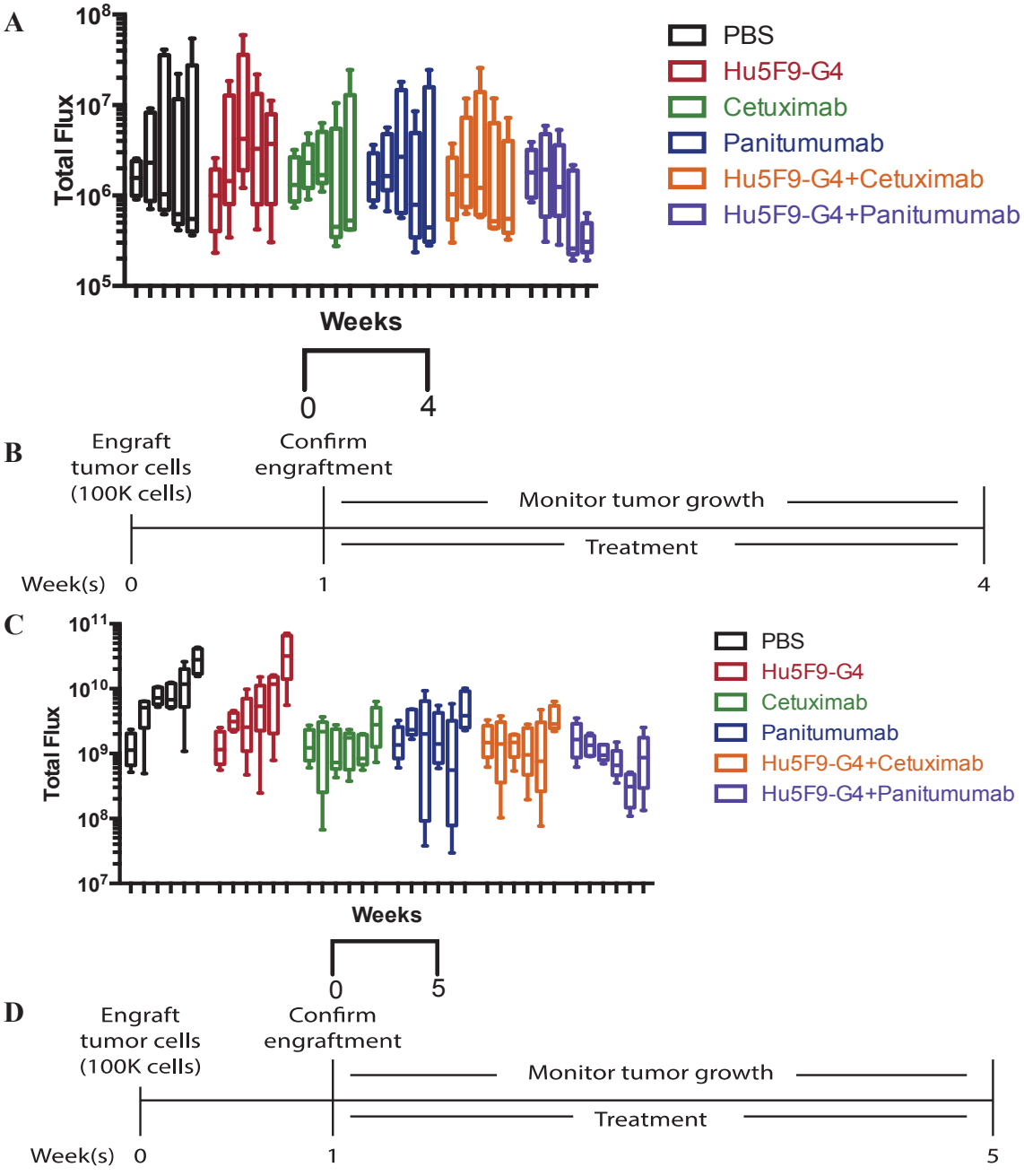

Figure 2.9. Combination therapy with Hu5F9-G4 and anti-EGFR antibodies in xenotransplanted NSG mice with primary liver metastasis of PDAC. (A) and (C) Total flux was graphed and is a surrogate for tumor growth. (B) and (D) After one week of engraftment, treatment was imitated with PBS, Hu5F9-G4, cetuximab, panitumumab, Hu5F9-G4 and cetuximab, or Hu5F9-G4 and panitumumab for 4 (B) or 5 (D) weeks. (A) Pa02C only responded to the combination of Hu5F9-G4 and panitumumab efficaciously compared to PBS controls. (C) Pa03C also shows little response to Hu5F9-G4, but does have some response to cetuximab, panitumumab, and Hu5F9-G4 and cetuximab. The Hu5F9-G4 and panitumumab cohort shows the greatest reduction in tumor load.

The Combination of Anti-CD47 Antibodies and Anti-EGFR Antibodies Have a Synergistic Effect on a Xenotransplated Model of CRC

In a xenotransplanted CRC model, we treated UM8 engrafted mice with Hu5F9-G4 and anti-EGFR antibodies cetuximab and panitumumab either alone as monotherapies or in combination. UM8 is a primary CRC tumor negative for *KRAS* mutations. The UM8 tumor bearing mice were resistant to Hu5F9-G4 treatment alone. However cetuximab, as a single agent, was able to inhibit tumor growth. Moreover, monotherapy panitumumab treatment slowly reduced tumor growth. Dramatically, Hu5F9-G4 in combination with either EGFR targeting antibody considerably reduces tumor burden in NSG mice (Figure 2.10).

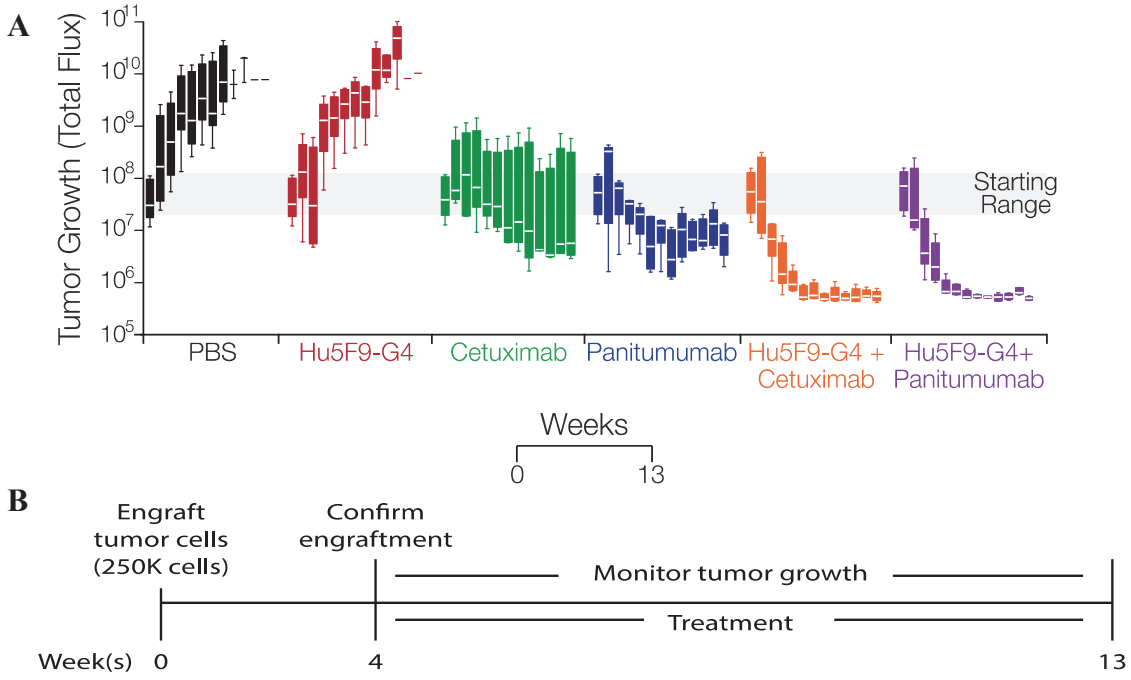

Figure 2.10. Combination therapy with Hu5F9-G4 and anti-EGFR antibodies in xenotransplanted NSG mice with primary CRC UM8. (A) Hu5F9-G4 therapy had no reduction in tumor growth as compared to PBS controls. Cetuximab treatment slightly reduced tumor burden and Panitumumab treatment further decreased tumors relative to Cetuximab. The combination treatments reduced tumors to the lower threshold of detection by bioluminescent imaging. (B) UM8 cells transduced with a luciferase-GFP gene were subcutaneously injected into the backs of NSG mice. Four weeks after tumor cell engraftment, PBS, Hu5F9-G4, cetuximab, panitumumab, Hu5F9-G4 and cetuximab, or Hu5F9-G4 and Panitumumab were administered. PBS and Hu5F9-G4 was administered every other day, while cetuximab and panitumumab were administered once a week for thirteen weeks. Tumor growth was measured by bioluminescence imaging.

Hu5F9-G4 Inhibits metastasis of Tumor in Both PDAC and CRC Xenotransplantation Models

In a PDAC xenotransplant model, we treated NSG mice engrafted with 50,000 Panc1 cells engrafted for 30 weeks and treatment was every other day in all cohorts (Figure 2.11B). Hu5F9-G4 was able to dramatically slow tumor growth as compared to PBS control mice. Cetuximab is also able to slow the tumor growth in this *KRAS* mutated tumor, but less so than Hu5F9-G4. Panitumumab has no effect on the tumor growth, while the combination groups showed a similar trend as the Hu5F9-G4 single agent cohort (Figure 2.11A, left). The three cohorts with Hu5F9-G4 treatment also survived much longer, some until the end of study, relative to PBS controls and anti-EGFR single agent groups (Figure 2.11A, right). In a separate experiment treated Panc1 mice with Hu5F9-G4 or PBS controls for 10 weeks with the intent of looking for metastasis. Under a dissecting scope, we examined the mice for GFP positive tumor cells

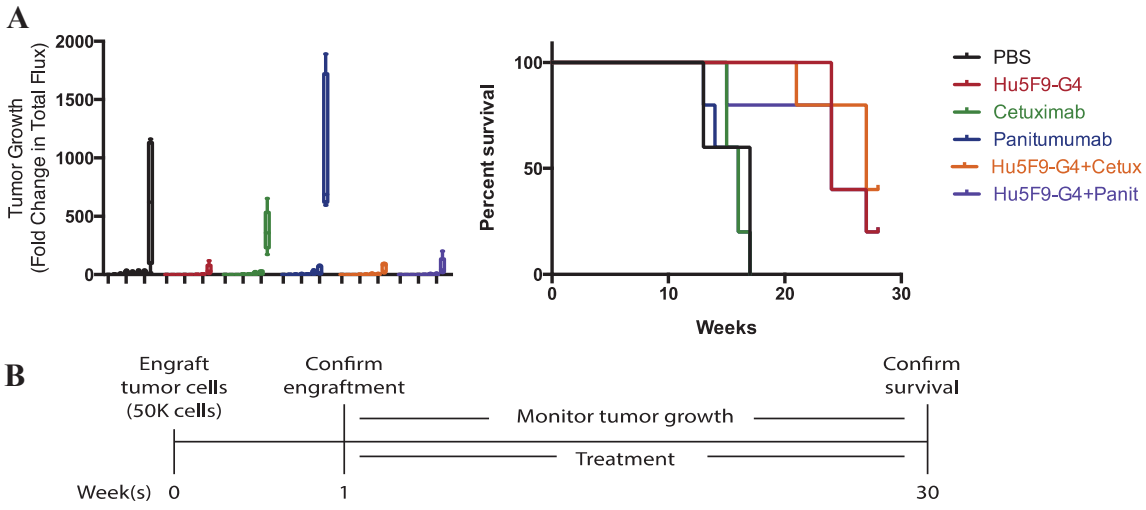

Figure 2.11. Combination treatment of Panc1 in NSG mice. (A) Tumor growth as measured by fold change over initiation of treatment at week 1 (left). Hu5F9-G4 was able to significantly slow tumor growth as compared to PBS control mice. Cetuximab was able to slow tumor growth at a lower rate than the Hu5F9-G4 cohort, while panitumumab had little effect. The combination of Hu5F9-G4 with an anti-EGFR antibody has a similar effect as Hu5F9-G4 monotherapies. Hu5F9-G4 and combination therapies increased survival as seen by the Kaplan-Meier survival plot (right). (B) A timeline indicating 50,000 cells were engrafted as treatment was started one week later, until week 30.

in major tissue groups, including liver, heart, lung, pancreas, stomach, kidney, axilla lymph node, and head . Astoundingly, we saw no metastasis in the anti-CD47 treated group where as in the PBS mice, there was massive tumor invasions in all tissues examined (Figure 2.12).

In a CRC xenotransplant model, we engrafted 50,000 DLD1 cells and treated for seven weeks (Figure 2.13B). The treatment schedule for the PBS and Hu5F9-G4 reagents were every other day, while cetuximab was only given once a week. Tumor growth as measured by bioluminescent imaging shows cetuximab had no effect on the *KRAS* mutated tumor. Hu5F9-G4 did slow tumor growth relative to PBS controls, while the combination of Hu5F9-G4 and cetuximab had the greatest effect on slowing tumor growth (Figure 2.13A). At seven weeks, the mice were all censored and examined for metastasis by fluorescent microscopy. Mice treated with Hu5F9-G4 had fewer lung and axial lymph node metastasis as compared to PBS controls (Figure 2.13C-D). Hu5F9-G4 treatment showed even fewer lymph node metastases and no lung metastasis. The combination group had the most dramatic effect, showing no metastases in either the lymph node or the lung.

Discussion

We show that CD47 is ubiquitously expressed on CRC and PDAC, while almost all the cancer lines screened also express EGFR. Hu5F9-G4 in vitro enables phagocytosis most CRC and PDAC as well, and in some cases synergies with an anti-EGFR antibody. Though investigated, no correlation was found between EGFR expression and/or CD47 expression predicting response to antibody blockade. Most likely “eat me” signals that have not been identified on solid tumors contribute to how the phagocytic effect beyond

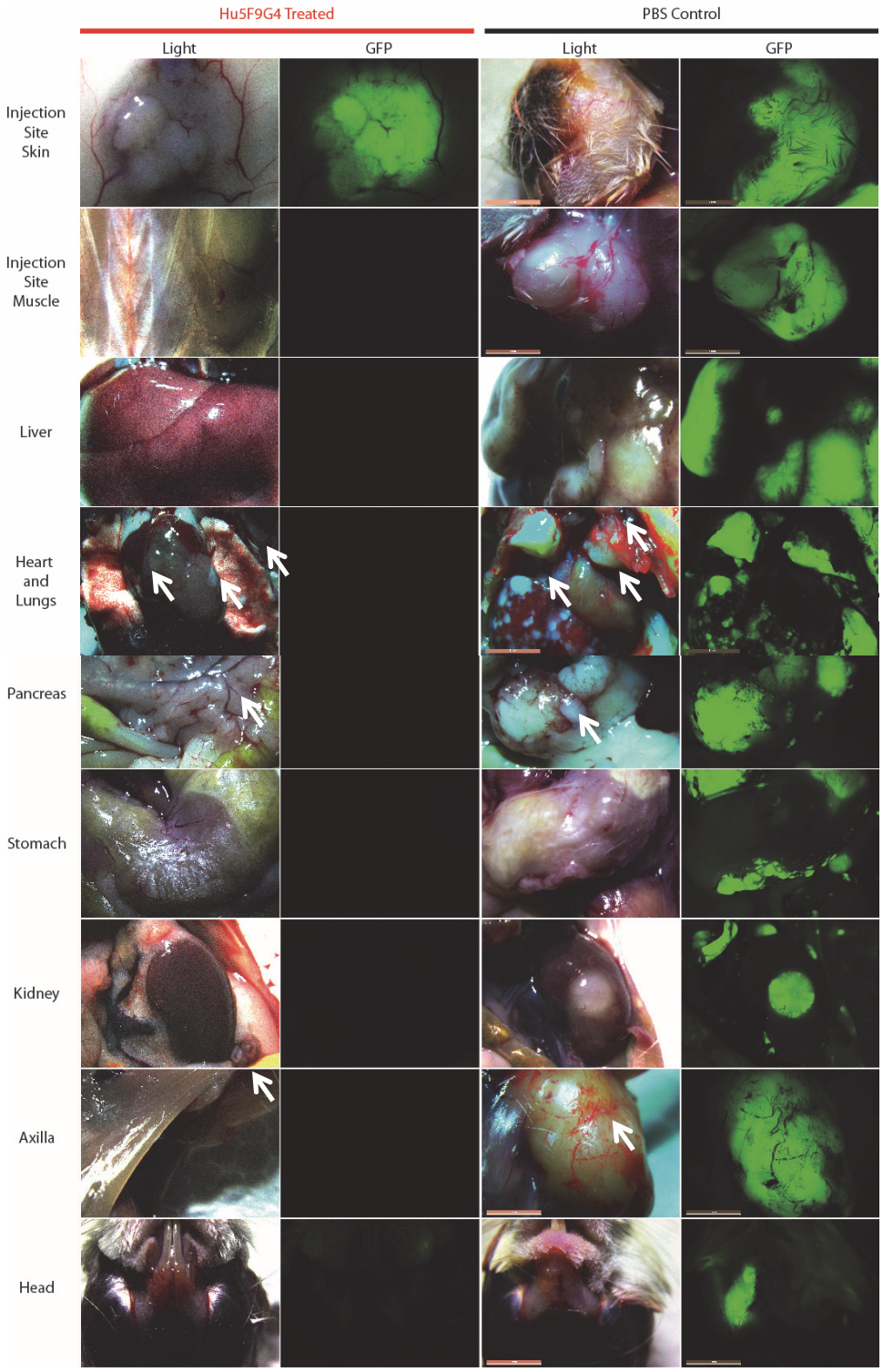

Figure 2.12. Fluorescence microscopy images of Panc1 mice treated with Hu5F9-G4. Images revealed an invasive primary tumor and diffuse metastases for untreated mice but only a small primary tumor and no evidence of metastatic disease in the censored Hu5F9-G4 treated mouse. White arrows are used to indicate organs of interest where organ structures are difficult to resolve.

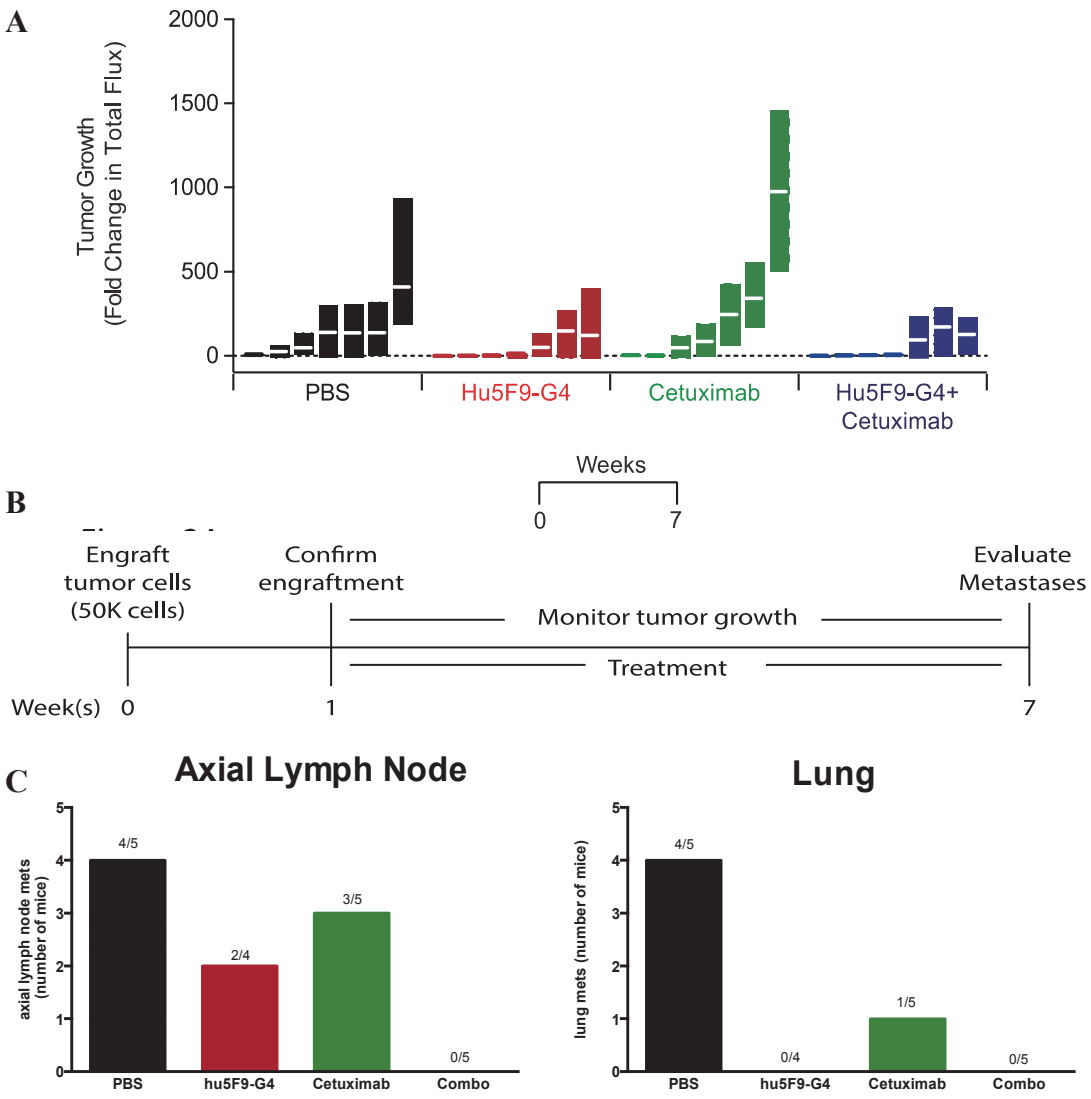

Figure 2.13. Combination therapy in the DLD1 CRC model. and prevents or eliminates metastasis. (A). Tumor growth as measured by fold change in total flux shows anti-EGFR and anti-CD47 antibodies in combination slows tumor growth the greatest among all cohorts. Hu5F9-G4 alone as a strong effect as well, while the cetuximab treatment ad no effect on tumor growth. (B) One week after tumor cell engraftment mice were treated with PBS, Hu5F9-G4, cetuximab, or Hu5F9-G4 with cetuximab, every other day (PBS, Hu5F9-G4) or weekly (cetuximab) for seven weeks. Tumor growth was measured by bioluminescence imaging. (C) Remarkably, mice treated with cetuximab had less lymph node and lung metastasis compared to PBS control cohort, but the therapeutic effect was less potent than with Hu5F9-G4. The strongest therapeutic effect had the combination Hu5F9-G4 with cetuximab. None of the mice in this cohort had any metastasis at the end of treatment.

signal blockade by anti-CD47 and FC receptor engagement with the addition of EGFR antibodies by macrophages. More studies need to be done to better elucidate pro-phagocytic signals. Furthermore, the addition of an anti-EGFR antibody can also

synergize with anti-CD47 antibodies to further enable programed cell removal. This enhanced effect is not a known mechanism of action for anti-EGFR therapy, where more conventionally anti-EGFR antibodies block the activation and downstream signaling of the EGF receptor.

In vivo treatment in some cases dramatically synergize with EGFR targeting mAbs, radically reducing tumor burden in xenotransplanted mouse models. In both CRC and PDAC models with KRAS mutations, sometimes an anti-EGFR antibody has an effect on tumor growth, but combination with CD47 always produced a better result. Signal blockade by anti-CD47 antibodies induce a strong antibody dependent cellular phagocytosis. While the two anti-EGFR antibodies do not always have the same effect, cetuximab and panitumumab both bind to EGFR but differ in their structure and isotype. Cetuximab is a chimeric (mouse/human) IgG1 while panitumumab is a fully human antibody with an IgG2 isotype. Given that cetuximab usually performed therapeutically better than panitumumab, perhaps the IgG1 played a role in activating the complement pathway and mediated antibody-dependent cellular cytotoxicity (ADCC) more strongly. We use a novel anti-CD47 monoclonal antibody that has not been reported on in a solid tumor setting. In some cases Hu5F9 works as monotherapy and in others cases, it works in synergy with Cetuximab or Panitumumab. Notably, this drastic tumor reduction is observed without the use of any cytotoxic agents as is standard of care in both CRC and PDAC.

For the first time in either CRC or PDAC, we show that Hu5F9-G4 is able to either prevent or eliminate metastasis in tumor bearing mice. The mechanisms by which this happens is unclear. From studies in the Weissman lab, we have observed that as HSC

are mobilized, they upregulate CD47 and egress the bone marrow into the circulatory blood system. Moreover our lab has observed that macrophages line the exterior of a tumor and phagocytosis their way into the tumor. Conceivably, by blocking CD47 on tumor cells, the tumor cells that do escape the primary site are unable to evade ADCP.

Conclusion

Monoclonal antibodies have been shown to be efficacious as a single agent and in combination for the treatment of cancer. In addition to in hematological malignancies, we show for the first time that an anti-CD47 therapy works in synergy with another antibody in a solid tumor setting. Combination therapy could also open new treatment options in all cases of EGFR+ CRC and PDAC, regardless of the patients KRAS mutational status. Moreover, in patients with marginally respectable tumors often surgery is not indicated, even though the treatment can be curative. Metastasis are too engrossed or too numerous to justify the high risk of surgery with the low reward of a cure. However, as an adjuvant or neoadjuvant treatment to surgery, Hu5F9-G4 could be administered remove tumors that surgery could not. Taken in entirety, Hu5F9-G4 could have a dramatic effect on the treatment of colorectal and pancreatic cancers.

CHAPTER THREE

Anti-CD47 Immunotherapy in Bridging the Innate and Adaptive Immune Systems

Portions of this chapter published as: McCracken MN, Cha AC, Weissman IL. 2015. Molecular Pathways: Activating T Cells after Cancer Cell Phagocytosis from Blockade of CD47 "Don't Eat Me" Signals. Clin Cancer Res 21:3597–3601.

Abstract

Cancer immunotherapy, including anti-CD47 blockade, tries to utilize the hosts own immune system to mount an immune response to cancer. To date, there have been a host of new therapies from cytokine treatments, to cell based vaccines, to antibody therapies which all recruit immune cells that have been somehow silenced by the tumor microenvironment. The Weissman lab's own studies into the adaptive immune system suggest that upon phagocytosis, mediated by anti-CD47 antibodies, the subsequent T cell presentation preferentially recruits cytotoxic T cells. Here, we propose two additional model systems to further investigate the role CD47 plays in antigen presentation and downstream T cell recruitment to target cancer cells.

Introduction

Background

Cancer immunotherapy tries to harness ones own immune system to help fight disease. Over the past two decades a multitude of immunotherapies have emerged which have show strong responses and long-lasting control over cancer. The earliest demonstration of immunotherapy is the allogeneic bone marrow transplants. This

treatment relies on the ability of allogeneic T cells to recognize minor antigen mismatch either from host self-antigens or host cancer antigens. This mismatch is the basis of graft-versus-tumor effects, which beneficially clears cancer, but also of graft-versus-host disease which leads to autoimmune disease (Thomas 1983; Negrin et al. 2000). More recent immunotherapies include cytokine therapy, DC based and T based cell therapy, and antibody-based therapy. IL-2 based cytokine therapy has been used to activate T cells in metastatic melanoma and renal cell carcinoma, leading to a significant response in 15% of patients (Rosenberg et al. 1987). DC vaccines against melanoma antigens in stage IV melanoma patients increased median survival to 20 months, where chemotherapy alone barely extends survival beyond 4 months (Fay et al. 2006). Sipuleucel-T was also recently FDA approved for use in prostate cancer using autologous peripheral blood mononuclear cells, assuming to contain APCs like DCs, increased overall survival by four months (Kantoff et al. 2010). Chimeric antigen receptor expressing T cells targeting the CD19 receptor of B cells in chronic lymphocytic leukemia patients produced durable remissions in a small number of patients (Porter et al. 2011). Ipilimumab targeting CTLA4 is able to release the brakes on anergic T cells and is the first in a host of new antibodies targeting T cell checkpoints (Sharma and Allison 2015a). It is also conceivable that T regulatory cells that are pro-tumorigenic might be depleted with ipiliumumab (W. Liu et al. 2006).

Tumors are able to evade immune recognition and removal through multiple processes including creating an immunosuppressive environment or direct tumor-immune cell interactions (Swann and Smyth 2007; Hanahan and Weinberg 2011; Chao, Weissman, et al. 2012; Sharma and Allison 2015a). One mechanism to avoid removal by

innate immune cells (macrophages and dendritic cells) is to upregulate "don't eat me" signals preventing phagocytosis (Jaiswal et al. 2009). In addition to preventing programmed cell removal (PrCR) by reducing total phagocytosis, antigen presentation from innate to adaptive immune cells is limited thereby restricting the cross-presentation to the adaptive immune cells (Swann and Smyth 2007; Chao, Weissman, et al. 2012). As a result, immunotherapies that increase tumor cell recognition by innate immune cells should also act as stimulation to the adaptive immune response in vivo.

To date, several strategies to block CD47:SIRP-α interaction have been developed including antibodies or antibody fragments against CD47 or SIRP-α (Kikuchi et al. 2005; Majeti, Chao, et al. 2009; Zhao et al. 2011), small peptides that bind CD47 or SIRP-α (Weiskopf et al. 2013; Ho et al. 2015), or systemic knockdown of CD47 expression (Zhao et al. 2011; Y. Wang et al. 2013; Soto-Pantoja et al. 2014). One advantage of antibodies that target CD47 is the increase in antibody dependent cellular phagocytosis (ADCP) which occurs when innate immune cells (macrophages and dendritic cells) Fcγ receptors (FcγR) bind to the Fc portion of the anti-CD47 antibody (Uno et al. 2007; Zhao et al. 2011; Weiskopf and Weissman 2015). To further increase antibody dependent cellular phagocytosis, anti-CD47 combination with additional tumor targeting antibodies has been tested pre-clinically and shown strong synergy in reducing total tumor burden in mice (Chao, Alizadeh, et al. 2010; Zhao et al. 2011; Weiskopf et al. 2013; Ho et al. 2015). The majority of these studies have been performed in NSG mice, which contain innate immune cells, but lack T, B and natural killer (NK) cells (Shultz et al. 2005). NK cells are the dominant cells responsible for antibody dependent cell-mediated cytotoxicity (ADCC), as a result the effects of NK cells after anti-CD47 treatment are not well studied

in this model (Uno et al. 2007; Zhao et al. 2011). Consequently, only a limited number of studies have investigated how CD47:SIRP-α blockade primes the adaptive immune response in immunocompetent systems.

Activating Adaptive Anti-Tumor Immunity In Vivo

Activation of the adaptive immune system, T and B cells, is antigen-specific and allows for a targeted immune response. T cells specificity comes from their T cell receptor (TCR) that recognizes a distinct peptide (antigen) when displayed on a major histocompatibility complex (MHC), also called human leukocyte antigen (HLA) in humans (27). T cells are subdivided into two major classes; CD8-cytotoxic T cells (TC or CTLs) or CD4-T helper (TH). Cytotoxic T cells can directly kill target cells when their TCR recognizes an 8-10 amino acid sequence that is displayed on MHC Class I (Schumacher and Schreiber 2015). In general, MHC Class I is expressed on all cells, including tumor cells, and present intracellular or endogenous peptides. Tumor reactive cytotoxic T cells recognize neo-antigens (peptides present within the cancer cell from mutations, that are not present on normal cells) allowing selective cytotoxicity of tumor cells (Schumacher and Schreiber 2015). Naïve T cells need an initial activation by APCs that have phagocytosed tumor cells and present these tumor neo-antigens. TCR/MHC recognition as well as engagement of additional costimulatory molecules occurs between the T cell/APC causing rapid expansion, cytokine production, and development of a memory T cell subset (Swann and Smyth 2007; Schumacher and Schreiber 2015; Sharma and Allison 2015b). Without initial activation by APCs and costimulation, T cells can become anergic rather than activated dampening the total anti-tumor T cell response (Murata et al. 2006; Swann and Smyth 2007; Hanahan and Weinberg 2011; Le et al.

2013; Schumacher and Schreiber 2015; Sharma and Allison 2015a). In cancer, activation of tumor reactive T cells can provide long-term tumor free survival by a continuous elimination of all malignant cells (Swann and Smyth 2007; Rosenberg and Restifo 2015; Schumacher and Schreiber 2015; Sharma and Allison 2015b). The memory T cell subset allows for a continued surveillance and identification of any cell expressing the tumor antigen at later times. Similar to lasting anti-viral responses, these tumor reactive T cells can last for decades providing the long-term tumor free survival seen in some patients treated with immunotherapies (Rosenberg and Restifo 2015). Therefore, a strong innate immune response is critical for mounting the maximum anti-tumor response (Chao, Majeti, et al. 2012). By increasing tumor cell phagocytosis with blockade of CD47:SIRP-α, an enhancement in antigen presentation and T cell priming should occur in vivo.

Pre-Clinical Evaluation for Activating the Adaptive Immune Response Following CD47 Blockade

In vitro, we have demonstrated that macrophages and dendritic cells increased their phagocytosis of tumor cells expressing the Ovalbumin antigen (Ova) when cells were treated with anti-CD47 (Tseng et al. 2013). In this study and others by our lab, macrophages were the dominant phagocytic cell after anti-CD47 treatment (Chao, Alizadeh, et al. 2011; Chao, Weissman, et al. 2012; Tseng et al. 2013). After phagocytosis, these macrophages secreted pro-inflammatory cytokines, and upregulated the costimulatory molecule CD86. These macrophages were then co-cultured with Ova specific T cells (OT-I CD8, OT-II CD4). OT-I cells were activated and primed by these macrophages, while interestingly, OT-II cells were not. OT-I cells could also be activated in vivo by adoptive transfer of macrophages (that had phagocytosed Ova expressing

tumor cells with the aid of anti-CD47 antibodies) providing them protection from the establishment of transplanted Ova tumors (Tseng et al. 2013). The same Ova expressing tumor cells co-incubated with the same types of macrophages in the absence of anti-CD47 blocking antibodies, but in the presence of anti-CD47 antibodies that do not interfere with CD47: SIRP-α interactions, do not cross-present Ova peptides to OT 1 cells in vitro or in vivo. These results demonstrate that anti-CD47 treatment induces macrophages to be efficient APCs to stimulate T cells in vitro and in vivo. In a separate study, the knockdown of CD47 has also been shown to synergize with radiotherapy by increasing the total quantity of tumor infiltrated CD8+ cells (Soto-Pantoja et al. 2014). In mice lacking T cells, this synergy was lost demonstrating that radiotherapy is an adjuvant to increase the adaptive immune response after CD47 blockade.

These studies provide strong evidence that CD47 blockade and tumor cell phagocytosis can efficiently prime CD8 T cells. It is still unclear however how CD4 cells, including regulatory T cells, are affected by CD47 blockade. Additionally, most adaptive immune studies have been performed with a defined tumor antigen. Ova for example, is known to effectively, if not even preferentially, cross-present to CD8 T cells (Li et al. 2001). Other limitations of our study include the xenograft system employed, where a human DLD1 tumor expressed a chicken protein, Ova. This was in turn engrafted in an immunodeficient NSG mouse, on a NOD background, that was subsequently transfused Ova specific T cells from another mouse strain, C57BL/6. The question is will CD47 blockade provide sufficient T cell priming in a more synergistic system and with naturally occurring antigens? We attempted to provide an answer for this question using two model systems.

Firstly, we wanted to work in a completely human system using all primary patient-derived cells. For this, we needed a naturally occurring cancer antigen where the dominant presenting peptide on MHC Class I and, ideally, MHC Class II was known. This would allow us to look at both the CD8 as well as the CD4 T cell responses, respectively. *NY-ESO-1*, also known at *CTAG1*, is a gene whose normal expression is restricted to the cytoplasm of germ cells. Therefore NY-ESO-1 expression on a cancer is restricted to the cancer, and not expressed normal cells or tissue. Cancers expression of NY-ESO-1 ranges from about ten percent in gastric cancer to forty percent on melanoma (Gnjatic et al. 2006). Bladder, breast, CRC, esophagus, hepatocarcinoma, head and neck, multiple myeloma, non-small cell lung, ovarian, prostate, and sarcoma cancers have all shown to express NY-ESO-1 within this range as well. Since its discovery in 1997, many vaccine studies have been attempted to try and re-educate the immune system to mount a response against, what in early development is, a self-antigen. In these trials, a large number of NY-ESO-1 specific CD8 T cells have been detected around the tumor, but they appear to be anergic. At the same time, a large number of CD4+CD25+ T regulatory cells also are seen, perhaps explaining both the initial education of CTLs as well as their lack of effectiveness at the local tumor site caused by T regulatory cell immune suppression (Gnjatic et al. 2006). Given the availability of tetramer regents specific for NY-ESO-1 as well as the cytoplasmic cancer specificity of the protein, we surmised that a human in vitro system could be developed. Anti-CD47 induced phagocytosis of cancer cells with naturally occurring NY-ESO-1 could be up taken and processed by macrophages and dendritic cells, then those APCs would present or cross-present the antigen to NY-ESO-1 specific CD4 and CD8 T cells, respectively.

Our second approach involved using syngenic animal models. Athough a couple of studies in mouse models of AML and breast cancers showed a response in vivo to CD47 blockade therapy, we tried to identify a syngeneic mouse system where the adaptive immune system could be studied as well. A methylcholanthrene (MCA) induced fibrosarcoma model was developed in the Schreiber lab. Tumors from immunodeficient mice were more immunogenic than their immunocompetent hosts. Recently, the Schreiber lab identified a neo-antigen, a point mutation in Spectrin-β2 was the cause of the MCA induced neo-epitope, which is responsible for the immunogenicity of d42m1 MCA sarcoma tumor. They demonstrate that treatment with anti-CTLA4 and/or anti-PD-1 antibodies enable immunocompetent mice to reject the tumor by way of CTL specific for neo-antigens *Alg8* and *Lama4.* This model would allow us, in an in vivo system, to show that CD47 blockade could not only reduces tumors by phagocytosis, but help us elucidate if T cells could be educated to provide an even more robust adaptive immune response.

Methods and Materials

Isolation of NY-ESO-1 Specific T cells

Primary patient peripheral blood mononuclear cells (PBMC) were obtained from the Cerundolo lab according Oxford University guidelines and procedures. Cells were sorted as previously described (Chen et al. 2010). In brief, NY-ESO-1 specific T cells were isolated from peptide-stimulated cultures and sorted by FACS for CD4+ and tetramer positive cells. CD8+ T cell clone 4D8 and LG2 B cells was a gift from the Cerundolo lab.

Growth of Primary T Cell Clones 4D8, C010, and C004

Cell were stimulated with irradiated allogeneic PBMC and LG2 cells 10 μg/ml phytohaemagglutinin and cultured for 12 days. RPMI-1640+GlutaMax (Invitrogen) supplemented with 10% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, 10 mM HEPES, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, non-essential amino acids and 100 U/ml recombinant human IL-2 (Peptrotech). T cells were restimulated every 4-6 weeks to maintain viability. Further details of the procedure can be found in (Chen et al. 2000).

FACS Analysis

All antibodies were obtained from BD Biosciences (San Jose, CA). Tetramers against human TCRs were obtained from TCMetrix (Epalinges, Switzerland). HLA-DR4/7 restricted tetramers were loaded with NY-ESO-1 119-143 peptide (PGVLLKEFTVSGNILTIRLTAADHR). The HLA-A2 restricted tetramer was loaded with a mutated NY-ESO-1 157-165 peptide (SLLMWITQ*V*). The tetramer for mLama4 was a kind gift from the laboratory of Robert Schreiber (WashU, St. Louis). Cell were analyzed by flow cytometry on a LSRFortessa or FACSCanto cell analyzer with high throughput sampler (BD Biosciences) and analyzed with FlowJo (Tree Star).

Generation of Monocyte Derived Macrophages

For human macrophages, leukocyte reduction system (LRS) chambers were obtained from the Stanford Blood Center from anonymous donors, and peripheral blood mononuclear cells were enriched by density gradient centrifugation over Ficoll-Paque Premium (GE Healthcare). Monocytes were enriched by incubating them on a plate in

RPMI+GlutaMax with 10% FBS (Thermo) and 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen) for 1-2 hours and washing the plate 3 times to remove non-adherent cells. Adhered cells were >90% CD14 positive. For dendritic cell generation, adhered cells were cultured in RPMI+GlutaMax with 10% FBS (Thermo), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen), 10ng/mL human IL-4 (Peprotech) and 100ng/mL GM-CSF (Leukine Sargramostim by Genzyme). Cultures were replenished at day 3 and day 5 then harvested at day 7-10. Macrophages were differentiated by culturing adherent cells with RPMI+GlutaMax with 10% FBS (Thermo), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen), 10ng/mL human M-CSF (Peprotech), then harvested at day 7-10. Mouse macrophages were generated with bone marrow cells isolated mice and differentiated in IMDM+GlutaMax supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, and 10 ng/mL murine M-CSF (Peprotech) for 7 days.

In Vitro Phagocytosis Assay

Phagocytosis assays were performed by co-culture of 50,000 macrophages with 100,000 tumor cells for two hours in serum-free medium, then analyzed using an LSRFortessa or FACSCanto cell analyzer with high throughput sampler (BD Biosciences). Antibodies were used at a concentration of 10 ug/ml. Primary human macrophages were identified by flow cytometry using anti- CD14 or anti-CD206 antibodies. Mouse macrophages were identified by anti-F4/80. All unlabeled target cells were labeled with 0.5 μM carboxyfluorescein succinimidyl ester (CFSE) according to manufactures protocols (Invitrogen). Dead cells were excluded from the analysis by staining with DAPI. Phagocytosis was evaluated as the percentage of GFP/CFSE+

macrophages using FlowJo (Tree Star) and was normalized to the maximal response in a given experiment. All conditions were performed with four technical replicates per experiment and error bars show standard deviations between the replicates.

In Vivo Tumor Engraftment, Growth, Treatment, and Analysis

One million tumor cells were suspended in PBS and engrafted subcutaneously on the flanks of 129 mice (Taconic). Tumor growth was measured by calipers at the greatest diameter and the greatest perpendicular diameter. Mice were treated by intraperitoneal (IP) injections with: PBS (100ul), MIAP410 (250 μg prime/750 ug maintenance), and/or CTLA4 (200ug). MIAP410 priming dose was given 3 days prior to treatment with the maintenance dose. CTLA4 was given every third day. Tumors were harvested and digested with Collagenase 1 (Sigma) for downstream preservation and analysis.

VITAL Assay

The VITAL assay was performed as previously described (Hermans et al. 2004). In brief, two cell populations of interest are labeled with CMTMR (10uM) or CFSE (333 nM). The labeled cells are co-incubated with effector T cells at a 3:1 target:effector ratio in a U-bottomed plate. After a 4-hour incubation, the plate was analyzed by FACS.

Lentiviral Expression Vector for Luciferase-GFP

The vector was previously described in detail (Chao, Alizadeh, et al. 2010). In brief, the pCDH-CMV-MCS- EF1-puro HIV-based lentiviral vector (Systems Bioscience) construct contains a ubiquitin promoter driving the expression of a luciferase-eGFP fusion product. The luciferase gene is the Luc2 (pgl4) version (Promega). The eGFP portion derives from the pIRES2-eGFP plasmid (Becton

Dickinson). Lentiviral production and concentration was accomplished using standard protocols.

Tumor Cell Lines and Luciferase-GFP$^+$ lines

DLD1 cells were obtained from ATCC and cultured according to their protocols. GFP-luciferase+ lines were generated by transduction using a pCDH-CMV-MCS-EF1 puro HIV-based lentiviral vector (Systems Biosciences) engineered to express an eGFP-luciferase2 (pgl4) fusion protein. Stable cell lines were created by sorting for GFP expression using a FACSAria II cell sorter (BD Biosciences). Trombelli, SK mel 29, and 4D8 were kind gifts from the laboratory of Vincenzo Cerundolo (Oxford). The MCA sarcoma lines WT 9069, RkIk 4862, D42M1.T3, D42M1.T9, H31M1, and F244 were all kind gifts from the laboratory of Robert Schreiber (WashU, St. Louis).

Monoclonal Antibodies for In Vitro and In Vivo

Hu5F9-G4 was purified using standard IgG purification procedures previously published (J. Liu et al. 2015). Hybridomas for B6H12 was obtained from ATCC, expanded and purified by BioXCell (West Lebanon, NH). Hybridomas for MIAP410 and MIAP470 were obtained from Eric Brown (Genetech) and purified on a protein G column using standard purification techniques. Isotype antibodies were obtained from eBiosciences. Anti-CTLA4 (9D9) antibody was purchased from BioXCell (West Lebanon, NH).

Western Blot for NY-ESO-1

Anti-NY-ESO-1 monoclonal antibody E987 was a kind gift from the Ludwig Institute for Cancer Research (New York, NY). Cells were lysed in Laemmli buffer and

probed with E987. The secondary antibody was anti-mouse IgG conjugated to horseradish peroxidase. We detected using a Roche ECL kit.

Results

Macrophages and Dendritic Cells Can Both Phagocytose Cancer Cells in the Contexts of CD47 Blockade

All the human in vitro phagocytosis assays to date only used human serum derived macrophages. Here we use cytokine-derived macrophages and dendritic cells from the same donor to show that DCs along with macrophages are able to phagocytose DLD1 colon cancer cells. The isotype control as well as the cells only controls show little eating of the target cells by IL-4/GM-CSF derived dendritic cells and M-CSF derived macrophages. Both the B6H12, a mouse anti-human CD47 blocking antibody, as well at Hu5F9-G4, a humanized anti-human CD47 blocking antibody, enable both dendritic cells and macrophages to phagocytose DLD1 tumor cells. In fact, dendritic cells may even phagocytose DLD1 cells slightly better than macrophages, at least with the B6H12 antibody (Figure 3.1).

Characterization of Primary Patient Derived T Cells

Two NY-ESO-1 specific CD4+ T cell clone was sorted from melanoma patients by FACS. One line is specific for the DR4 allele of HLA class II, C010, while another sorted line is specific for the DR7 allele, C010. Post sorting, we expanded the T cell clones using a mixture of co-cultured cells, NY-ESO-1 peptide, IL-2 and phytohaemagglutinin (PHA). Irradiated peripheral blood mononuclear cells as well as LG2, a Epstein-Barr virus-transformed B cell line, were used as supportive cells to help

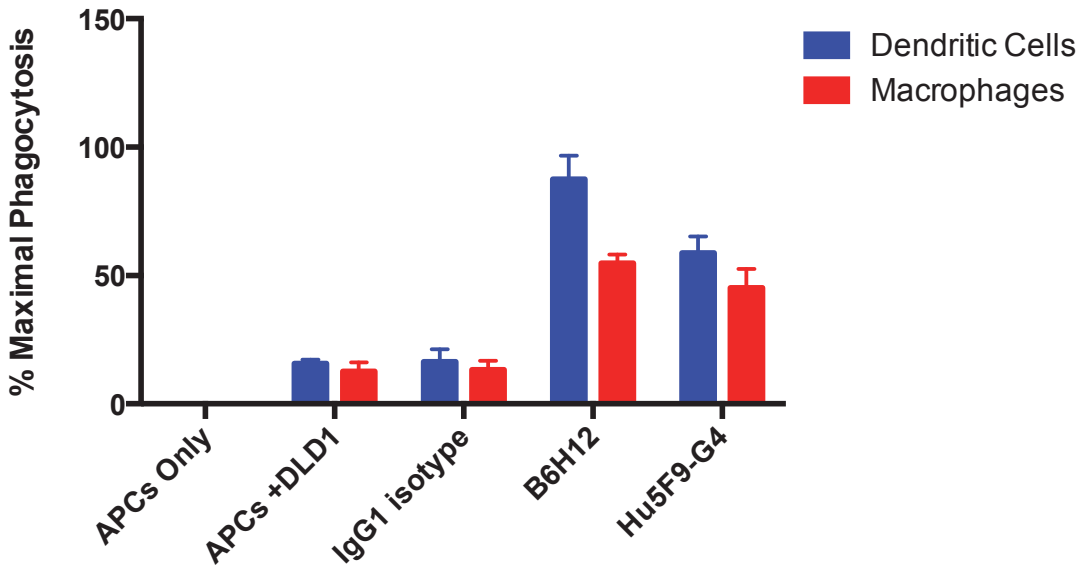

Figure 3.1. *In vitro* phagocytosis of DLD1 cells by macrophages and dendritic cells. Cultures of APCs alone show no eating of target cells. Both APCs cultured with DLD1s alone and with an isotype control antibody show minimal engulfment of DLD1 cells by APCs. In the context of CD47 blocking antibodies B6H12 and Hu5F0-G4, the dendritic cells and macrophages are able to strongly phagocytos the DLD1 cells. The dendritic cells are able to eat the DLD1s at a slightly better efficiency than the macrophages.

expand the T cell lines. Additionally, IL-2, PHA, and NY-ESO-1 peptide 121-138 was added to the cultures to provide proper signaling to initiate cell division. After ex vivo expansion, C010 stained with a tetramer recognizing HLA-DR4 NY-ESO-1 and C004 staining for a tetramer recognizing the HLA-DR7 NY-ESO-1 (Figure 3.2).

Cytotoxic T Lymphocyie 4D8 can kill an NY-ESO-1 expressing Melanoma Cell Line

With the CD8+ T cell line 4D8, we were able to show it effectively killing Trobelli, a melanoma know to express NY-ESO-1. The VITAL in vitro fluorometric CTL cytotoxicity assay was used to measure 4D8's ability to kill target cells. SK mel 29 cells, which are negative for NY-ESO-1 were labeled with CMTMR while Trombelli was labeled with CFSE. Upon co-culture with CTL 4D8, only the Trombelli cells are killed,

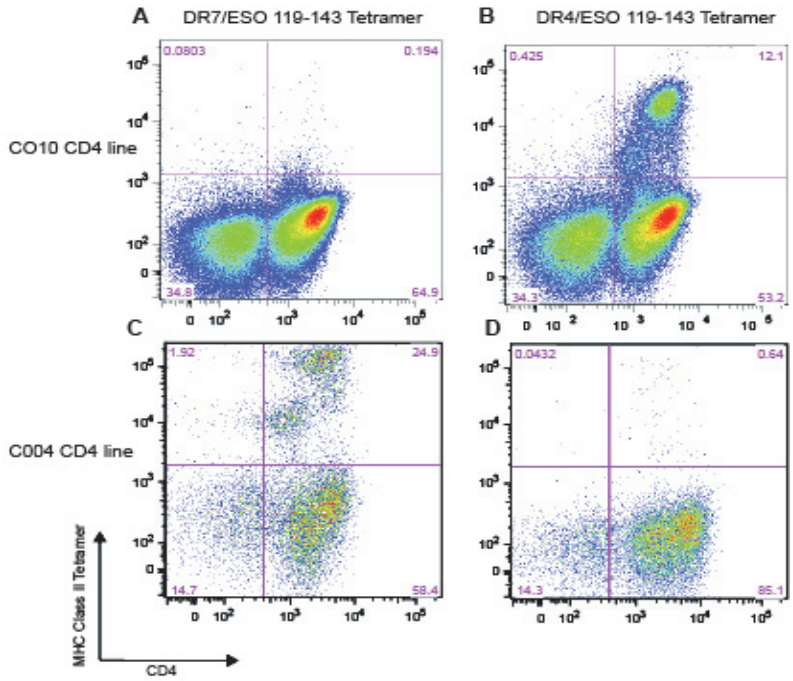

Figure 3.2. FACS analysis showing NY-ESO-1 specific CD4+ T cells after clonal expansion. In all FACS plots, cells in the lower left quadrant are the CD4- PBMCs and LG2 cells, while CD4+ cells in the lower right quadrant are both the PBMCs as well as the NY-ESO-1 specific T cell lines. (A) The HLA-DR7 tetramer does not recognize C010 as demonstrated by the lack of cell in the upper right quadrant. B. The HLA-DR4 tetramer does recognize C010 as demonstrated by the population of cells in the upper right quadrant. (B) The HLA-DR47 tetramer does recognize C004 as demonstrated by the population of cells in the upper right quadrant. D. The HLA-DR4 tetramer does not recognize C004 as demonstrated by the absence of cells in the upper right quadrant.

demonstrating that 4D8 is able to effecitvely and specificlly kill NY-ESO-1 melanoma cells that are HLA-A2 positive (Figure 3.3).

Expression of Activation Markers Upon 4D8 Restimulation

The expansion of the CTL line 4D8 requires a host of co-culturing reagents which actively simulate the T cells in order to induce their cell division. When performing an antigen presentation assay the effects of the co-culturing could confound analysis of T cell clonal expansion. Here, we look for cell surface expression of T cell activation makers by FACS in both 3 week resting 4D8 T cells and co-culture stimulated 4D8 T cells. HLA-DR remains unchanged in a population of T cells regardless of their active

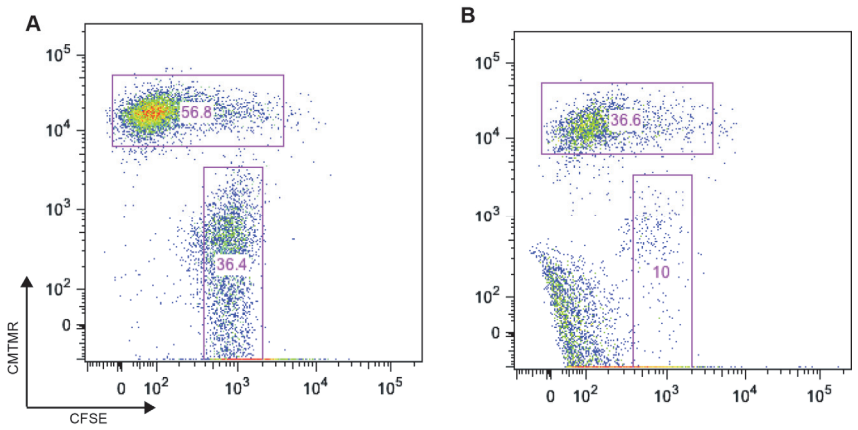

Figure 3.3. VITAL assay showing 4D8 killing Trombelli cells. SK mel 29, an HLA-A2 positive, NY-ESO-1 negative melanoma line was labeled with CMTMR. Trombelli, an HLA-A2 positive, NY-ESO-1 positive melanoma line was labeled with CFSE. A. A representative FACS plot which only has the two cell lines and no CTLs shows that the melanoma lines are viable. B. When the melanoma cell lines are co-cultures with 4D8 in a 3:1 target to effector ratio, the CFSE labeled Trombelli cells are poorly detectable by FACS, demonstrating that 4D8 is an effective CTL.

culturing or resting status. Upon active culturing, CD69 is strongly upregulated as compared to the resting cells, and at both states is upregulated relative to unstained controls. Several late stage activation markers, known to be involved in T cell regulation, remain unchanged during co-culturing conditions, including ICOS, ICOS-L, 4-1BB, CD25, CD71, and PD-1 (Figure 3.4). Additional markers like LAG-3, TIM-3, CD38, and CD40L would also be used to screen for T cell activation in subsequent profiling assays.

In Vitro Phagocytosis of Melanoma Cell Lines by Macrophages is Ineffective

The melanoma cells we tested are not eaten any better in the context of CD47 blocking antibodies than their controls. Trombelli and SK mel 29 are both HLA-A2 positive melanoma cell lines. Trombelli is also positive for NY-ESO-1. There is no effect on phagocytosis by macrophages in the context of CD47 blocking antibodies. The mouse isotype and 2D3 non-blocking controls look similar to the mouse anti-human CD47 blocking antibody B6H12. Similarly humanized CD47 blocking antibody Hu5F9-G4 does not increase phagocytosis over the human isotype control antibody (Figure 3.5).

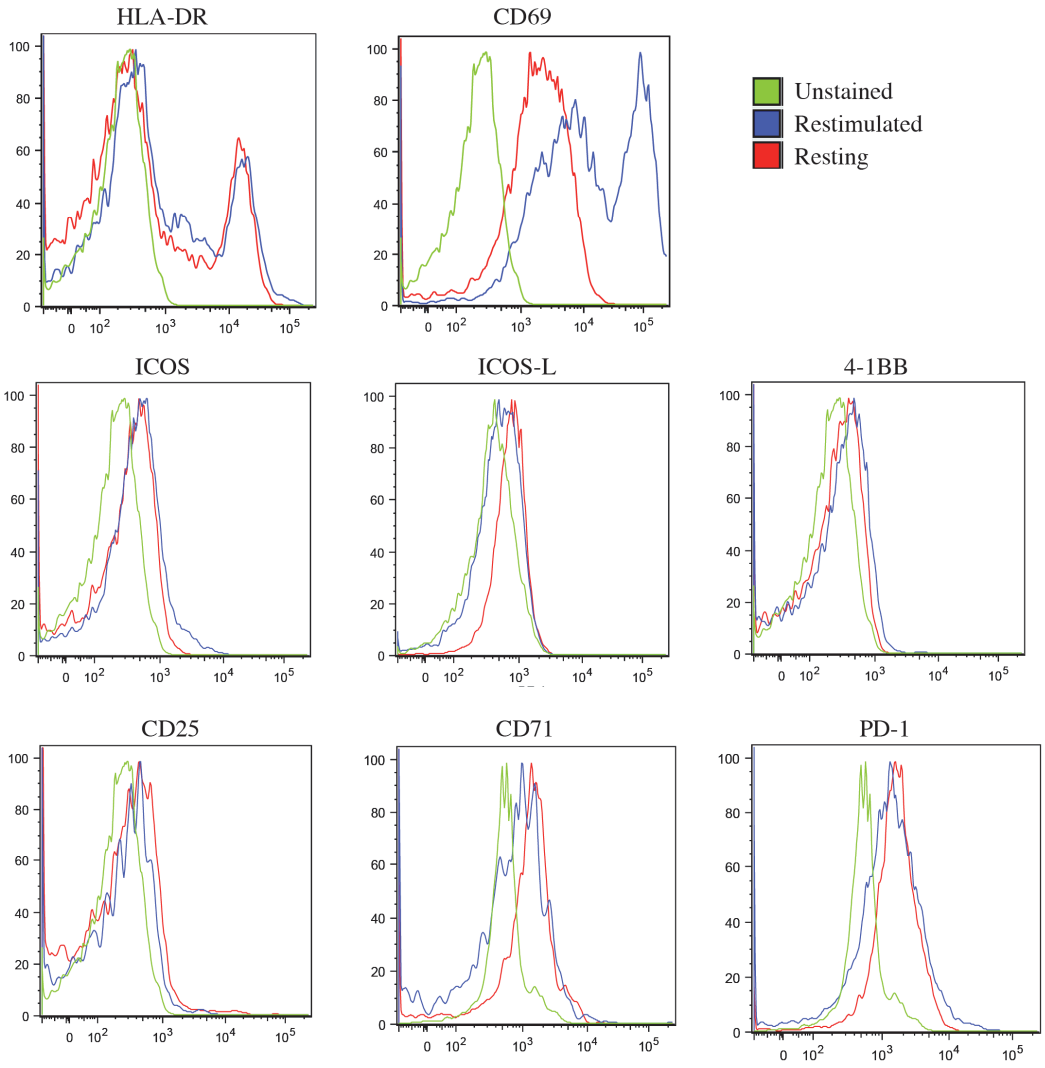

Figure 3.4. FACS histograms of 4D8 T cell surface activation markers by FACS analysis. The green lines denote unstained 4D8 control cells. The blue lines are of 4D8 cells, which have been stimulated by T cell culture conditions. The red line is staining of 4D8 cells which have been resting in culture for 3 weeks. A host of later stage activation markers remain downregulated even after restimulation including ICOS, ICOS-L, 4-1BB, CD25, CD71, and PD-1. CD69 only is upregulated during restimulation while HLA-DR remains HLA-DR remains upregulated irrespective of culture conditions.

Western Blot in a Multitude of Tumor Lines Shows No NY-ESO-1 Expression

Because an in vitro phagocytosis assay of melanoma cell lines had little effect, we screened other cancer cell lines that may have NY-ESO-1 expression. Trombelli and SK mel 29 were included as positive and negative controls showing that our antibody against NY-ESO-1 correctly targeted the protein. Sk mel 28 is another melanoma line that others

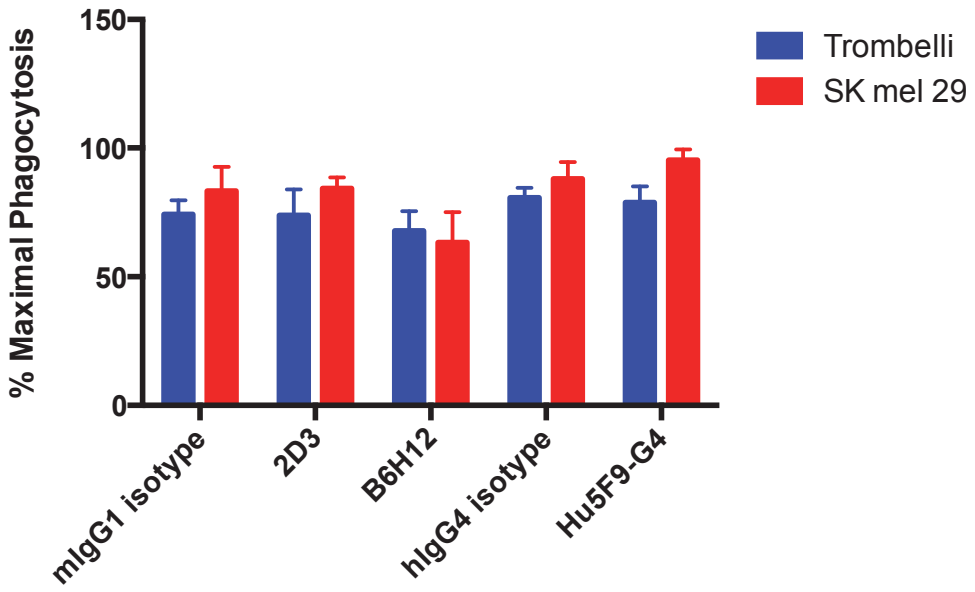

Figure 3.5. *In vitro* phagocytosis of melanoma lines by macrophages by CD47 blockade is ineffective. Mouse anti-human antibody B6H12 do not phagocytos any better than its mouse IgG1 isotype control and 2D3 anti-CD47 non-blocking antibodies. Similarly, the humanized Hu5F9-G4 does not effectively enable phagocytosis over its human IgG4 isotype control.

in lab had shown had some phagocytosis activity with a CD47 blocking antibody so this was also included in the screen. HCT 116, HT29, LS 174T, and DLD1 are all CRC lines that were available in house. MOV30V, ES2, OVACO13, and SKOV3 are ovarian cancer lines. CAPAN-1 is a PDAC cell line. Lastly, 647V is a bladder cancer line. All were negative for NY-ESO-1 protein expression (Figure 3.6).

In vitro Phagocytosis of MCA Sarcoma Lines Show Some Efficacy with CD47 Blockade

We wanted to make sure MCA sarcomas could be phagocytosed by mouse macrophages. In this case, TM4 mice bone marrow, expressing dTomato, was used to differentiate M-CSF macrophages engulfing a multitude of MCA sarcoma lines provided by the Schreiber lab. WT9069, RkIk 4862, and D42M1.T9 are MCA sarcomas on a 129 mouse background, while H31M1 and F244 are on a C57BL/6 background. Here two different mouse anti-human CD47 antibodies that cross-react with mouse CD47 were

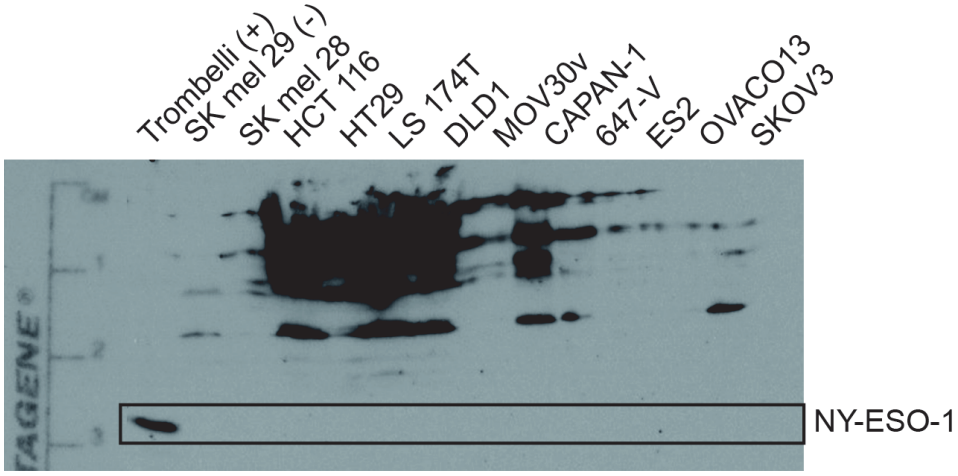

Figure 3.6. Western blot analysis for NY-ESO-1 protein expression on cancer cell lines. The positive control cell line Trombelli expressed NY-ESO-1 as detected by antibody E978, while the negative control line SK mel 29 did not. All other tested cell lines also had undetectable levels of NY-ESO-1 protein expression.

used to block CD47's interaction with SIRPα. Both WT9069 and RkIk 4862 were able to be phagocytosed by macrophages in the context of both CD47 antibodies, relative to isotype controls. H31M1 and F244 also shows efficacy in the context of MIAP470 but not MIAP410. Lastly. D42M1.T9 could not be phagocytosed by macrophages when co-incubated with either antibody (Figure 3.7).

MCA Sarcomas Regress In Vivo with Anti-CTLA4 and Anti-CD47 Antibodies

A MCA sarcoma line, D42M1.T3 was provided by the Schreiber lab and engrafted subcutaneously on the back of 129 mice. The mice were treated with a priming dose of anti-CD47 clone MIAP410 at engraftment. Three days later at the nadir of anemia, maintenance dose treatment every other day was initiated. Seven days post engraftment, a three-dose course every third day was initiated of anti-CTLA4 treatment (Figure 3.8B). The anti-CD47 only treated group showed some efficacy relative to PBS control treated mice. The anti-CTLA4 treated mice were the earliest to respond to treatment with the checkpoint antibody. The combination of anti-CD47 and anti-CTLA4

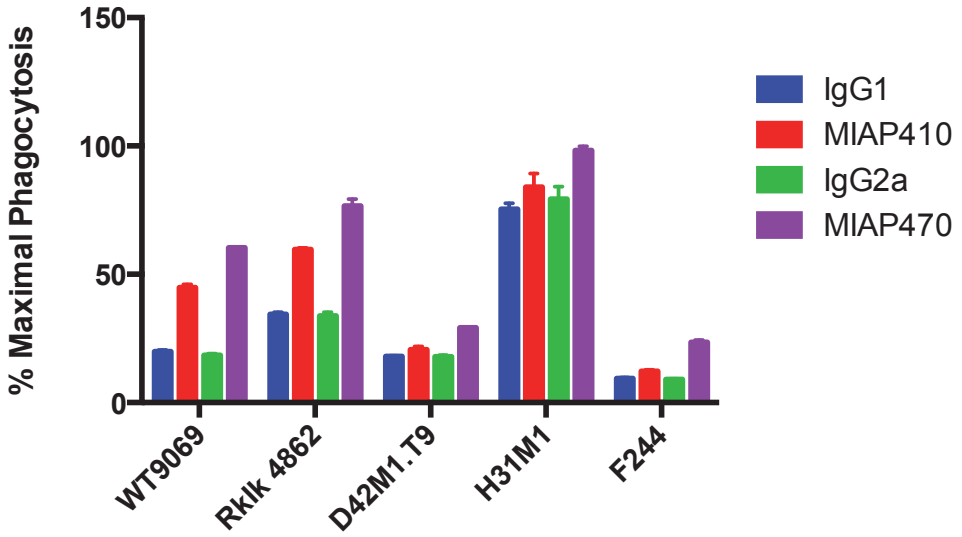

Figure 3.7. *In vitro* phagocytosis of MCA sarcoma lines by macrophages shows some efficacy. MIAP410 and MIAP470 are mouse anti-human antibodies that cross react with mouse CD47 of IgG1 and IgG2a subclasses, respectively. WT9069 and RkIk respond to both antibodies relative to isotype controls. H31M1 and F244 show slightly better eating with MIAP470. D42M1.T9 does not respond to CD47 blockade.

treated mice initially progressed, but then regressed to almost pre-engraftment levels of tumor burden (Figure 3.8A).

MCA Sarcomas Regress In Vivo with Anti-CTLA4 at a Third the Dose with Anti-CD47 Antibodies

We were not sure if the regression in tumor by the combination group in the previous study was a result of anti-CTLA4 antibody alone or did anti-CD47 antibody play a role in the tumor's regression as well. To test this, the cell engraftment numbers and schedule of antibody treatment remained the same as previously described, but the dose of CTLA4 was reduced by a third. This time, the anti-CD47 treated group performed as poorly as the PBS control treated mice. The anti-CTLA4 treated mice again responded the best, and the combination of anti-CTLA4 and anti-CD47 treatment trended similarly to the anti-CTLA4 treated mice but was starting to break through treatment at the end of study (Figure 3.9A). Upon FACS analysis of digested tumors, the

combination treatment group had a similar number of tumor infiltrating lymphocytes (TIL) specific for the known rejection neo-antigen mLama4, while anti-CD47 therapy did not increase the CTL response relative to PBS controls (Figure 3.9B). Tumor associated macrophages (TAM), as detected by F4/80, were also analyzed and that anti-CD47 antibody decreased the number of macrophages and CTLA4 therapy decreased TAMs even further (Figure 3.9C).

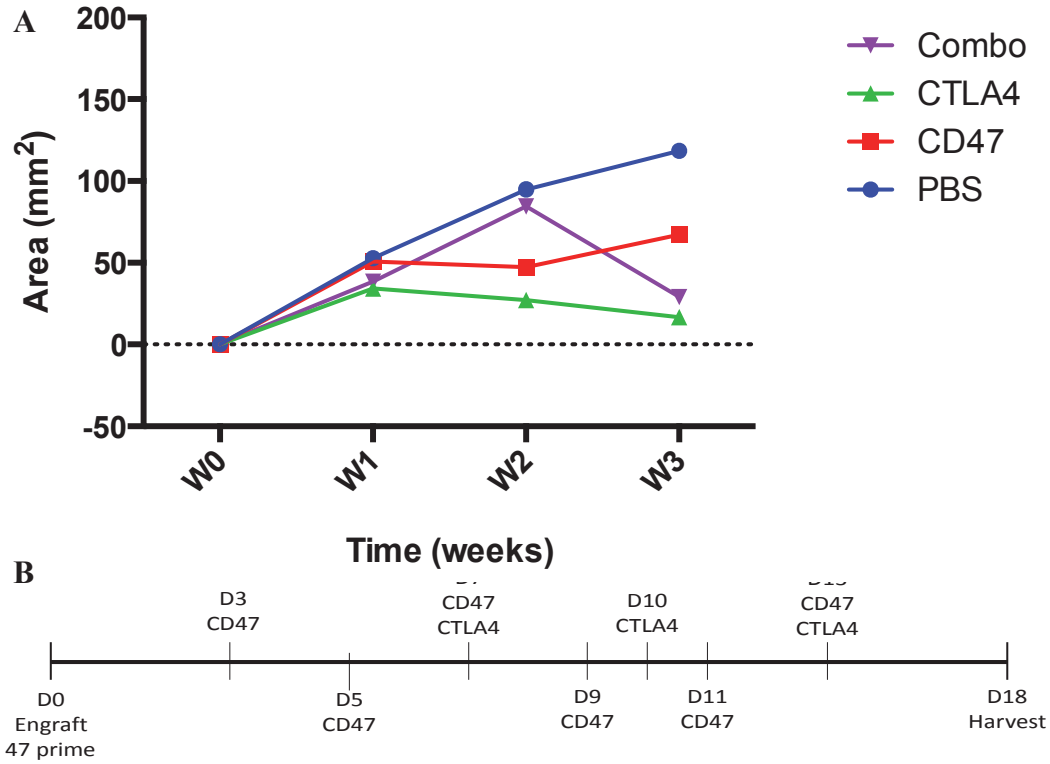

Figure 3.8. In vivo treatment of 129 mice with one million MCA sarcoma cells, D42M1.T3. A priming dose of 250ug of anti-CD47 MIAP410 was given followed by a maintenance dose of 750ug. 200ug of anti-CTLA4 antibody was given as well A. The graph shows the tumor burden of each treatment group as measured by area of tumor. The anti-CTLA4 treated group had the most immediate effect. The combination group had a delayed response relative to anti-CTLA4 treated mice alone, but as strong of a regression. Anti-CD47 seemed to slow the tumor growth as well. B. A timeline illustrating the schedule of antibody treatment.

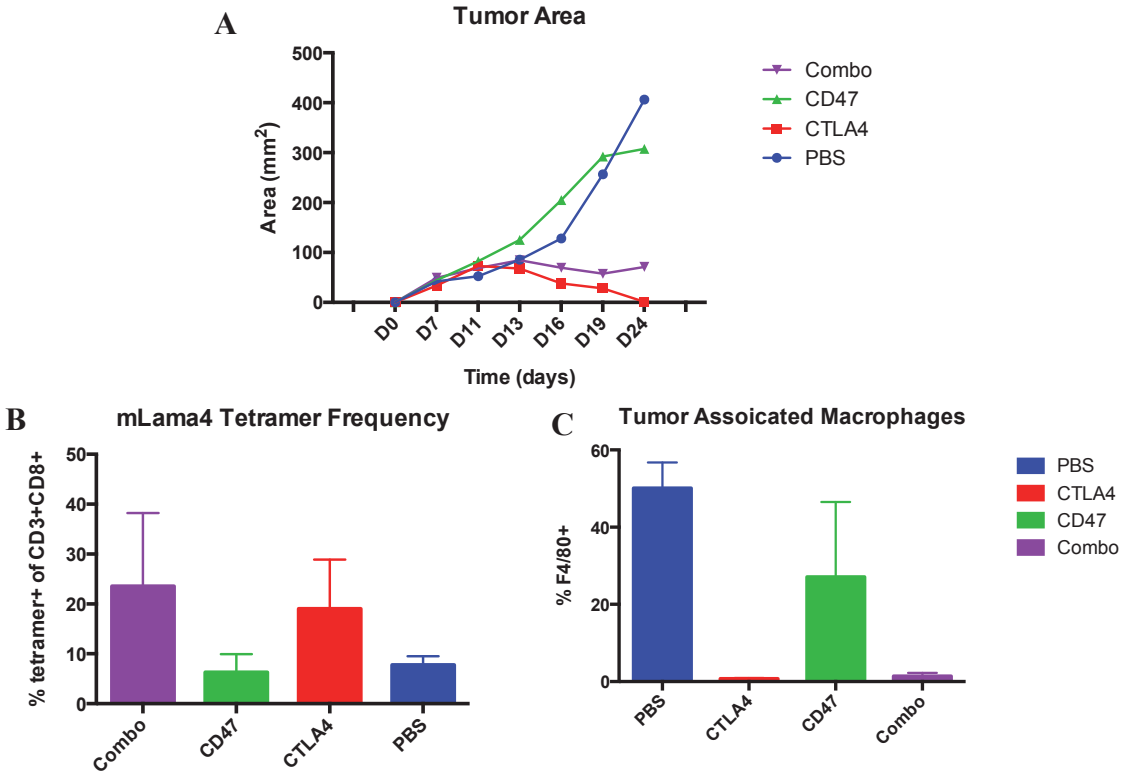

Figure 3.9. *In vivo* low CTLA4 dose treatment of 129 mice with one million MCA sarcoma cells, D42M1.T3. A priming dose of 250ug of anti-CD47 MIAP410 was given followed by a maintenance dose of 750ug. 67ug of anti-CTLA4 antibody was given as well (A) The graph shows the tumor burden of each treatment group as measured by area of tumor. The anti-CTLA4 and combination treated group had a similar effect. The combination group did not reduce tumor burden as much relative to anti-CTLA4 treated mice. Anti-CD47 responded similarly to PBS. (B) Upon tumor analysis, the combination and anti-CTLA4 treated mice showed similar levels of mLama4 specific T cells by FACS analysis of tumors. The anti-CD47 treated group did not show an increase in TILs relative to the PBS control group. (C) FACS analysis for TAMs showed the PBS group had the most macrophages, while the anti-CD47 group had a lower number. Intriguingly, the CTLA4 and combo group had very low levels of TAMs.

Discussion

Results from the Ova studies with anti-CD47 antibodies made us want to explore the potential role of the adaptive immune system further as it relates to CD47-SIRPα blockade. Dendritic cells are professional antigen presenting cells that are characterized to be better able to present antigen to a fewer number of T cells to illicit a T cell response (Palucka and Banchereau 2012). The ultimate goal was to have an APC phagocytose a tumor, and then present the processed antigen to both CD4 and CD8 T cells. Given that

all the work in the Weissman lab was done with macrophages using a non-specific mix of human serum to differentiate, we set out to look at the phagocytic ability of DC and macrophages in a more defined differentiation protocol. Interestingly, we found that human IL-4 DCs were better able to phagocytose DLD1 cancer cells, which could be powerful knowledge for downstream APC-T cell antigen presentation assays.

Knowing the type of APC we wanted to use in our studies, we wanted to find a biologically relevant tumor antigen with reagents that would be useful in in our studies. Initial thoughts were to look at other model antigens like CMV or Flu, but like Ova these would be model antigens which may not elicit the responses as a true cancer antigen. Melanoma antigens have been studied extensively with so many candidates, including MART-1, MAGE, and gp100. In the end, we chose NY-ESO-1 because of its expression in a broad range of cancers, as well as its already discovered dominant peptide on common MHC class I allele, HLA-A2, and being an antigen where its MHC class II peptides were studied. That led us to sorting primary patient CD4 T cells which recognized NY-ESO-1 peptide with both HLA-DR4 and HLA-DR7 alleles.

We initially started using melanoma lines provided by the Cerundolo lab, Trobmelli and SK mel 29, since they had previously been characterized against the 4D8 T cell clone also provided. Even having two different HLA-DR clones, given the very large diversity of HLA-DR haplotypes, we knew it would be difficult to find a donor that matched, and therefore donor derived APCs that would be a match.

Then we wanted to make sure we could readout a clonal T cell response by more than just cell divisions, as typically assessed by half-fold dilutions CFSE signal on CFSE labeled T cells. Complicating factors we had to provide the primary T cells with some

stimulation in order to expanded the cells to quantities needed for our experiments. Our initial screen provided at least 6 candidate markers we could use to look at T cell activation regardless of the co-culture stimulus.

We next ran an in vitro phagocytosis assay on the melanoma cell lines, Trombelli and SK mel 29. The two cell lines, nor any subsequent melanoma lines phagocytosed well (data not shown), so we screened for other potential cancer types that could express NY-ESO-1. Upon screening for NY-ESO-1 expression by western blot, none of the cell lines we had available in house were positive for the protein of interests, and the project was put on hold.

Understanding the immune response in a fully competent mouse system was also important given that the Weissman lab had been primarily using an immunodeficient mouse model, NSG. The Schreiber lab, at the time, unpublished data identifying a rejection antigen on one of their MCA sarcoma lines. We endeavored to use this model to better understand the immune response in a syngeneic system. Since a tetramer was also available against CTLs that provided the dominant adaptive response against the tumor, we hoped that we also combine anti-CD47 therapy with a checkpoint antibody, anti-CTLA4, to model both anti-CD47's ability to better enable phagocytosis by APCs, and then to further overcome the immunosuppressive signaling of primed T cells against the sarcoma.

Initially in vitro phagocytosis assays showed that some MCA sarcoma lines were able to be phagocytosed with CD47 blockade therapy and so we progressed to in vivo modeling. Initial studies showed that although anti-CD47 therapy alone didn't regress tumors, the combination therapy looked similar to the checkpoint therapy alone. Unsure

if this was a single agent effect or a combination therapy effect, we ran another pilot experiment with the intent to harvest tumors and looked for tumor infiltrating immune cells. There was no increase in TILs with CD47 therapy, but there was a large increase in the CTL population when anti-CTLA4 treatment was involved. Upon macrophage analysis, there was a reduction in TAMs with anti-CD47 therapy, and an even further reduction with anti-CTLA4. Though the reasoning for the TIL and TAM frequencies could not be fully explained, there was definitely some immune response.

We first hypothesized that the CTLA4 may have been too effective at such a high dose or the MCA sarcoma was such an aggressive tumor that a lower initial tumor burden was needed. In unpublished data, we titered down CTLA4 from 200ug per dose down to 1/32 the dose and found that at a quarter the original dose, 50 ug, we could recapitulate a similar effect as the original dose. Secondly, perhaps the anti-CD47 therapy could not overcome the standard 1 x 10^6 cell engraftment. We titered down the initial engraftment cell numbers to 200,000 cells, when the tumors would get spontaneously rejected by the mouse, but no effect on tumor growth was seen over PBS treated controls.

MIAP410 is an antibody that was raised against human CD47 as its target. However, upon screening, it was discovered that this antibody would also cross react with mouse CD47. Testing by others in the lab showed that this cross-reactive antibody bound more weakly to mouse CD47 as compared to human CD47. Moreover other syngenic mouse tumors did not respond to MIAP410 as well, including a melanoma model (B16) and a colon cancer model (CT28). As such, we endeavored to other mouse CD47 antibodies. MIAP301, an rat anti-mouse CD47 antibody bound stronger than MIAP410 to mouse CD47 but was still ineffective in vivo. We set forth to generate a

novel anti-mouse CD47 antibody with limited success. Recombinant CD47 was used to immunize Armenian hamsters. Initially hybridomas were positive for binding to recombinant mouse CD47 by ELISA, but upon screening of subclones by FACS none were positive for binding. Based on prior experience in the lab, we knew that binding to native protein expressed on the surface of cells was the highest screening threshold, because the native protein maintains the true 3-dimentional structure, properly folded. When the hamster hybridomas failed, we tried a few immunization techniques in CD47 knockout mice. We again tried to immunize the mice with recombinant CD47 protein. Also, we tried immunizing mice with human AML HL-60 cells that had the human CD47 protein knocked down, while inserting the mouse CD47 gene. Lastly, we tried hydrodynamic immunization, where in a mammalian expression vector encoding for the mouse CD47 gene is injected into the mouse, and with the help of an adjuvant the plasmid gets transduced into liver cells and the mouse started to generate proteins *in situ* (Sheehan et al. 2006). All these attempts at generating antibodies failed to generate viable clones against CD47, as screen for by FACS. Part of the reason this could be is the nature of CD47 itself. Since CD47 up regulation prevents phagocytosis, a B cell would never uptake the antigen enough to generate an antibody response. We are still exploring other mouse CD47 blocking reagents, but generating a good high binding reagent is necessary before moving forward with the MCA sarcoma model.

Conclusions

Ultimately, technical hurdles prevented us from better understanding how anti-CD47 therapy may regulate the adaptive immune system. In the human in vitro project, cancers that are NY-ESO-1 positive and negative need to be identified. Those cancer

lines will also need to be screened for proper HLA matching and deliberate mismatching before further studies can take place. In the in vivo MCA sarcoma project, further development is needed to identify a strong anti-mouse reagent before greater progress can be made.

CHAPTER FOUR

Conclusions

Portions of this chapter published as: McCracken MN, Cha AC, Weissman IL. 2015. Molecular Pathways: Activating T Cells after Cancer Cell Phagocytosis from Blockade of CD47 “Don't Eat Me” Signals. Clin Cancer Res 21:3597–3601.

Abstract

Recent advances with immunotherapy agents for the treatment of cancer has provided remarkable, and in some cases, curative results. Our laboratory has identified CD47 as an important “don’t eat me” signal expressed on malignant cells. Blockade of the CD47:SIRPα axis between tumor cells and innate immune cells (monocytes, macrophages, and dendritic cells) increases tumor cell phagocytosis in both solid tumors (including, but not limited to bladder, breast, colon, lung, pancreatic) and hematological malignancies. These phagocytic innate cells are also professional antigen presenting cells, providing a link from innate to adaptive anti-tumor immunity. Preliminary studies have demonstrated that APCs present antigens from phagocytosed tumor cells, causing T cell activation. Therefore, agents that block CD47:SIRP-α engagement are attractive therapeutic targets as a monotherapy or in combination with additional immune modulating agents for activating anti-tumor T cells in vivo.

Background

CD47- A "Don't Eat Me" Signal on Cells

CD47, a transmembrane protein found ubiquitously expressed on normal cells to mark "self" has increased expression in circulating hematopoietic stem cells (HSCs), red blood cells (RBCs), and a high proportion of malignant cells. Although CD47 has multiple functions in normal cell physiology, in cancer it acts primarily as a dominant "don't eat me" signal (Figure 26) (Jaiswal et al. 2009; Chao, Weissman, et al. 2012). On tumor cells pro-phagocytic signals may be present, but if the tumor cells are expressing CD47 it can bind with SIRP-α on phagocytic immune cells preventing engulfment (Figure 26) (Chao, Jaiswal, et al. 2010; Zhao et al. 2011; Chao, Weissman, et al. 2012; Feng et al. 2015). CD47:SIRP-α engagement results in activation of SIRP-α by which phosphorylation of immunoreceptor tyrosine-based inhibition (ITIM) motifs leading to the recruitment of Src homology phosphatase-1 (SHP-1) and SHP-2 phosphatases preventing myosin-IIA accumulation at the phagocytic synapse preventing phagocytosis (Figure 26) (Tsai and Discher 2008). This inhibitory mechanism of CD47 expression is seen in a broad range of malignancies and is therefore an attractive therapeutic target for all tumors expressing CD47 (Kikuchi et al. 2005; Jaiswal et al. 2009; Majeti, Chao, et al. 2009; Chao, Alizadeh, et al. 2010; Chao, Alizadeh, et al. 2011; Chao, Tang, et al. 2011; Zhao et al. 2011; Edris et al. 2012; Kim et al. 2012; Willingham et al. 2012; Tseng et al. 2013; Weiskopf et al. 2013; Mäbert et al. 2014; Soto-Pantoja et al. 2014; Ho et al. 2015). In pre-clinical models, disruption of CD47:SIRP-α axis results in enhanced phagocytosis, tumor reduction, and recently has been demonstrated as a means to cross present tumor

antigens to T cells (Figure 4.1) (Chao, Alizadeh, et al. 2010; Tseng et al. 2013; Soto-Pantoja et al. 2014).

Clinical Translational Advances

CD47 Blocking Agents in Clinical Development

Translation of CD47 blocking therapies into clinical use is new, with no FDA approved drugs. Therefore, little is known about potential side effects, resistance, or what combination therapies will be most efficacious. To date, several academic and industry labs have CD47 blocking agents under development with open enrollment or planned clinical trials in targeting both hematological and solid tumors. In the USA, two phase 1 dose escalation trials are currently underway with anti-CD47 antibodies as a monotherapy for the treatment of advanced solid tumors and hematological cancers (ClinicalTrials.gov Registry Number: NCT02216409, NCT02367196). No outcome data has been reported yet, but those currently in progress will be used to determine the toxicity and maximum tolerated dose with antitumor efficacy as a secondary measurement for this phase. Additional agents being developed within pharmaceutical companies are high affinity SIRP-α mimics including a fusion protein "SIRPαFc" that combines a portion of SIRP-α fused to the Fc region of an antibody to retain ADCP.

Potential Adverse Events or Resistance Mechanisms in CD47 Blockade

In clinical applications, anti-CD47 is not predicted to have adverse drug interactions or side effects. Anticipated off-target effects of CD47:SIRPα blockade include potential allergic reactions to humanized antibodies, or removal of non-malignant CD47 expressing cells (Chao, Weissman, et al. 2012). In particular, CD47 expression on

red blood cells is a mechanism that prevents programed cell removal (Oldenborg et al. 2000). Initial dosing of anti-CD47 is therefore expected to cause a significant reduction in total red blood cell count. To lessen this effect, a priming dose of anti-CD47 can be given to remove "aged" red blood cells and to stimulate erythropoiesis (J. Liu et al. 2015). Newly developed red blood cells should not be significantly affected during the remaining anti-CD47 therapy. If red blood cell counts remain low after priming and during therapy, erythropoietin or transfusions can also be given to alleviate this.

An additional concern of a new drug is potential resistance including loss of expression, amplification, mutations, or alternative non-CD47 related tumor cell adaptations. To date no common mechanisms have been described pre-clinically. Loss of CD47 by tumor cells is not expected, and if this occurs it will mimic the blocking effect of anti-CD47. Amplification will not cause total resistance but may be one mechanism to dampen efficacy if therapeutic antibody dosing is not at an antigen saturating level. Mutations in CD47 may occur, but these need to prevent antibody binding while retaining the CD47:SIRPα interaction. Alternatively, the upregulation of additional "don't eat me" or "immune tolerance" signals can cause resistance (Chao, Majeti, et al. 2012). These changes may be induced after anti-CD47 therapy, or might be present initially and will be selected for during therapy. Identifying the resistance mechanism to anti-CD47 clinically can provide information on additional future drug targets, or combination therapies to reduce the identified resistance.

Identifying Synergistic Combination Therapies with CD47 Blockade

Based on preclinical studies, anti-CD47 (and SIRPαFc) is expected to have efficacy as a monotherapy, with the most promising preclinical data in acute

myelogenous leukemia (Uno et al. 2007; Jaiswal et al. 2009; Majeti, Chao, et al. 2009; Chao, Alizadeh, et al. 2011; Zhao et al. 2011; Kim et al. 2012; Willingham et al. 2012; Tseng et al. 2013; Y. Wang et al. 2013; Ho et al. 2015; Xiao et al. 2015). Combining CD47 blocking agents with additional immunotherapies is believed to further increase efficacy in a wider range of cancers and provide potential cures. A previous study demonstrated that the combination of anti-CD20 (Rituximab) with anti-CD47 therapy cured NSG mice transplanted with human B cell non-Hodgkin lymphoma (Chao, Alizadeh, et al. 2010). Single agents increased survival, but could not induce a full clearance of the transplanted lymphoma. These antibodies synergized by combining Fc receptor (FcR)-dependent tumor targeting (anti-CD20) and blocking CD47:SIRPα (Figure 4.1). In solid tumors, blocking CD47 with a high affinity soluble SIRPα in combination with anti-Her2 provided a significant decrease in total tumor cell volume of breast cancers within the mouse mammary fat pad (Weiskopf et al. 2013). The robust effect of combining these antibodies in both solid and blood malignancies should lead to alternative combination therapies mimicking this method for other cancers.

In particular, combination immunotherapies may provide the proper stimulation to both innate and adaptive immune cells to induce total tumor eradication and lasting anti-tumor T cell memory. Immunotherapies are being developed to increase the anti-tumor response by blocking inhibitory signals, agonizing stimulatory signals, systemic cytokines, vaccinations, or adoptive cell therapy (ACT) with expanded tumor infiltrated lymphocytes (Rosenberg and Restifo 2015; Sharma and Allison 2015b). Combining an increase in phagocytosis/tumor cell removal by CD47 blocking agents should synergize with immunotherapies such as anti-CD40, IL-2, anti-CTLA4, anti-PD-1, anti-PD-L1, or

adoptive cell therapy. CD40 is widely expressed on innate immune cells and when targeted with an agonistic antibody it can directly activate APCs increasing MHC expression and costimulatory molecules that then activate an adaptive immune response through antigen cross presentation (French et al. 1999; Mangsbo et al. 2015). Anti-CD40 has been efficacious when combined with systemic IL-2 which can non-specifically activate T cells in vivo (Murphy et al. 2003). Combining anti-CD47 with anti-CD40 should increase total APC activation and phagocytosis increasing total tumor cell removal, with each antibody targeting two separate pathways in innate immunity. The upregulation of MHC by CD40 stimulation can help phagocytosed tumor antigens be cross presented to naïve T cells (French et al. 1999).

Alternatively, to target both innate and adaptive immunity, anti-CD47 (or anti-CD47/anti-CD40 combination) can be combined with checkpoint inhibitors (anti-CTLA4, anti-PD-1, and anti-PD-L1). Combination of these agents is predicted to increase antigen cross presentation and anti-tumor T cell development. Checkpoint inhibitors have been highly efficacious as single agents or when used in combination with each other, and are able to increase the T cell response by blocking "negative" feedback inhibition (Sharma and Allison 2015b). These checkpoint antibodies have also been tested with co-administration of dendritic cell vaccines or radiotherapy as adjuvants to initiate an immune response (Murata et al. 2006; Twyman-Saint Victor et al. 2015; Sharma and Allison 2015b). The dendritic cell vaccines aim to stimulate and activate naïve T cells by TCR/MHC and costimulatory signaling, while the checkpoint inhibitor blocks T cell exhaustion (van den Eertwegh et al. 2012; Le et al. 2013). A problem with checkpoint inhibitor antagonist antibody treatment is that all active or exhausted T cells will be

stimulated, both anti-tumor and anti-self autoimmune T cells. Anti-CD47 could be added in addition to these regimens to allow macrophages to activate and prime anti-tumor T cells. This could lead to selective amplification of anti-tumor T cells, and subsequent anti-checkpoint blockade antibodies could conceivably be used at lower doses in order to amplify anti-tumor and not amplify anti-self T cells. Within the tumor, anti-CD47 therapy will increase the phagocytosis and these macrophages and may provide additional stimulation for TILs reducing the previously immunosuppressive tumor microenviroment (Swann and Smyth 2007; Soto-Pantoja et al. 2014; Sharma and Allison 2015b); and both these macrophages and TILs are likely to enter draining lymph nodes to amplify existing and to induce new T cell responses. The removal of tumor cells by phagocytosis is also beneficial to reduce total tumor burden. Tumor cells will be engulfed entirely after anti-CD47 allowing for the presentation of tumor antigens on macrophages to T cells in vivo.

Conclusions

CD47 blocking agents are expected to be well tolerated, efficacious, and broadly applicable for cancer therapies. In addition to leukemia, lymphoma, and a host of solid tumors, we demonstrate strong activity of an anti-CD47 blocking monoclonal antibody, Hu5F9-G4, to enable phagocytosis of both colorectal and pancreatic cancers. Furthermore, in multiple xenotransplantion models anti-CD47 therapy we are able to slow growth and reduce tumor burden. Strikingly, Hu5F9-G4 was able to help inhibit or eradicate metastasis in CRC and PDAC tumor bearing mice. For the first time in the solid tumor setting, we were able to demonstrate that an anti-CD47 antibody in

combination with an anti-EGFR antibody is able to dramatically shrink tumors in animal models.

We proposed a few model systems to better understand the role of anti-CD47 therapy as it relates to the adaptive immune system. Initial studies using the Ova model system reveals that an anti-CD47 antibody could preferentially skew towards a cytotoxic T cell response against cancer. To better understand these initial observations, we setup a fully human in vitro system to understand how anti-CD47 mediated phagocytosis could present native cancer antigen NY-ESO-1 to NY-ESO-1 specific CD4 and CD8 T cells. Furthermore, to be able to study downstream T cell responses, we establish a syngeneic model system with the expectation to not only observe how anti-CD47 enabled phagocytosis effects T cell response but to also understand if the addition of checkpoint antibodies could further enhance anti-tumor activity.

As discussed, a strong synergy is predicted when combining CD47 blockade with alternate immunotherapies. If clinical studies reflect the current pre-clinical data, the inhibition of CD47:SIRPα should activate adaptive immunity without directly targeting T cells. Clinical trial and future studies will demonstrate if the proposed therapies with anti-CD47 can truly activate both innate and adaptive immunity to allow for significant tumor reduction, and ideally, cures.

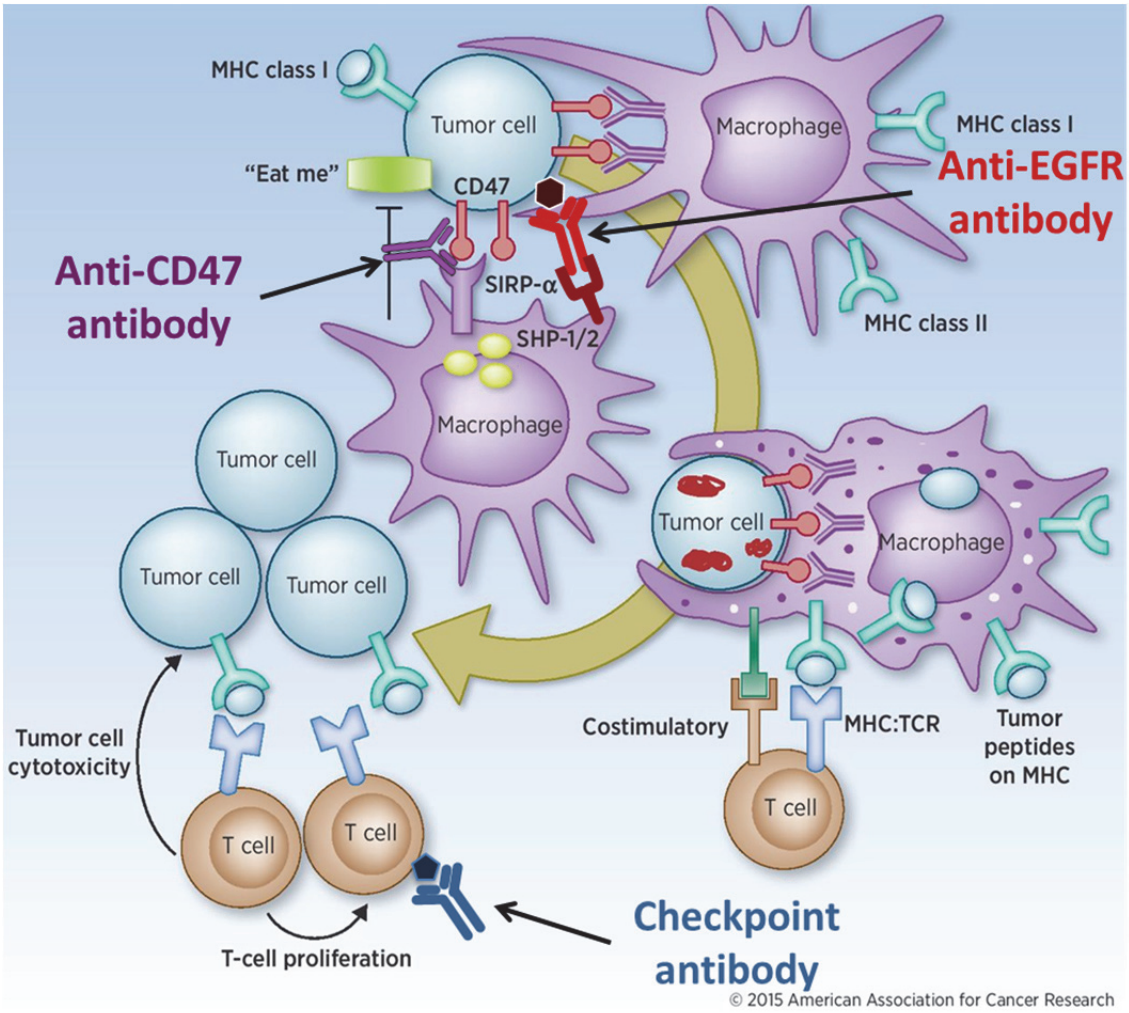

Figure 4.1. CD47's role in both the innate and adaptive immune system. Tumor cells display MHC class I, surface markers of 'self', anti-phagocytic-'don't eat me' and phagocytic-'eat me' signals. Engagement of tumor cells CD47 ('don't eat me' signal) with macrophages SIRP-α causes activation and phosphorylation of SIRP-α ITIM motifs and the recruitment of SHP-1 and SHP-2 phosphatases preventing myosin-IIA accumulation at the phagocytic synapse inhibiting tumor cell phagocytosis. By blocking the CD47:SIRP-α engagement with antibodies (or alternate strategies) an in increase tumor cell phagocytosis by APCs is observed. The engulfed tumor cells are then processed and tumor associated antigens are presented by these APCs on their MHC. The engulfment can be enhanced by additional antibodies targeting the tumor cells, like anti-EGFR, whereby the Fc region binds to the FcR on APCs. Naïve tumor reactive T cells can then engage with MHC on APCs presenting tumor neo-antigens with additional costimulatory molecules. These tumor specific T cells are then activated, expand, and are able to cause antigen specific tumor killing, and maybe enhanced with additional checkpoint antibodies.

APPENDIX

APPENDIX

Table A.1. KRAS mutational status in codons 12 or 13 in all cell lines used in previously described study. The top section lists colorectal cancer lines used in the studies, while the bottom lists pancreatic cancers. WT corresponds to tumor lines that are WT to the KRAS mutation while * correspond to tumor lines with the codon 12 or 13.

CRC	SW620	LS174T	CACO2	SW480	DLD1	CT116	SW48	HT29	UM8
KRAS Mutation Status	*	*	WT	WT	*	*	WT	*	WT

PDAC	CAPAN-1	MIAPaCa-2	Pa03C	CFPAC-1	PA02C	BxPC3	PANC-1	Pa01C	
KRAS Mutation Status	*	*	*	*	*	WT	*	*	

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