R E V I E W

Antibody targeting of phosphatidylserine for the detection and immunotherapy of cancer

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Abstract: Phosphatidylserine (PS) is a negatively charged phospholipid in all eukaryotic cells that is actively sequestered to the inner leaflet of the cell membrane. Exposure of PS on apoptotic cells is a normal physiological process that triggers their rapid removal by phagocytic engulfment under noninflammatory conditions via receptors primarily expressed on immune cells. PS is aberrantly exposed in the tumor microenvironment and contributes to the overall immunosuppressive signals that antagonize the development of local and systemic antitumor immune responses. PS-mediated immunosuppression in the tumor microenvironment is further exacerbated by chemotherapy and radiation treatments that result in increased levels of PS on dying cells and necrotic tissue. Antibodies targeting PS localize to tumors and block PS-mediated immunosuppression. Targeting exposed PS in the tumor microenvironment may be a novel approach to enhance immune responses to cancer.

Keywords: immunosuppression, tumor microenvironment, immunotherapy, imaging, phosphatidylserine, bavituximab

Introduction

Major advancements to our understanding of immune response regulation have led to the development of therapeutic antibody checkpoint inhibitors for the treatment of cancer.[1,2] The approval of the anti-cytotoxic T-lymphocyte antigen-4 (CTLA-4) antibody ipilimumab for the treatment of advanced melanoma in 2011 was the critical turning point to the surge in the development of antibody-based therapeutics to immune checkpoint inhibitors.[3,4] The subsequent development of antibodies blocking the programmed death-1 (PD-1) pathway, first with US Food and Drug Administration-approved agents nivolumab and pembrolizumab and followed by other approved antibodies, has made a significant impact on the treatment of melanoma and other tumor types.[5–11] However, many patients respond weakly or are unresponsive to antibodies targeting immune checkpoints. This has driven new treatment modalities of combining existing therapies and a search to identify other tumor-associated immunoregulatory targets.[12–14]

Rationale for phosphatidylserine (PS)-targeting immunotherapy for cancer

In eukaryotic cells, an asymmetric distribution of phospholipids exists across the bilayer membrane, where the positively charged phospholipids phosphatidylcholine (PC) and sphingomyelin are maintained on the outer membrane leaflet and the negatively charged amino-phospholipids PS and phosphatidylethanolamine are localized

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in the inner membrane leaflet.[15–17] This asymmetry is actively maintained by the regulated activity of ATP-dependent and -independent enzymes, collectively known as flippases, floppases, and scramblases.[18,19] Under normal physiological conditions, PS exposure serves as an "eat me" signal that attracts macrophages for the engulfment of apoptotic cells.[20–22] Viable immune cells including B cells, T cells, monocytes, macrophages, and dendritic cells (DCs) transiently express PS but escape phagocytosis possibly via a PS exposure threshold.[23–25] In the tumor microenvironment, various biochemical pathways associated with apoptosis result in the flipping of PS to the external membrane, including the generation of reactive oxygen species,[26,27] caspase activation,[28] and Ca^{++} influx due to cell activation.[29] Moreover, the interaction between cells with exposed PS and immune cells elicits highly regulated and redundant immunological responses by triggering immunosuppressive pathways that prevent local and systemic immune activation. The immunosuppressive properties of PS exposure weaken innate and adaptive immune responses and subsequently facilitate tumor cell evasion of immune surveillance.[30–32] PS exposure foreshadows, or appears "upstream" of the myriad of immunosuppressive signals that follow, and is recognized as a pharmacologically targetable immunological control point.

PS exposure in the tumor microenvironment is largely contributed to by necrotic tissue and apoptotic cells as a result of pathological conditions or therapy but is also observed on viable endothelial cells[33] and extracellular vesicles derived from tumors,[34] stroma, and leukocytes.[34–36] PS is also exposed on the surface of infectious agents and cells infected with a variety of bacterial, viral, and parasitic pathogens and also creates noninflammatory conditions associated with diminished host immune responses,[37–41] a process termed "apoptotic mimicry".[42] Multiple PS receptors have been identified. These receptors vary in structure, direct or indirect PS binding, cell type expression, and signaling pathways. Most PS receptors are involved in the stimulation of anti-inflammatory responses for quiescent removal of apoptotic cells[22,30] but can contribute to proinflammatory responses under certain conditions.[43] PS receptors of particular importance for PS recognition and immune suppression in the tumor microenvironment are T cell/transmembrane, immunoglobulin, and mucin (TIM)[44] and Tyro, Axl, and Mertk (TAM) gene families.[45,46] Tim-1, Tim-3, and Tim-4 are associated with Th2 stimulation, Th1 receptor-mediated immunosuppression, and apoptotic cell engulfment by dendritic cells and macrophages.[47–51] Members of the TAM gene family are receptor tyrosine kinases (RTKs) expressed on leukocytes and on many tumor types.[52] TAM RTK binding to PS occurs via γ-carboxylated bridging proteins Gas6 or Protein S. The γ-carboxylated GLA domain of Gas6 and Protein S binds directly to exposed PS, and the receptor-binding domain interacts with the TAM receptor. TAM receptor activation on macrophages triggers the engulfment of PS⁺ target cells and promotes an anti-inflammatory "M2" phenotype.[53] The activation of TAM RTKs on tumor cells is linked to chemoresistance and epithelial plasticity.[54] The blocking of vitamin K-dependent γ-carboxylation of Gas6 inhibits Axl activation on tumor cells and reduces tumor progression and metastasis in preclinical tumor models.[55] PS binding to TAM RTKs on tumor cells also upregulates programmed death-ligand 1 (PD-L1) expression on tumor cells.[56] Thus, blocking the PS-mediated activation of TIM and TAM RTK pathways can enhance anticancer immune responses (Figure 1).

The origins of PS targeting

In a study published in 1998, Ran et al[57] covalently linked a monoclonal antibody to murine VCAM-1, a marker of tumor endothelium, to the extracellular domain of the human tissue factor, a protein involved in blood coagulation.[57] This vascular-targeting agent, termed as "coaguligand", was designed to induce thrombosis in tumor blood vessels, resulting in catastrophic destruction of the tumor vasculature. When administered to tumor-bearing mice, the coaguligand-induced thrombosis of VCAM-1⁺ tumor vasculature; however, there was no sign of thrombosis in normal organs, including VCAM-1⁺ vasculature of the lungs and heart. Since PS is required for tissue factor-induced coagulation,[58–60] Ran et al hypothesized that PS was exposed on tumor vasculature but not on the endothelial cells of normal organs. Indeed, PS and VCAM-1 co-localize on tumor blood vessels, whereas regardless of the presence of VCAM-1, externalized PS is not detected on heart or lung vasculature.

Following up on the findings that PS is externalized on tumor vasculature, Ran et al[33] generated a new monoclonal antibody, 9D2, specific to anionic phospholipids. 9D2, an immunoglobulin M, was generated by injecting rats with bEnd.3 endothelial cells that had been treated with hydrogen peroxide, a condition that induced PS externalization. The 9D2 and annexin V did not compete with one another for PS, indicating that they bind different epitopes. Moreover, unlike annexin V, the interaction of 9D2 with PS does not require calcium. The authors found that 9D2 and annexin V specifically localized to tumor blood vessels in mice but not to the endothelium of the 10 normal organs examined. The authors then investigated which factors of the tumor
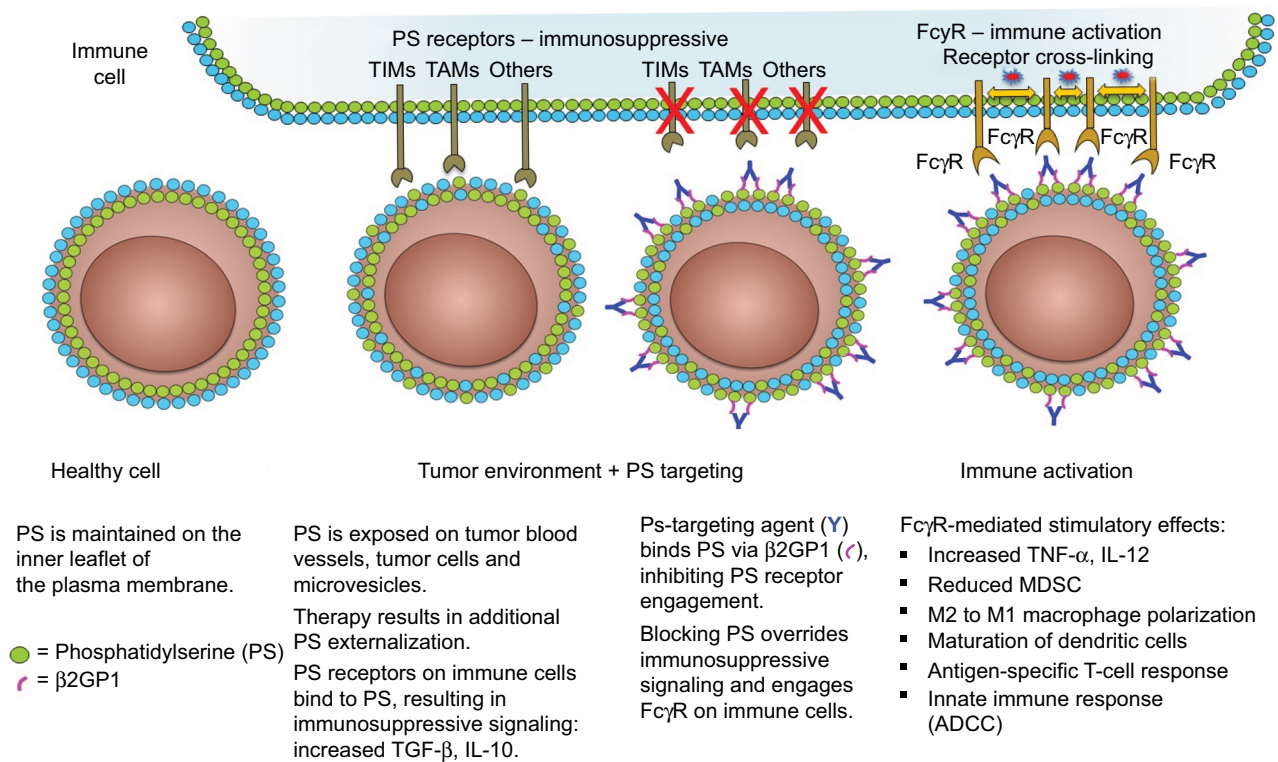
Figure 1 PS-targeting antibodies interact with exposed PS in the tumor microenvironment to activate immune cells.
Notes: PS exposure in the tumor microenvironment activates PS receptors in immune cells and causes these cells to adopt an immunosuppressive phenotype. PS-targeting antibodies 2aG4, 3G4, bavituximab, 1N11, and mch1N11 bind exposed PS via β2GP1. Binding of the PS-targeting complex blocks the interaction between PS and the PS receptors on immune cells and activates these cells via their FcγR.
Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; β2GP1, beta 2 glycoprotein-1; FcγR, Fc gamma receptor; IL, interleukin; MDSC, myeloid-derived suppressor cell; PS, phosphatidylserine; TAMs, Tyro3, Axl, Mer receptor tyrosine kinases; TIMs, transmembranes, immunoglobulins, and mucins; TGF, transforming growth factor; TNF, tumor necrosis factor.

microenvironment might be responsible for endothelial PS externalization. In in vitro experiments, they exposed endothelial cells for 24 hours to various factors that were known to be present in the microenvironment of many tumors. The most potent factor was hydrogen peroxide, causing nearly maximal ^{125}I-annexin V binding. Other factors such as hypoxia/reoxygenation, thrombin, and acidity were found to induce moderate PS externalization. Inflammatory mediators such as IL-1-α, IL-1-β, tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) caused a small increase in PS exposure. The cells remained attached and viable under all the above conditions, and PS asymmetry was re-established after removal of the stressor. The authors concluded by suggesting that anionic phospholipids such as PS may be a target for tumor therapy and imaging. They proposed exploring the idea of using annexin V or antibodies to anionic phospholipids for the delivery of drugs, radionuclides, or coagulants to tumor vessels.

In a subsequent study, the Thorpe Laboratory developed an immunoglobulin G (IgG)-targeting PS and hypothesized that it might exert antitumor activity by damaging tumor vasculature.[61] This new antibody, a mouse IgG3 monoclonal antibody named 3G4, did not bind enzyme-linked immunosorbent assay (ELISA) plates coated with anionic phospholipids in the absence of serum. The binding was rescued when β2-glycoprotein 1 (β2GP1) was added, suggesting that this serum protein is involved in the interaction between PS and 3G4. In vitro binding of 3G4 to PS was blocked with liposomes made from anionic phospholipids but not by those made from neutral phospholipids. The authors found that 3G4 localized to tumor vessels, caused vessel destruction, and inhibited tumor growth in multiple models without causing toxicity. For example, in mice bearing orthotopic MDA-MB-231 tumors, 40% of tumor vessels were bound by 3G4; no staining was observed in normal tissues from 17 organs. A reduction in tumor growth was noted, ranging between 50 and 90%, but complete regression was not observed. The inhibition of tumor vasculature by 3G4 was shown by reduced plasma volume and decreased vascular density. The 3G4 caused monocytes/macrophages to bind to, and destroy, tumor blood vessels in an antibody-dependent manner. This was the first indication that targeting PS altered the immune microenvironment of tumors. In addition, targeting exposed PS on tumor endothelial cells resulted in tumor-associated

macrophages adopting an M1-like phenotype. Based on these data, the authors hypothesized that a chimeric or humanized version of 3G4 might have anticancer activity in humans through antibody-dependent cell-mediated cytotoxicity (ADCC) of PS+ tumor endothelial cells by macrophages. The 3G4 was shown to target PS by dimerizing two molecules of β2GP1, thus stabilizing its interaction with externalized PS (Figure 2);[61,62] β2GP1 (or apolipoprotein H) is a glycoprotein present in the circulation at concentrations of 100–200 mg/mL and consists of four short repeat domains and a fifth domain that contains the phospholipid-binding site.[63,64] The physiological function of β2GP1 remains unknown, although it may function in homeostatic buffering of the coagulation system.

Combining PS-targeting antibodies with chemotherapies

In the second published study on the mouse monoclonal antibody 3G4, Huang et al[65] combined 3G4 with chemotherapy. First, they showed that subtoxic concentrations of docetaxel induced the externalization of anionic phospholipids in endothelial cells in vitro. Additionally, in vivo experiments demonstrated that docetaxel induced enhanced anionic phospholipid exposure in tumor blood vessels, but not in normal organs. The major finding of the study was that 3G4 significantly enhanced the therapeutic efficacy of docetaxel against the growth and dissemination to the lung of MDA-MB-435 human tumors in mice without increased toxicity. Antivascular effects of docetaxel plus 3G4 were

significantly enhanced compared to the individual therapies. Similarly, the authors found more apoptotic endothelial cells in tumors treated with the combination than in those treated with single therapy. The authors concluded by suggesting that clinical evaluation of PS targeting in patients was warranted. Shortly, thereafter, a chimeric version of 3G4, bavituximab, entered clinical trials.

Additional preclinical studies in orthotopic mouse models of pancreatic cancer demonstrated that 3G4 in combination with gemcitabine was significantly more effective at controlling primary tumor growth and reducing metastatic burden than either agent alone.[61] The authors also found that 3G4 alone or in combination with chemotherapy reduced microvessel density and increased macrophage recruitment into tumors. The combination treatment caused a 14-fold increase in macrophage infiltration over controls (single therapies caused 4.2- and 1.76-fold increases over controls). Second-generation PS-targeting antibodies 2aG4 and bavituximab were derived from 3G4, and separately, 1N11 was isolated for subsequent preclinical and clinical studies. Preclinical tumor studies showed that mouse PS-targeting antibodies 3G4, 2aG4, and mouse chimeric 1N11 (mch1N11) (human variable regions of 1N11 fused to mouse IgG2a-kappa constant regions) localize with the same specificity to PS-expressing tumors and tumor blood vessel endothelial cells and elicit strong antitumor effects, especially when combined with treatments that expose PS in tumors.

To better understand how PS targeting functions to reduce PS-mediated immune suppression, Yin et al[32] investigated the

β2GP1 is an abundant serum protein; antibody induced dimerization of β2GP1 increases affinity for PS by >1000-fold
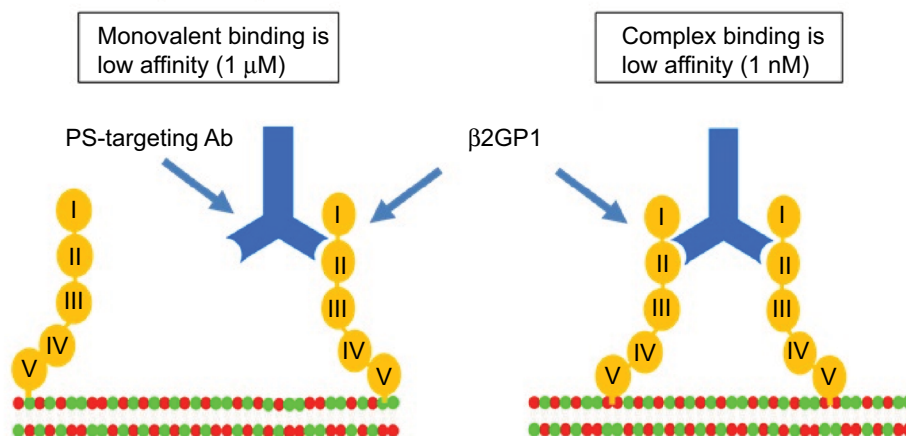

Figure 2 PS-targeting antibodies interact with β2GP1 and PS to form a high avidity complex.
Notes: PS-targeting antibodies 2aG4, 3G4, bavituximab, 1N11, and mch1N11 bind circulating monomeric β2GP1 with low affinity. When sufficient surface exposure is present on a target cell, PS-targeting antibody interacts with two molecules of β2GP1 to form a high avidity (1 nM KD) complex. Domain V of β2GP1 interacts with PS exposed on the target cell. KD, equilibrium dissociation constant.
Abbreviations: Ab, antibody; β2GP1, beta 2 glycoprotein-1; PS, phosphatidylserine.

efficacy of 2aG4, an IgG2a, class-switched version of 3G4, in prostate cancer xenografts. Similar to previous studies, the authors showed that combining PS-targeting antibody 2aG4 with docetaxel effectively inhibited tumor growth in two orthotopic models of prostate cancer. They also observed that M1-like macrophages localized to blood vessel remnants and were likely responsible for their destruction. Tumors treated with a control antibody had fewer macrophages, and the ones present exhibited an M2 phenotype and were not associated with blood vessels. Furthermore, only macrophages isolated from 2aG4-treated tumors synthesized nitric oxide, a characteristic of M1 macrophages. These macrophages were also able to drive ADCC-mediated killing of tumor cells in vitro. Additionally, the authors showed that treatment of tumors with 2aG4 transformed the tumor microenvironment from immunosuppressive to immunostimulatory by showing that tumors from 2aG4-treated mice had a decrease in myeloid-derived suppressor cells (MDSCs), an increase in macrophages and mature DCs, a shift in cytokine balance toward immune stimulation, along with the repolarization of macrophages from M2 to M1 phenotype. This macrophage repolarization could be replicated in vitro by culturing macrophages from PC3 tumors (mostly of the M2 phenotype) with 2aG4. The M1 polarization of macrophages in vitro required the Fc of 2aG4 since 2aG4 F(ab')$_2$ failed to repolarize M2 to M1 macrophages. Although these studies were performed in immunocompromised mice, the authors found a significant increase in the number of mature DCs in tumors from 2aG4-treated mice compared to mice treated with the control IgG. The PS-targeting antibodies discussed earlier have been studied extensively in a variety of rodent tumor models, including breast, hepatocellular, and pancreatic cancers, showing enhanced antitumor responses when combined with standard-of-care chemotherapies utilized for human cancer (Table 1).[65–67]

Combining PS-targeting antibodies with radiation

Irradiation in combination with PS-targeting antibodies also enhances tumor targeting of the antibodies and correlates with increased antitumor responses in mice bearing human lung tumors,[68] an F98 glioma rat brain tumor model,[69] and a B16 mouse melanoma model (Table 2).[70] Combining PS targeting with radiation was first explored in a study by He et al[68] where it was shown that radiation induced PS externalization on human vascular cells (HUVECs) in vitro. Similarly, they observed a 6.5-fold increase in PS-positive blood vessels

in subcutaneous (SC) tumors after irradiation in severe combined immunodeficiency (SCID) mice. A therapy experiment was then conducted in nude mice bearing SC A549 human lung tumors, a relatively radio-resistant cell line. Compared to untreated mice or mice that were treated with 2aG4 alone or radiation + C44 control antibody, the mice treated with 2aG4 and radiation had significantly slower tumor growth. A similar antitumor effect was observed using the H460 human lung carcinoma cell line. Tissue sections from the treated A549 tumors had greatly reduced blood vessel density in the group that received radiation + 2aG4 compared to single agent and control groups. Additionally, tumors from combination treatment were infiltrated by macrophages to a greater extent than those from the three other groups and macrophages were seen in tumor vasculature 48 hours after treatment, suggesting that they are responsible for the decrease in vasculature density. An in vitro ^{51}Cr release assay further supported this possible mechanism by demonstrating that 2aG4 mediates ADCC of irradiated HUVEC by RAW264.7 macrophages. This cytotoxicity was shown to depend on the Fc part of the antibody. Because no additional toxicity was detected, the authors concluded that bavituximab + radiation should be considered for use in patients.

In a subsequent study, He et al[69] investigated the combination of 2aG4 and radiation in an immunocompetent rat model of glioblastoma. Immunocompetent Fischer rats were injected with F98 rat glioma cells into the right caudate nuclei, and these cells developed into a solid tumor invading into the surrounding normal brain. A dose of 10 Gy of radiation to the whole brain of tumor-bearing rats caused the externalization of PS on tumor blood vessels and F98 tumor cells within 24 hours. Normal brain blood vessels were not affected. PS exposure also occurred in vitro following 10 Gy of radiation to endothelial cells. A survival experiment was performed to compare single agent and combination radiation and 2aG4 therapies. Rats receiving combination therapies had a longer median survival than the other groups, and 13% had tumor regressions up to 230 days after tumor implantation, whereas rats from all other groups survived a maximum of 8 weeks following the implantation. Strikingly, rats with regressed tumors rejected a re-challenge of F98 tumor cells into the contralateral hemisphere, suggesting that the rats were immune to F98 tumor cells. When looking at macrophages, the authors saw a large increase in these cells in tumors in the combination therapy group and this was associated with a destruction of tumor vasculature. Additionally, they showed that irradia-

Table 1 Summary of preclinical tumor models evaluating the combination of PS-targeting antibodies with chemotherapy or radiation

Tumor name	Tumor type	PS-targeting antibody/dose regimen	TGI/survival/regression PS targeting	Chemotherapy or radiation	TGI/survival/regression chemotherapy or radiation	TGI/survival/regression combination	Reference
MiaPaca-2, Pan02	Human pancreatic	3G4	MiaPaca-2, 31% TGI; Pan02, 36% TGI	Gemcitabine	MiaPaca-2, 48% TGI; Pan02, 40% TGI	MiaPaca-2, 69% TGI; Pan02, 61% TGI	Beck et al (2005)[66]
F98	Rat glioma	2aG4	0% survival	Radiation 10 Gy	0% survival	13% survival	He et al (2009)[69]
A549, H460	Human lung	2aG4 or 3G4, 100 µg, tiw ×3	41% TGI; 73% TGI	Radiation, 2 Gy ×5 days	14% TGI; 80% TGI	80% TGI; >99% TGI	He et al (2007)[68]
MDA-MB-435	Human breast	3G4, 100 µg, biw	50% TGI	Docetaxel, 10 mg/kg, biw	70% TGI	93% TGI	Huang et al (2005)[65]
BT-474, HCC-1428	Human breast	2aG4, 100 µg, tiw ×4	BT474, 30% TGI, 5.6% reg; HCC-1428, 40.4% TGI	Prima-1, 50–75 mg/kg, 100 µg, tiw ×3	BT474, 50% TGI, 11.1% reg; HCC-1428, 43.5% TGI	BT474, 75% TGI, 27.8% reg; HCC-1428, 66% TGI	Liang et al (2010)[71]
MDA-MB-435	Human breast	3G4, 100 µg, biw	65% TGI	–	–	–	Ran et al (2005)[61]
Meth A	Mouse fibrosarcoma	3G4, 100 µg, biw	90% TGI	–	–	–	Ran et al (2005)[61]
MDA-MB-231	Human breast	3G4, 100 µg, biw	75% TGI	–	–	–	Ran et al (2005)[61]
L540cy	Hodgkin's lymphoma	3G4, 100 µg, biw	50% TGI	–	–	–	Ran et al (2005)[61]
PC3	Human prostate	2aG4, 4 mg/kg, biw	~30%	Docetaxel, 5 mg/kg, biw	~14%	>95%	Yin et al (2013)[32]
TRAMP	Mouse prostate GEMM	mch1N11, 5 mg/kg, biw	0% survival	Castration	0% survival	~50% survival	Yin et al (2012)[115]

Notes: Preclinical models were conducted in immunocompetent or immune-deficient animals with antibodies 3G4, 2aG4, or mch1N11. TGI was determined based on percent inhibition of treatment group compared to control for either the last day of measurement or when animals reached tumor volume limit.
Abbreviations: biw, two times weekly; GEMM, genetically engineered mouse model; PS, phosphatidylserine; TGI, tumor growth inhibition; tiw, three times weekly.

Table 2 Summary of preclinical tumor models evaluating the combination of PS-targeting antibodies with ICI, OV, and ACT

Tumor name	Tumor type	TGI PS-targeting antibody (%)	ICI, OV, or ACT	TGI ICI, OV, or ACT alone (%)	TGI PS targeting + ICI, OV or ACT (%)	Complete tumor regression ICI, OV or ACT; combination (%)	Reference
B16F10	Melanoma	40–58	a-CTLA-4	47	72	ND	Freimark et al[94]
			a-PD-1	42	65		
K1735	Melanoma	29	a-CTLA-4	13	68	ND	Freimark et al[94]
			a-PD-1	69	87		
E0771	Triple-negative breast	55	a-PD-1	71	90	20; 60	Gray et al[95]
			a-PD-L1	42	57	ND	Unpublished observations
		38	a-LAG-3	43	66	0	Gray et al[99]
		38	a-PD-1 + a-LAG-3	36	>99	80	
EMT-6	Breast	0	a-PD-1	0	57	ND	Gray et al[95]
B16F10	Melanoma	ND	ACT	ND	ND	40; 80	Hirschhorn-Cymerman et al[100]
B16F10	Melanoma	33	a-PD-1 + radiation	83	94	40; 60	Budhu et al[70]
MDA-PATC53	PDAC	50	OV	75	88	ND	Dai et al[74]

Note: TGIs were determined on the day that controls reached the maximum allowable tumor volumes.
Abbreviations: ACT, adoptive cell therapy; a-CTLA-4, anti-cytotoxic lymphocyte-associated protein 4; ICI, immune checkpoint inhibitors; LAG-3, lymphocyte activation gene-3; ND, not determined; OV, oncolytic virus; a-PD-1, anti-programmed death-1; PDAC, pancreatic ductal adenocarcinoma; PS, phosphatidylserine; TGI, tumor growth inhibition.

tion and 2aG4 induced hallmarks of adaptive immunity in vitro. The 2aG4 improved antigen presentation by rat DCs and triggered the generation of T cells that secreted IFN-γ in response to F98 cells and showed antigen-specific cell cytotoxicity.

Combining PS-targeting antibodies with immune activators and checkpoint inhibitors

Other novel approaches to induce PS exposure in tumors have demonstrated a combination antitumor effect with PS-targeting antibodies (Table 2). Reactivation of mutant p53 (mtp53) to a wild-type form using the small molecule "p53 reactivation and induction of massive apoptosis" (Prima-1) in nude mice bearing mtp53 human breast cell tumors restored the p53-directed apoptosis pathway and enhanced the exposure of PS in tumors.[71] The mutation of the p53 tumor suppressor gene is the most common genetic alteration in human cancer, and the majority of mtp53 alleles in breast cancer cells are defective in DNA binding, cell cycle checkpoints, and the DNA damage-induced apoptosis.[72,73] The combination of 2aG4 and Prima-1 resulted in greater tumor growth inhibition and tumor regressions than single agents alone.[71] The oncolytic adenovirus Delta-24-RGD conditionally replicates in tumor cells with an abnormal p16/RB/E2F pathway, leading to cell lysis. PS exposure is enhanced in infected cells and increases the antitumor activity when combined with mch1N11.[74]

In summary, various therapies that induce tumor cell damage and subsequent PS exposure have the potential to enhance their therapeutic activity when combined with PS-targeting antibodies. Enhanced immune responses by PS-targeting antibodies are the result of direct killing by ADCC and immune reprogramming of suppressive cell types, such as MDSCs, M2-macrophages, and regulatory T cells (Tregs), with concomitant maturation of DCs and expansion of activated effector T cells. Reprogramming is thought to be mediated by the blockade of PS receptor signaling on immunosuppressive cell types and the engagement of antibody with activating Fc receptors (FcRs) on macrophages and DCs (Figure 1). The contribution of FcR activation is demonstrated by the work of DiLillo and Ravetch,[75] where they have elegantly demonstrated that presentation of tumor antigen:antibody complexes engage with activating FcγRs on DCs, promoting DC maturation, the presentation of tumor antigens to T cells, and the development of long-term immunity. Further studies have demonstrated that FcR uptake of antibody:tumor immune complexes by DCs is important for T-cell cross priming and tumor rejection.[76,77]

In recent years, considerable attention has been given to understanding cancer immunobiology and immunotherapy, driven by the clinical success of inhibitors to immune check-

point PD-1 and its ligands PD-L1 and PD-L2.[78,79] A common signature for responsiveness has emerged, including the presence of CD8^{+} tumor-infiltrating lymphocytes (TILs), PD-L1 expression on tumors and immune cells, IFN-γ production and elevated expression of IFN-γ-induced genes, and high mutational load in tumor cells.[80,81] Despite improvements in patient antitumor responses and survival, only a subset of patients benefits from PD-1/PD-L1 blockers.[82] Several mechanisms of resistance to immune checkpoint inhibition have been identified, some of which have been integrated into decisions for next generation immunotherapies.[2] IFN-γ is also capable of chronic stimulation of signaling pathways in activated T cells, leading to exhaustion[83] and the production of immunosuppressive indoleamine 2,3-dioxygenase 1 (IDO).[84,85] Activated TILs can coexpress multiple immune checkpoints, including CTLA-4, lymphocyte activation gene-3 (LAG-3), and Tim-3,[86–88] resulting in higher immunosuppression than single-positive cells. Chemotherapies and irradiation, both inducers of PS externalization, are well documented to upregulate PD-L1.[89–91] Aside from the tolerogenic signals that develop in the tumor microenvironment, genetic mutations that select for growth or "cancer immuno-editing" are recognized as an immune escape mechanism.[92,93] Based on these and other observations, improvements in immunotherapy of cancer will likely include combinations of these targeted therapies.[2] Recently, studies in immune-competent mice bearing EMT-6 or E0771 breast or B16 melanoma tumors revealed that the combination of PS-targeting mch1N11 and anti-PD-1 (α-PD-1) or anti-CTLA-4 (α-CTLA-4) showed greater antitumor effects than single-agent therapies (Table 2).[94,95] Additionally, mch1N11-based combination therapies enhanced the levels of CD4^{+} and CD8^{+} TILs, elevated the fraction of cells expressing the proinflammatory cytokines including IL-2, IFN-γ, and TNF-α, and increased the ratio of CD8^{+} T cells to MDSCs and Tregs in tumors and spleens. These results are similar to the studies that demonstrated that the combined blockade of PD-1 and LAG-3 was more effective than single-agent therapy.[96–98] LAG-3 is expressed on T cells where it negatively regulates effector T-cell activity. Based on the induction of LAG-3 on T cells by mch1N11 and α-PD-1, the inclusion of anti-LAG-3 to mch1N11 and α-PD-1-targeted therapies increased the tumor growth inhibition to 99% with tumor regressions occurring in 80% of mice.[99] Animals with regressed tumors were resistant to a re-challenge of E0771 tumors but not syngeneic B16 tumors. Gene signatures of tumors obtained from animals treated with the mch1N11, α-PD-1, and anti-LAG-3 triple combination therapy revealed marked increases in antigen presentation pathways and a decrease in tumor growth-

promoting genes. A separate strategy to include PS-targeting antibodies in combination therapies was demonstrated by the adoptive transfer of tumor-specific T cells in a mouse model of melanoma.[100] Adoptive cell therapy (ACT) using ex vivo manipulated, tumor-specific T cells is also subjected to immunosuppressive conditions in the tumor microenvironment. The combination of mch1N11 with melanoma-specific (Trp1) CD4^{+} T cells resulted in the regression of advanced tumors in mice, with fewer side effects than mice treated with the same tumor-specific T cells combined with anti-OX40 antibodies. These results suggest that PS-targeting antibodies can reduce immunosuppression observed in ACT and minimize adverse events. ACTs such as tumor-derived T cells[101] and chimeric antigen receptor T-cell therapy[102] may require blockade of immunosuppressive signals, such as those induced by PS. Taken together, these data show that antibody-mediated PS blockade enhances the antitumor efficacy of multiple targets of immune checkpoint therapy.

Imaging solid tumors with PS-targeting antibodies

The monitoring of PS exposure in tumors is a potential predictor of successful therapy. The detection of exposed PS as an approach to detect tumors is compelling since many forms of treatment, including chemotherapy and radiotherapy, enhance PS exposure on cell membranes of the tumor endothelium and tumor cells.[33,65,68] Histological detection of exposed PS in a biopsy is confounded by specimen collection bias and artefactual exposure of PS in the intracellular compartment. However, the imaging of PS in vivo can potentially be used as a general cancer imaging agent for detection, staging, and treatment monitoring,[103] in particular with therapeutic approaches that induce cancer cell apoptosis.[104,105] The early detection of PS exposure in tumors would provide guidance to continue treatment, and conversely, the lack of enhanced PS exposure could provide a basis to change a therapy. Annexin V has been evaluated as a probe to detect PS but tumor uptake is limited by a short circulating half-life (7 minutes), whereas antibody-based probes with longer half-lives offer the potential for increased tumor uptake.

Full-length PS-targeting antibodies bavituximab and PGN635 (1N11) have been evaluated in preclinical studies as imaging agents. In animal studies, [74]As-labeled full-length bavituximab was successfully used to detect tumors in the R3227-AT1 rat Dunning prostate model.[106] Full-length PGN635 labeled with [89]Zr was evaluated by positron emission tomography (PET) imaging in mouse tumor xenograft models to measure responses to proapoptotic therapies.[107] A high accumulation

of ^{89}Zr-PGN635 was observed in treated tumors undergoing apoptosis, reaching 30% injected dose/per gram tissue and tumor-to-blood ratios of up to 13. Further technologies using PS-targeting antibodies have been developed by attaching 1N11 to liposomes containing magnetic nanoparticles for magnetic resonance imaging for diagnostic evaluation or encapsulated with arsenic trioxide with therapeutic activity.[108,109] The combination of a PS imaging agent together with a therapeutic drug may provide a unique opportunity to simultaneously detect, monitor, and treat tumors. PGN650, a F(ab')$_2$ antibody fragment derived from PGN635, has been used to image human tumor xenografts in mice with near-infrared (NIR) optical imaging and PET. NIR dye-labeled PGN650 injected in mice with SC human U87 glioma tumors had a tumor:normal tissue probe ratio (TNR) of 2.5 at 24 hours post injection.[110] The treatment of SC tumors with 12 Gy irradiation enhanced tumor uptake of NIR dye-labeled PGN650 with a TNR of 4.0 when measured 24 hours following injection. The treatment of mice bearing orthotopic BT-474 human breast tumors with docetaxel enhanced the uptake of NIR dye-labeled PGN650 compared to untreated tumors.[111] Compared to unlabeled PGN650, ^{124}I-labeled PGN650 was shown to have similar binding activities in vitro and to target human PC3 SC and orthotopic tumors in mice as demonstrated by microPET (Figure 3).[112] Histological evaluation of tumor-bearing mice treated with NIR-labeled PGN650 showed that the imaging agent targeted tumor vasculature and tumor cells.[110,111] Based on the results from the preclinical imaging studies, a Phase 0 clinical study was conducted using ^{124}I-labeled PGN650 as a PET imaging agent to monitor safety, pharmacokinetics, radiation dosimetry, and tumor targeting.[113] Patients with solid tumors received ~140 MBq (3.8 mCi) ^{124}I-PGN650 intravenously and underwent PET/CT ~1, 3, and either 24 or 48 hours later to establish tracer kinetics. The safety of ^{124}I-PGN650 was established for human PET imaging; however, the tumor targeting in these patients was less than previously observed in animal studies. An example of whole-body PET imaging of a patient's tumor is shown in a recent publication,[113] which demonstrates the retention of the probe in the tumor and correlates with the targeting of ^{18}F-deoxyglucose, a marker for high cellular metabolism.

Clinical and translational studies of PS-targeting antibodies in cancer patients

Based on data from preclinical efficacy and safety studies, efforts were made to develop a PS-targeting antibody for human trials. Bavituximab is a chimeric monoclonal antibody constructed from the variable region of murine antibody
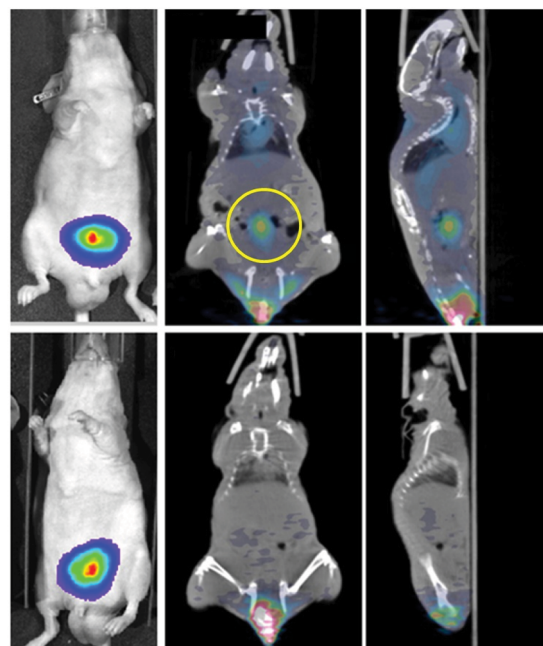

Figure 3 PET imaging of PS exposed in tumors.
Notes: Mice implanted with tumors (PC3-luc) are injected with ^{124}I-labeled 1N11 F(ab')$_2$ (PGN650) or control F(ab')$_2$ probes and imaged by bioluminescence in left panels and PET in middle and right panels. The yellow circle marks the tumor uptake of ^{124}I-PGN650. Reproduced from Stafford JH, Hao G, Best AM, Sun X, Thorpe PE. Highly specific PET imaging of prostate tumors in mice with an iodine-124-labeled antibody fragment that targets phosphatidylserine. *PLoS One.* 2013;8(12):e84864.[112]
Abbreviations: Ctrl, control; F(ab')$_2$, bivalent antigen-binding fragment of an antibody; I-124, iodine 124; luc, luciferase; PET, positron emission tomography; PS, phosphatidylserine.

3G4 and fused to human IgG1 kappa constant regions. Bavituximab and 3G4 target PS in a high-affinity complex with β2GP1 in an identical manner. Bavituximab has been administered to over 700 patients in clinical trials evaluating the antibody as monotherapy and in various combination regimens in patients with multiple tumor types, chronic hepatitis C virus, and HIV infection. To date, studies have shown promising signs of activity and an acceptable safety profile (Table 3). Bavituximab has been evaluated in several investigator-sponsored trials in patients with solid tumors including late-stage clinical trials in patients with locally advanced or metastatic non-small cell lung cancer.

As previously discussed, resistance to checkpoint therapy occurs in up to 70% of patients and is, in part, attributed to low levels of PD-1 and PD-L1 on TILs and tumor cells in the tumor microenvironment.[82] In a translational study utilizing 3D ex vivo-cultured tumor microspheres from non-small-cell lung cancer patients, microspheres expressing low levels of PD-L1 incubated with bavituximab increased the production of immune-activating cytokines (IFN-γ, GM-CSF, and TNF-α) and decreased levels of immunosuppressive cytokine IL-10. In addition, tumor microspheres with low PD-L1

Table 3 Summary of clinical trials evaluating bavituximab in cancer

Indication	Phase trial design	N	Experimental regimen	Results	Reference
Refractory advanced solid tumors	I, single arm, dose escalation; company sponsored	26	Bavituximab monotherapy (0.1, 0.3, 1.0, 3.0 mg/kg)	Well-tolerated, pharmacokinetics support weekly dosing	Gerber et al[116]
Refractory advanced solid tumors	I, single arm; company sponsored	14	Bavituximab + chemotherapy for indication	Well-tolerated in combination	Digumarti et al[117]
Second-line advanced breast cancer	II, single arm; company sponsored	46	Bavituximab (3 mg/kg) + docetaxel	Overall response rate: 61%; median progression-free survival: 7.4 months; median overall survival: 20.7 months	Tabagari et al[118]
Front-line non-small-cell lung cancer	II, single arm; company sponsored	49	Bavituximab (3 mg/kg) + carboplatin–paclitaxel	Overall response rate: 41%; median progression-free survival: 6.0 months; median overall survival: 12.4 months	Digumarti et al[119]
Advanced pancreatic cancer	IIb, randomized, open label; company sponsored	70	Bavituximab (3 mg/kg) + gemcitabine versus gemcitabine	Overall response rate: 28.1 vs 12.9%; median overall survival: 5.6 vs 5.2 months	Pandya et al[120]
Second-line non-small-cell lung cancer	IIb, randomized, double blind;[a] company sponsored	121	Bavituximab (3 or 1 mg/kg) or placebo + docetaxel[a]	Overall response rate: 17.1 vs 11.3%;[a] median progression-free survival: 4.2 vs 3.9 months;[a] median overall survival: 11.7 vs 7.3 months[a]	Shtivelband et al[121]
Second-line non-small-cell lung cancer	III, randomized, double blind; company sponsored	582	Bavituximab (3 mg/kg) or placebo + docetaxel	Manuscript in preparation	
Front-line HER2-negative breast cancer	I, single arm	14	Bavituximab (3 mg/kg) + paclitaxel	Overall response rate: 85%; complete response: 15%	Chalasani et al[122]
Front-line stage IV non-small-cell lung cancer	I, single arm	25	Bavituximab (3 mg/kg) + carboplatin/pemetrexed	Overall response rate: 35%; median progression-free survival: 4.8 months; median overall survival: 12.2 months	Grilley-Olson et al[123]
Front-line hepatocellular carcinoma	I/II, single arm	48	Bavituximab (0.1, 0.3, 1.0, 3.0 mg/kg) + sorafenib	Median time to progression: 6.7 months; median overall survival: 6.2 months	Yopp et al[124]

Note: [a]Placebo and 1 mg/kg bavituximab arms were combined for analysis and compared to 3 mg/kg bavituximab arm.

expression treated with bavituximab expressed increased IFN-γ levels in culture accompanied with an increase in M1 macrophage gene expression with a corresponding decrease in M2 macrophage gene expression.[114] These translational data suggest that PS blockade may increase the proportion of patients who benefit from α-PD-1/PD-L1 checkpoint therapy.

Conclusion

PS is well-recognized as a cell surface marker of apoptotic cells which provides signals to specific receptors for non-inflammatory efferocytosis by phagocytes. The same signals are usurped in the tumor microenvironment by the exposure of PS on tumor blood vessel endothelium and tumor cells, contributing to immunosuppression and tolerance of tumor growth. Specific receptors that bind PS, including TIMs and TAMs, on immune cells and tumors, trigger these immunosuppressive pathways. The uptake of PS-targeting antibodies by tumors is readily demonstrated in preclinical models and initial clinical studies. Multiple preclinical

studies serve as proof of concept that the antibody-mediated blockade of PS in tumors can reactivate innate and adaptive immune responses in the tumor microenvironment. A combination of PS-targeting antibodies with approved immune activating therapies such as chemotherapy, radiation, and immune checkpoint inhibitors (including antibodies targeting CTLA-4, PD-1, and PD-L1) and with novel therapies such as oncolytic viruses has the potential to treat a variety of different tumor types. These data support clinical trial evaluation of the PS-targeting antibody, bavituximab, in multiple oncology indications.

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Disclosure

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