Enhanced phosphatidylserine synthase 1 expression induces polarization of tumor-associated macrophages by externalizing phosphatidylserine on cancer cell surface

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Directed by Professor Byoung Chul Cho

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This certifies that the Master's Thesis of Kim Do Hee is approved.

Thesis Supervisor: Byoung Chul Cho

Thesis Committee Chair: Chae Gyu Park

Thesis Committee Member: Hye Ryun Kim

The Graduate School
Yonsei University

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To my parents,
Wol Seop Kim and Hyang Suk Ha

and my love,
Ji Hwan Yook

" The truth will set you free. "

John 8:32

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I wish everyone's who supported me good health and a happy life.

Sincerly, Do Hee Kim.

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ABSTRACT

Enhanced phosphatidylserine synthase 1 expression induces tumor-associated macrophages polarization by externalizing phosphatidylserine on cancer cell surface

Kim Do Hee

Department of Medical Science
The Graduate School, Yonsei University

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Even though cancer immunotherapy faced a new era with immune checkpoint inhibitors (ICIs) with durable and unprecedented effects, respond rates of patients with non-small cell lung cancer only have been reached to 7-27%. This response rate indicates presence of resistance mechanisms against ICIs. Because of complexity and heterogeneity of tumor microenvironment (TME), the resistance mechanisms were not fully understood. The most abundant and pivotal components in TME are tumor associated macrophages (TAMs), the macrophage population largely derived from bone marrow (BM) precursors and related to M2 anti-inflammatory phenotype. Phosphatidylserine exposed on apoptotic cell surface promotes M2 polarization of macrophage. Non-apoptotic, viable cancer cells expose elevated level of phosphatidylserine on cell surface. However, the mechanisms how cancer cells externalize

phosphatidylserine remain uncertain. Furthermore, not every phosphatidylserine is functionally equivalent and no direct investigation was executed with phosphatidylserine exposed on viable cancer cell surface. As a pilot study, phosphatidylserine synthase 1 (PSS1) expression was profiled in patient transcriptome data. PSS1 expression was enhanced in tumor compared to normal tissue. The high expression of PSS1 was related to high infiltration of macrophages and low infiltration of $CD8^+$ T cells. Therefore, this study hypothesized that cancer cells expose phosphatidylserine by increasing phosphatidylserine synthesis, and the exposed phosphatidylserine induces M2 polarization from infiltrated BM precursors and resident macrophages. To investigate this, PSS1 overexpressing cancer cell line was established. Using established cell line, the differentiation of bone marrow derived macrophage (BMDM) and BM precursor were investigated. Phosphatidylserine exposed by overexpressing PSS1 not only promoted BMDM differentiation into TAM, but promoted BM precursor differentiation into TAMs and Myeloid-derived suppressor cells (MDSCs). Taken together, cancer cell externalize phosphatidylserine on cell surface by enhancing PSS1 expression in order to promote TAM and MDSC polarization.

Keywords: macrophage, immunotherapy, phosphatidylserine, lung neoplasm, microenvironment

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I. INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality and common types of cancer in the world [1,2]. Non-small-cell lung cancer (NSCLC) is the major population of lung cancer patients, accounts for about 80% of primary lung cancer [1,3]. According to their histological feature, NSCLC can be classified into squamous cell carcinoma (LUSD), lung adenocarcinoma (LUAD), and large cell carcinoma [4]. In the last few decades, precision therapy and immune checkpoint inhibitor changed paradigm of lung adenocarcinoma treatment, rolling on standard cures, followed in traditional platinum based chemotherapy, radiotherapy, and surgery [2]. Even though precision medicine and immune check point inhibitors profoundly improved treatment outcome, the 5-year survival rate of NSCLC still remains low (23%) compared to other leading cancer sites [5]. Therefore, efficient treatment approaches are staying in urgent demand [6-9].

Table 1. FDA-approved immune checkpoint blockade therapies

Drug	Target	Manufacturer	FDA approval	Indication
Ipillimumab	CTLA-4	Bristol-Myers Squibb	2011	Melanoma
Nivolumab	PD-1	Bristol-Myers Squibb	2014	Melanoma Classical Hodgkin lymphoma Renal cell carcinoma Squamous cell carcinoma of head and neck CRC Small cell lung cancer Urothelial carcinoma Non-small cell lung carcinoma Hepatocellular carcinoma Cervical cancer
Pembrolizumab	PD-1	Merck	2014	Classical Hodgkins lymphoma Hepatocellular carcinoma Melanoma Non-small cell lung cancer Squamous cell lung carcinoma Squamous cell carcinoma of head and neck Urothelial carcinoma
Atezolizumab	PD-L1	Genentech, Roche	2016	Non-small-cell carcinoma Urothelial carcinoma
Avelumab	PD-L1	Pflzer, Merck KGaA	2017	Merkel cell carcinoma Urothelial carcinoma
Duvalumab	PD-L1	AstraZeneca	2017	Urothelial carcinoma Non-small cell carcinoma
Cemiplimab	PD-L1	Sanofi Genzyme	2018	Cutaneous Squamous cell carcinoma

PD-1, programmed cell death protein; PD-L1, programmed cell death ligand-1; CTLA-4, cytotoxic T lymphocyte-associtaed antigen 4.

Immune checkpoint inhibitor is one of the cancer immunotherapy whose goal is to harness the patient's immune system to recognize and attack tumor cells [10]. This therapy targets immune checkpoints, which is a negative feedback mechanism of the immune system preventing the immune system from onset of autoimmunity [10,11]. For instance, cytotoxic T-lymphocyte antigen 4, also known as cluster of differentiation 152 (CD152) found in regulatory T cells (Tregs) and dendritic cells, is an immunoglobulin superfamily that transmits an inhibitory signal to T cells [12,13]. CTLA-4 exerts its inhibitory function through multiple mechanisms including competition with costimulatory molecule B7 ligands, CD80 and CD86 presented on the antigen presenting cells. Not only antagonizing costimulatory molecules, CTLA-4 directly regulates immunity using cytoplasmic tail interacting with signaling molecules in T cell [14]. Several studies showed that cancer cells persistently expression of CTLA-4 and CTLA-4 expression is related to clinical outcome [15-17]. The blockade of this negative immune regulation provides novel immunotherapy strategy for cancer patients. U.S. 2011, Food and Drug Administration (FDA) approved the ipilimumab, antibody against CTLA-4 as a therapy for cancer patients [18]. On the back of first approval of ipilimumab, antibody against programmed cell death 1 (PD-1), and programmed cell death ligand 1 (PD-L1) were approved as cancer immunotherapy (Table 1) [19-25]. Treatment with Immune checkpoint inhibitor showed unprecedented and durable clinical responses in a wide range of tumor types [18,26-37].

Although treatment of immune checkpoint inhibitor shows promising outcome in multiple cancer types, only subsets of patients respond to therapy whilst some patients initially respond but ultimately relapse. The patients with advanced NSCLC treated with ICI as a first line therapy reported only 7-27% as respond rate in definition for primary resistance as progressive disease (PD) as best response [7,38,39]. These variations in the response to ICIs implicates the existence of resistance mechanisms. Resistance to ICIs can be classified into two groups: (1) tumors that have no respond at all (primary

resistance) and (2) tumors that initially respond, but relapse overtime (acquired resistance) [40-43]. The resistance mechanisms of ICIs are not fully investigated, but cancer-immunity cycle helps the understanding of the resistance mechanisms. The concept of cancer-immunity cycle is a succession of process to establish anti-tumor immunity. This stepwise process is the essential process to control tumor growth. At first, this process initiates by innate immune cells like dendritic cells that uptake neo-antigen, originated from cancer cells, as a result of genomic instability. These innate cells reduce neo-antigen to fragments and bind the antigen to major histocompatibility complex (MHC) to present antigen on their surface. Next, this antigen presenting cells should be migrated into lymph node. At the lymph nodes, antigen presenting cells prime and activate T cells. T cell activation process is supported by three activation signal, recognition of antigen by the antigen-specific T cell receptor, stimulation by co-stimulatory molecules that presented on antigen presenting cells, and cytokines stimulation that secreted by antigen presenting cells such as IL-2. After activation and proliferation, antigen specific T cells migrate from lymph nodes to tumor residue. The chemokines, selectin and integrin are involved in migration. In tumor residue, T cells recognize tumor antigen specifically with T cell receptor. Finally, T cell tumoricidal function occurs supported with perforin and granzyme B [44]. If any defects in this series of steps occur, anti-tumor immunity lose their function, so that the tumor can be tolerant to immune checkpoint inhibitors.

The factor causing resistance also can be classified as intrinsic or extrinsic to tumor cells [42,43,45]. Tumor cell intrinsic resistance mechanisms are related to capacity of cancer cells that present antigen or secrete cytokine. The cancer cells bearing low tumor mutation burden or lack of neo-antigens are hard to be directed by T cells. Cancer types that known as bearing high levels of tumor mutation burden shows the highest response rate to ICIs [46]. Current studies showed that NSCLC and melanoma patients that bearing heavy mutation burden were associated to higher response to anti-PD-1 and

anti-CTLA-4 therapy [47]. Cancer cells can also modulate antigen processing pathway [43,48]. Loss of function mutation of antigen presenting machinery also impairs anti-tumor immune response. For example, gene alteration in β2-microglobulin, components of MHC class I, was related to lower patient survival [47,49]. As well, the NSCLC patients established acquired resistance against ICI treatments showed evolution of neoantigen landscape. Matched analysis of demonstrated that tumor cells eliminated mutation associated neoantigens during drug treatments [50]. Genomic alteration in cancer cells can bring secretion of immuno-modulatory cytokine. For instance, PTEN loss that increases activation of AKT-PI3K pathway, promotes secretion of VEGF and lowers level of T cell infiltration [51]. WNT/β-catenin pathway is also known as related to resistance mechanism against ICIs. Alteration in WNT/ β-catenin pathway leads to defect of CCL4 secretion. The defects of CCL4 secretion give rise to decreased infilteration of CD103+ dendritic cells. Tumor cells can escape from anti-tumor immunity by altering response to cytokine. Loss of function mutation of Janus kinase 1 and 2 (JAK-1, JAK-2) results in loss of response to IFN-γ, associated with both primary and acquired resistance to ICIs [45,52].

The extrinsic mechanisms against ICIs are mainly involved with tumor microenvironment (TME) [53-57]. Tumor microenvironment is the environment surrounding the tumor. In the tumor niche, not only malignant cells exist but blood vessels, immune cells, fibroblasts, pericytes, adipocytes, and the extracellular matrix (ECM) exist and involvedly interact to promote tumorigenesis and evade host immunity. Because malignant cells rapidly proliferating, tumor niche is highly hypoxic, acidic and lack of nutrients. In tumor microenvironment, to supply oxygen and nutrients, pathological angiogenesis is established. The hypoxic condition induces cancer cells to secrete vascular endothelial growth factor A (VEGFA). The endothelial cells sense this cytokine by vascular endothelial growth factor receptor 2 (VEGFR2), results in sprout of new vascular. Genetic aberrations in tumor cells also contribute to neovascularization.

Activation of MAPK, PI3K, and protein kinase C pathway contribute in HIF1α secretion, that activates angiogenesis. However, this dysregulated angiogenesis usually failed to mature and fester hypoxia in tumor microenvironment. Fibroblasts in tumor microenvironment are heterogeneous group that serves several functions supporting tumor growth, called carcinoma associated fibroblasts (CAFs). CAFs promotes angiogenesis by producing VEGF, fibroblast growth factors (FGFs), and platelet-derived growth factor (PDGF) and support Reverse Warburg effect of cancer cells by producing lactate as a product of glycolysis. Also, CAFs are responsible to produce collagens, elastin, proteoglycans, glycosaminoglycans, compounds of extracellular matrix (ECM). ECM blocks cytotoxic CD8+ T cells to infiltrate in tumor residue. Immunosuppressive cells, Regulatory T cells (Tregs), Myeloid-derived suppressor cells (MDSCs), and Tumor-associated macrophages (TAMs) that present in tumor microenvironment lose their cytotoxic activity and maintains suppressive status of anti-tumor immunity by secreting inhibitory cytokines, exposing immune checkpoint inhibitors on their surface, and contributing to modulation of tumor metabolism [58]. As other factors known as related to resistance to ICIs, gut microbiome, and urea cycle have been reported [48,59,60]. However, mechanism of resistance to ICI are not fully understood since the complexity and heterogeneity of tumor microenvironment and cancer cell biology [45,58,61,62].

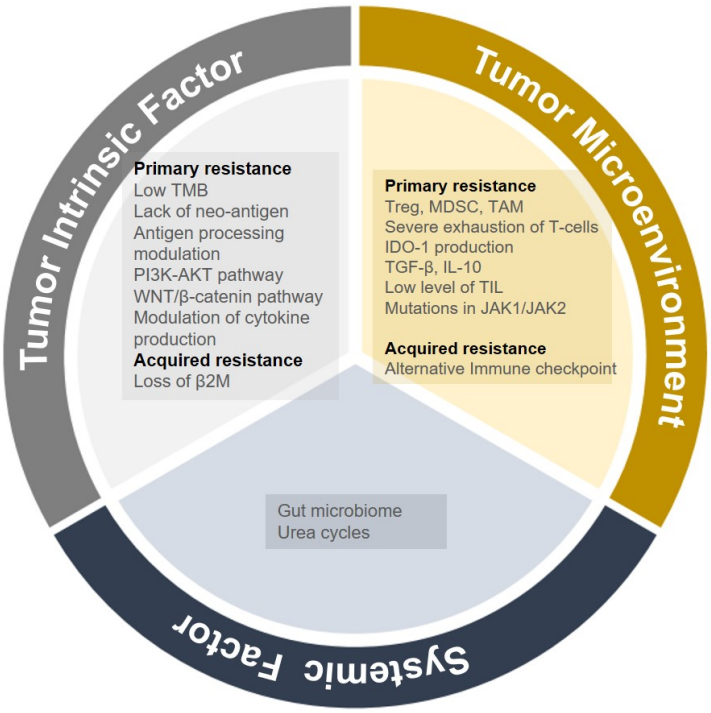

Figure 1. Resistance mechanism against immune checkpoint inhibitor.

The most abundant components of the tumor microenvironment is monocytes and macrophages, form 30-50% of the tumor mass [63,64]. In current study, genome profiling of 15,000 cancer patients data certificated that macrophages were not only the most abundant population, but important biomarker which has strong association to clinical outcomes [65]. In consistence, macrophages in tumor residue promotes tumorigenesis from initiation through to angiogenesis and systemic dissemination [66]. High level of macrophage infiltration in tumor is associated to low response to stand-of-care therapeutics covering chemotherapy, irradiation and angiogenic inhibition [67]. Besides, increased levels of infiltration of macrophage in tumor microenvironment are associated to metastasis [68,69].

In tumor microenvironment, unlikely to conventional antigen presenting cells, macrophages were educated into tumor-associated macrophages (TAMs) by tumor cells [70-72]. While some TAMs were originated from Ly-6G$^+$ circulating monocytes, and some were tissue resident macrophages, a high proportion of TAMs are originated from bone marrow derived monocytes. Cancer cells secrete cytokines and chemokines to recruit monocytes in tumor residue [73]. CCL2 (MCP-1), CCL3 (MIP1α), CCL4 (MCP1β), and CXCL12 (SDF1α) revealed as tumor-derived chemoattractants. IL-6 and colony stimulating factor 1 (CSF1) has been recovered to promote monocyte migration into tumor residue in mouse models [72]. After recruitment into tumor residue, monocytes were differentiated into heterogeneous populations of myeloid derived suppressor cells (MDSCs). MDSCs induce regulatory T cell differentiation and suppress CD4$^+$ T cells, CD8$^+$ T cells and natural killer (NK) cells [74-76]. Monocytic subtype of MDSCs (M-MDSCs) differentiated into TAMs [77].

TAMs commonly express characteristic molecules, haemoglobin scavenger receptor 1 (CD163) and macrophage mannose receptor 1 (CD206) and related to suppression of adaptive immunity. The pivotal immunosuprresive roles of tumor

associated macrophages are expressing anti-inflammatory cytokines that can suppress T cell recruitment and activation [66,72]. For instance, IL-10 and TGF-β from TAMs suppress the anti-cancer immune responses of T cells, NK cells in tumor microenvironment [78,79]. TAMs also expression immune checkpoints on their surface suppressing the anti-tumor immune response executed by T cells. Besides, hypoxia-inducible factors (HIF-1), vascular endothelial growth factor A (VEGF-A), adrenomeullin and basic fibroblast growth factor secreted by TAMs promotes tumor angiogenesis [63]. Besides, metabolic activity of TAMs inhibits cytotoxic T cells, including depletion of arginine, expression of IDO1, production of reactive oxygen species [70-72,79].

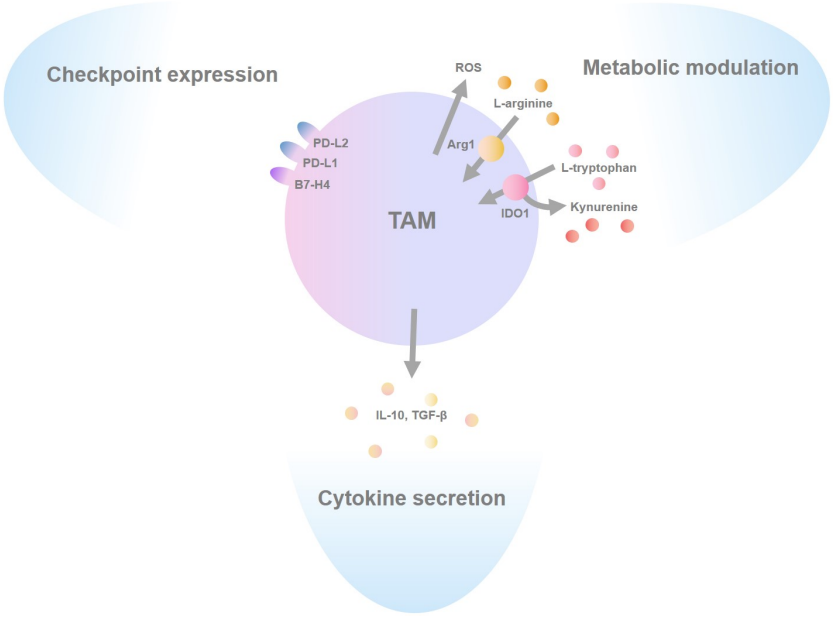

Figure 2. Mechanisms of tumor-associated macrophage mediated T cell suppression.

Macrophages can be categorized into two main groups called classically activated macrophages (M1) and alternatively activated macrophages (M2) based on their polarization status. M1-type macrophages highly produce proinflammatory cytokines such as TNF-α, IL-12 and inducible nitric oxide synthase (iNOS) and express MHCII. In contrast, M2-type macrophages produce IL-6, IL-10, IL-18, transforming growth factor β, arginase and CCL2. The tumor associated macrophages are strongly related to the M2-type polarization [53,80-83]. The cellular stress conditioned generated by tumor cells, including metabolic stress, hypoxia though to influence macrophage polarization. TAM polarization also be driven by anti-inflammatory cytokines, such as IL-10, IL-4. However, the mechanisms polarizing TAMs are largely unrevealed. One of a major mechanism driving macrophage to anti-inflammatory M2 phenotype is phagocytosis [84-91]. Phagocytosis of apoptotic cell drives macrophages to anti-inflammatory phenotype. The coverall signal of apoptotic cell clearance is phosphatidylserine [84]. The recognition of phosphatidylserine by macrophage promotes immuno-suppressive cytokines, IL-10, TGF-β and facilitates expression of indolamine 2,3-deoxygenase 1 (IDO1) that well known as its nature tend immune modulation roles [92]. In the context, the secretion of pro-inflammatory cytokines is downregulated, for instance, IL-6, TNF-α, IL-12 secretion are decreased.

Phosphatidylserine is negatively charged glycerophospholipid in eukaryotic membranes. Glycerophospholipid is composed of glycerol backbone esterified with two fatty acyl chains on their sn-1 and sn-2 site [84]. These fatty acyl chains possess various length and saturation. The factor distinct types of glycerophospholipid is head groups that link to sn-3 site. The covalent attachment of choline, serine, inositol, and ethanolamine on this esterified backbone makes phosphatidylcholine, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine, individually [93]. The synthesis of glycerophospholipid occurs at mitochondrial-associated membranes (MAMs), that refers structure locating between endoplasmic reticulum and

mitochondria. MAMs contains many enzymes including two glycerophospholipid synthesizing homologous enzymes, phosphatidylserine synthase 1 (PSS1), and phosphatidylserine synthase 2 (PSS2). PSS1 converts phosphatidylcholine to phosphatidylserine whether PSS2 converts phosphatidylethanolamine to phosphatidylserine. The synthesis of phosphatidylserine occurs in calcium dependent manner [93]. Osh6p and Osh7p transport phosphatidylserine from MAMs to the plasma membrane, after synthesis from MAMs. Osh6p and Osh7p are included to Oxysterol-binding protein (OSBP)-related protein (ORP) oxysterol-binding homology (Osh) [94,95], fueled by phosphatidylinositol 4-phosphate (PI4P) gradient. PI4P is plentifully localized at plasma membrane and trans-Golgi, rather than endoplasmic reticulum. ORP/Osh proteins exchange PI4P with sterol. After transportation to plasma membrane, phosphatidylserine abundantly localize in membrane facing the cytosol.

Phosphatidylserine serves an essential role in several signaling pathways. In the cytoplasmic leaflet, anionic head of phosphatidylserine provides electronic charge that performs role of binding motif for several molecules performing signal transition. Three major pathways, phosphatidylinositol 3-kinase (PI3k)/Akt, protein kinase C and Ras and Rho family GTPases were defined to be phosphatidylserine-dependent. The protein kinase C and proto-oncogene tyrosine-protein kinase Src contains Ca^{2+} dependent C_2 domains that binds to phosphatidylserine. Phosphatidylserine mediating signal transduction pathways are regard as a critical part in neuropathophysiology, neurotransmitter release and neurotransmitter receptor function [93,94]. Phosphatidylserine also affects conformation of the tau protein and asymmetry loss of phosphatidylserine was observed in Alzheimer disease, implicating association to Alzheimer's disease [94].

Even though phospholipid can flip-flop from one leaflets of membrane to other leaflets of lipid bilayer, the asymmetry of phosphatidylserine is constant because of some transmembrane enzymes contributing to this asymmetry. Flippases are

transmembrane lipid transporter proteins that are members of ABC transporter family. ATP11A, ATP11B, ATP11C, ATP8A1 and ATP8A2 were discovered as transporter of phosphatidylserine in mammalian cells [84,96]. These enzymes transport phosphatidylserine from the outer membrane from the inner membrane in ATP and calcium dependent manner. Phosphatidylserine is the most abundant negatively charged glycerophospholipids, locating at inner side of plasma membrane. The outer layer of plasma membrane is composed of 86% phosphatidylcholine and others. In the case of inner membrane, 47% is phosphatidylcholine and 23% is phosphatidylserine [97]. However, when the cell undergoes apoptosis, the flippase lose their function and phosphatidylserine actively expose to the outer membrane by scramblase. Scramblase is the enzyme whose enzymatic function is antithetic to flippase. Scramblase randomizing all types of phospholipids between leaflets. Transmembrane 16F (TMEM16F) and Xkr8 (ced-8) are found and characterized as scramblases. ATP11C has three caspase recognition site that cut by caspase-3 irreversibly. Xkr8 has low activity in normal condition because of inhibitory sequence of its C-termini but in apoptosis, caspase 3/7 cut this site to activate the enzyme. These results of accumulation of phosphatidylserine to outer membrane of lipid bilayer. After exposure, phosphatidylserine recognized by macrophages and monocytes by interacting with adaptor proteins, gas 6, protein S, and MFG-E8 and stimulating receptors including Tyro3, Axl, Mer, Tim-1, Tim-3, Tim-4, integrin αVβ3, integrin αVβ5, RAGE and BAI1 [98-100]. The externalization of phosphatidylserine is well known eat-me signal that facilitates efferocytosis and tolerate-me signal that prevent local and systemic immune activation stimulated by apoptotic cell [101]. The recognition of phosphatidylserine by receptor for advance glycation end products (RAGE) activates Ras-related C3 botulinum toxin substrate (Rac1) by Diaphanous-related formin 1 (Dia1) [102]. The recognition of phosphatidylserine mediated by MFG-E8 increases SOCS3 expression, followed by decrease of STAT3 phosphorylation [100]. Interaction of phosphatidylserine and BAI1 results in activation of ELMO-Dock180-CrkII complex. ELMO and Dock180 are

promotes exchange of GDP to GTP of Rac. Other phosphatidylserine receptors, stabilin 2 and T-cell immunoglobulin and mucin domains-containing protein 4 (Tim-4) are also known as to lead the activation of Rac. Rac is the GTPase that contributes to change the morphology of macrophage, which are considered to responsible to cell engulfment. The activation of Axl upregulates SOCS1 and SOCS3 results in suppression of JAK-STAT signaling pathway, thereby inflammatory cytokines, TNF-α, IL-1 β, and IFN-α secretions are regulated. Activation of Mertk inhibits NF-κB and reduces IL-1β and TNF-α production. This dominant and evolutionarily conserved immunosuppressive signal has been hijacked by numerous viruses and parasites to evade host immune system [86,87]. Meanwhile, according to the previous reports, viable, non-apoptotic cancer cells display enhanced Phosphatidylserine on the outer leaflet [96,103-107]. Hence, phosphatidylserine is respected as global immunosuppressive signal that drives suppression of tumor microenvironment [84,98,107-110].

However, phosphatidylserines exposed on cell surface are not functionally equivalent [111,112]. The fatty acid composition, saturation, and oxidative status of phosphatidylserine were diverse. And oxidized phosphatidylserine is more efficient to induce phagocytosis by macrophages [113]. In the same context, bridge proteins mediating phosphatidylserine recognition by macrophage, milk fat globule-EGF factor 8 protein (MFG-E8), Gas6 has higher affinity to oxidized phosphatidylserine than non-oxidized phosphatidylserine [113]. Asp-to-Gly point mutation at amino acid position 409 in transmembrane protein 16F (TMEM16F or anoctamin 6), one of the Ca-dependent scramblase, constitutively active this enzyme, results in bidirectionally exchange of phospholipid. The mouse lymphoma cells bearing Asp-to-Gly mutation consistently expose phosphatidylserine on their surface in normal condition. But these cells were not engulfed by mouse peritoneal macrophages and splenic dendritic cell [104]. Viable monocyte and mature macrophage also externalize phosphatidylserine. But, this phosphatidylserine does not induce phagocytosis [114,115].

In oncology field, immune suppression mechanism mediated by phosphatidylserine was investigated by inducing apoptosis to cancer cells [100,114,116,117]. After treatment of TC1 syngeneic mouse model with platinum based chemotherapy, annexin V was treated by intravenous injection to block phosphatidylserine on apoptotic cancer cells recognition. As a result, CD4+ and CD8+ T cells in tumor microenvironment were increased by Annexin V treatment whether regulatory T cells were decreased [117]. In addition, cancer cells are highly express MFG-E8, promoting phosphatidylserine recognition. Treatment of apoptotic cancer cells on macrophage increases M2 polarization compared to non-apoptotic cancer cells. And pre-treatment of anti-MFG-E8 antibody decreased macrophage polarization induced by apoptotic cancer cells [100]. Artificially enhance expose level of phosphatidylserine on B16F10 melanoma cell lines by fusing phosphatidylserine containing liposome with B16F10 cells elevated the engulfment by dendritic cells [116]. Although several studies investigated the immune suppressive function of phosphatidylserine exposed on apoptotic cancer cell surface, the function of phosphatidylserine exposed on viable cancer cell by its nature is elusive. And the function of phosphatidylserine exposed on cancer cell during myeloid cell differentiation has not been explored.

After studies demonstrated that viable cancer cell consistently expressing phosphatidylserine on their surface, several attempts followed to utilize phosphatidylserine as a biomarker of cancer [103,104,106]. Phosphatidylserine targeting antibody (Bavituximab), lysosomal protein, phospholipid Saposin C dioleoylphosphatidylserine (SapC–DOPS), peptide-peptoid hybrid PPS1, PS-binding 14-mer peptide (PSBP-6) and hexapeptide (E3) have been explored for cancer imaging or cancer cell specific cytotoxic effect. Using Bavituximab enables engagement of Fc-γ receptors in tumor microenvironment, thus leading to M1-phenotype macrophage polarization and an increase in pro-inflammatory cytokines [118]. Using liposome containing Sposin C, small, non-enzymatic glycoprotein that induce degradation of fatty

acid to ceramide, apoptosis inducer, has strong affinity with phosphatidylserine, tumor cells were selectively targeted [119-123].

Although, there is sufficient theoretical background of immune suppression function of phosphatidylserine and association between phosphatidylserine and cancer cells, function of phosphatidylserine in tumor microenvironment is explored in part and immune evasion mechanisms mediated by phosphatidylserine exposed on viable cancer cells were elusive [84,110]. Therefore, the goal of this study was to examine the cancer-immune modulation mechanisms mediated by phosphatidylserine externalization. In a previous study, the elevated level of phosphatidylserine synthase 1 (PSS1) expression in cancer was investigated with cancer patient data of The Cancer Genome Atlas. Furthermore, high expression level of PSS1 was related to poor prognosis and higher macrophage infiltration. Considering the machinery sustaining phospholipid asymmetry is flippase enzyme, the increase of total phosphatidylserine might increase the expose level of phosphatidylserine. Therefore, we hypothesized that cancer cells expose phosphatidylserine by increasing phosphatidylserine synthesis, and the exposed phosphatidylserine induces M2 polarization from infiltrated BM precursors and resident macrophages. In this case, cancer cell apoptotic mimicry is camouflage mechanism against macrophage and savotage mechanism that cancer cells taken over and educate immune cells to maintain suppressive status in tumor microenvironment. Clarifying this immune modulation function of phosphatidylserine would be cornerstone of novel therapeutic approach to cancer cell treatment.

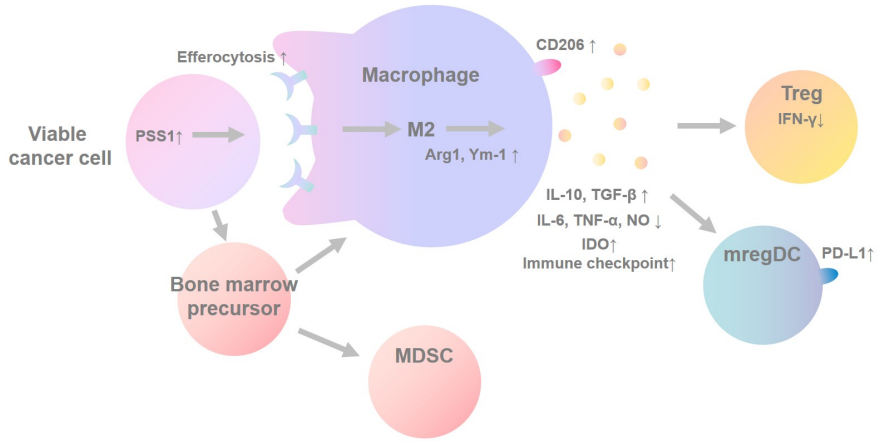

Figure 3. Schematic representation of efferocytosis mediated by phosphatidylserine.

II. MATERIALS AND METHODS

2.1 Cell culture

TC1 mouse lung adenocarcinoma cell line was obtained from the American Type Culture Collection. Cells were cultured in RPMI supplemented with 10% FBS and 5% penicillin and streptomycin. All cells were maintained at 5% CO_2 incubator at 37℃.

2.2 Lentiviral activation particles transduction

TC1 mouse cells seeded 1 x 10^5 per well in 6 well plate. After overnight adherence, media was changed to fresh total medium with polybrene (Millipore Corp, Molsheim, France) at a final concentration of 5 μg/ml. Cells were transfected with 20 μl of Control CRISPR activation plasmid (sc-418211-ACT, Santa Cruz Biotechnology, CA, USA) and PSS1 CRISPR activation plasmid (sc-422474-ACT, Santa Cruz Biotechnology, CA, USA), respectively for 48 hours. After transfection, cells were reseeded and selected with puromycin (Gibco BRL, MD, USA).

2.3 Isolation of bone marrow derived macrophage (BMDM)

Bone marrow progenitor cells were isolated from 6-weeks-old male C57BL/6 mice Femur and tibia. Isolated cells were once suspended and filtered with 20 μm strainer. Bone marrow derived macrophage was differentiated in RPMI supplemented with 10% FBS, 1% AA and 10% L929 cell-conditioned medium for 7 days. Media was changed on day 3 and 5. Cells were cultured in a humidified incubator at 5% CO2 and 37℃.

2.4 *In vitro* phagocytosis assay

Before 24 hours before assay, bone marrow derived macrophages were washed once with PBS and fasted with RPMI without FBS and L929 cell-conditioned medium. 1 x 10^5 bone marrow derived macrophages were seeded in a 6 well plate. After then, 1 x 10^4 CFSE stained mock and PSAT TC1 cells were added. This co-culture system

was incubated for 30 minutes and then cells were collected and stained with fluorescence-conjugated antibodies, APC/Fire 750 anti-mouse F4/80 (BM8, eBioscience) analyzed by Flow cytometry. The number of phagocytic macrophages was estimated by counting the number of double positive cells (CFSE+ APC/Fire 750+).

2.5 Flow cytometric analyses of annexin V binding

Cells were seeded 1 x 10^5 25T flask one day before assay. After 24 hours, cells were trypsinized, resuspended in complete medium, spun down and washed once with PBS and once with annexin V binding buffer. Cells (1 x 10^5) were incubated with 5 μl annexin V FITC (Invitrogen) and 2 μg/ml propidium iodide (PI) in a final volume of 100 μl at room temperature in dark for 15 minutes. AnnexinV FITC binding was measured by flow cytometry after adding 500 μl of annexin V binding buffer, using BD Fortessa. Data was analyzed by BD FACS Diva. For analyzing the annexin V FITC signal from living cells, PI positive dead cells were gated out and annexin V FITC signal was obtained from PI negative forward scattered cells.

2.6 RT-PCR

The total RNA of mock and PSAT was harvested with Trizol reagent (Invitrogen, Carlsbad, USA). The RNA samples were equivalently transcribed to cDNA with reverse transcription premix (Elpis-Biotech, Daejeon, Korea). Synthesis of each cDNA was performed in a total volume of 20 μl for 60 minutes at 42℃ and terminated by incubation for 5 minutes at 94℃. PCR was performed in a 20 μl total mixture containing 100 pM primer pairs, 1.0 μl of the 20 μl total reverse transcription PCR product, PCR buffer, deoxyribonucleotides, and Taq polymerase, according to the manufacturer's recommendations (Bioneer, Daejeon, Korea). Amplications were for 35 cycles, 20 seconds at 95℃ for denature, 30 seconds at 62℃ for annealing, and 30 seconds at 72℃ for elongation. Final elongation time was 5 minutes at 72℃. 5 microliters of the total 20 μl of PCR product was analyzed by 2% agarose gel electrophoresis with safe-pinky

DNA Gel staining solution (genDEPOT, TX, USA). To provide a quantitative control for reaction efficiency, PCRs were performed with primers coding for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers used to detect GAPDH and PSS1 are indicated in Table 2.

2.7 Immunoblotting

The cells were lysed with lysis buffer supported with protease inhibitor PMSF. The cell lysates were centrifuged to remove precipitate. The concentration of protein was determined by Pierce BCA protein assay kit (Thermoscientific, MA, USA), according to manufacturer's protocol. Equivalent amount of total protein was denatured by using sample volume of 5× sample loading buffer (250mM Tris-HCl pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol, 50mM sodium fluoride, and 5mM sodium orthovanadate). The collected samples were separated at 12% SDS-PAGE gel and transferred onto nitrocellulose membranes. The membranes were blocked with 5% BSA in Tris-buffered saline (TBS) for 1 hour at room temperature, and then incubated with primary antibodies in 5% BSA in TBS overnight at 4 °C on a shaker. Primary antibodies used: PSS1 and β-actin were purchased from cell signaling technology (Danvers, Massachusetts, USA). The membranes were washed 3 times with TBST and incubated with the secondary antibodies, diluted in TBS for 1 hour at room temperature. After washing with TBST, the membranes were exposed to enhanced chemiluminescence 10 (ECL) solution to visualization protein. The chemiluminescence signals were captured using LAS-4000.

2.8 Thin layer chromatography

Total cellular lipids from indicated cells were extracted by classical chloroform/methanol extraction, Folch method [124]. Concentration of total lipid was quantified with sulfo-phospho-vanillin (SPV) assay. Equal amounts of lipids were loaded onto a TLC plate and lipids were separated by TLC. Egg yolk PC

(Sigma-Aldrich) and PS (Avanti Polar Lipid) were run as molecular standards. PS was estimated by acquiring TLC band intensities of PS and PC, using ImageJ software.

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2.9 Cell cycle assay

The cells were trypsinized and then fixed with 70% ethanol in PBS and incubated on ice for 30 minutes and then washed twice with ice-cold PBS. Fixed cells were suspensioned with 100 μg/ml RNase A (Invitrogen, Paisley, UK) in PBS. After incubation for 3 hours at 37℃, the cells were stained with propidium iodide (BD Biosciences, Franklin Lakes, NJ) at 25 °C in the dark for 5 minutes. Thereafter, DNA contents of the stained cells were analyzed by a flow cytometer, using BD Fortessa. Data was analyzed by BD FACS Diva.

2.10 Flow cytometry

The isolated cells were washed once in permeabilization buffer (Biolegend, San Diego, CA) and suspensioned in fixation buffer for 1 hour at room temperature according to manufacturer's protocol. After washing with permeabilization buffer, cells were blocked with Mouse BD Fc blocker (BD Bioscience, San Jose, CA) for 15 minutes at room temperature. The cells were stained with fluorescence-conjugated antibodies, mF4/80 (BM8, eBioscience), mCD206 (C068C2, eBioscience), mIL-12 (C15.6, Biolegend), mCD11c (N418, eBioscience), mCD11b (GL1, invitrogen), mLy-6G (1A8, invitrogen). Flow cytometric analyses were performed using BD Fortessa and FlowJo software.

2.11 ELISA

1×10^6 BMDM cells were seeded in 100 mm culture dish and then cultured with 1×10^5 cancer cells for 72 hours. Collected supernatant was analyzed for determining protein level of IL-10, TNF-α, TGF-β. Protein levels for IL-10 were determined by ELISA kit (Mabtech, Nacka, Sweden), TNF-α were determined by ELISA kit (Biolegend, San Diego, California), TGF-β were determined by ELISA kit (R&D system,

MN, USA) according to the manufacturer's instructions. Plates were read at 450 nm in an ELISA reader (SpectraMAX 190 microplate reader).

2.12 Gene set enrichment analysis (GSEA)

To examine the significantly-enriched genes by comparing the transcriptome data from PSS1 highly expressing tumor bearing patient from data of PSS1 lowly expressing tumor, the transcriptome data was prepared by pooling data of TCGA and lung adenocarcinoma patient data from yonsei university. Total 651 patient data was divided to top 10% expressing PSS1 (n=65) and down 10% expressing PSS1 (n=65). The gene sets were downloaded from the Molecular Signatures Database (MSigDB) and C2 (curated gene sets: chemical and genetic perturbation (CGP) and C7 (immunologic signature gene sets) were used to assay. GSA packages in R software was used for analysis.

2.13 Data analysis

Each set of results shown is representative of at least 3 separate experiments. Results are given as means ± SEM. Differences between groups were tested by analysis of variance followed by a post hoc test and an unpaired two-tailed Student's test and considered to be significant when $p<0.05$.

Table 2. Mouse primer sequences RT-PCR

Target gene	Forward 5' → 3'	Reverse 5' → 3'	Amplicon size (bp)
PSS1	GCAGGACTCTGAGCAAGGATG	GGCGAAGTACATGAGGCTGAT	152
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	123

III. RESULTS

3.1 Profiling of PSS1 gene in The Cancer Genome Atlas (TCGA) data

The profiling of the transcriptome data of The Cancer Genome Atlas (TCGA) was performed. The expression level of PSS1 was higher in various cancer types compared to adjacent normal tissue, and statistical significance (p value < 0.05) was shown in BLCA (Bladder urothelial carcinoma), BRCA (Breast invasive carcinoma), CHOL (Cholangiocarcinoma), COAD (Colon adenocarcinoma), ESCA (Esophageal carcinoma), HNSC (Head and neck squamous cell carcinoma), HNSC-HPVneg (Head and neck squamous cell carcinoma-HPV negative), KIRC (Kidney renal clear cell carcinoma), KIRP (Kidney renal papillary cell carcinoma), LUAD (Lung adenocarcionma), LUSC (Lung squamous carcinoma), READ (Rectum adenocarcinoma), STAD (Stomach adenocarcinoma), THCA (Thyroid carcinoma), and UCEC (Uterine corpus endometrial carcinoma) tumor (Fig. 4A). Correlation between PSS1 expression and Tumor infiltrating lymphocytes was determined by analyzing of Tumor IMmune Estimation Resource (TIMER) database (Fig. 4B). Lower infiltration of $CD4^+$ T cell, $CD8^+$ T cell were associated with higher expression of PSS1, whether higher macrophage infiltration was related to high expression of PSS1. The clinical outcome of patients highly expressing PSS1 was worse than patients lowly expressing PSS1 (Fig. 4C).

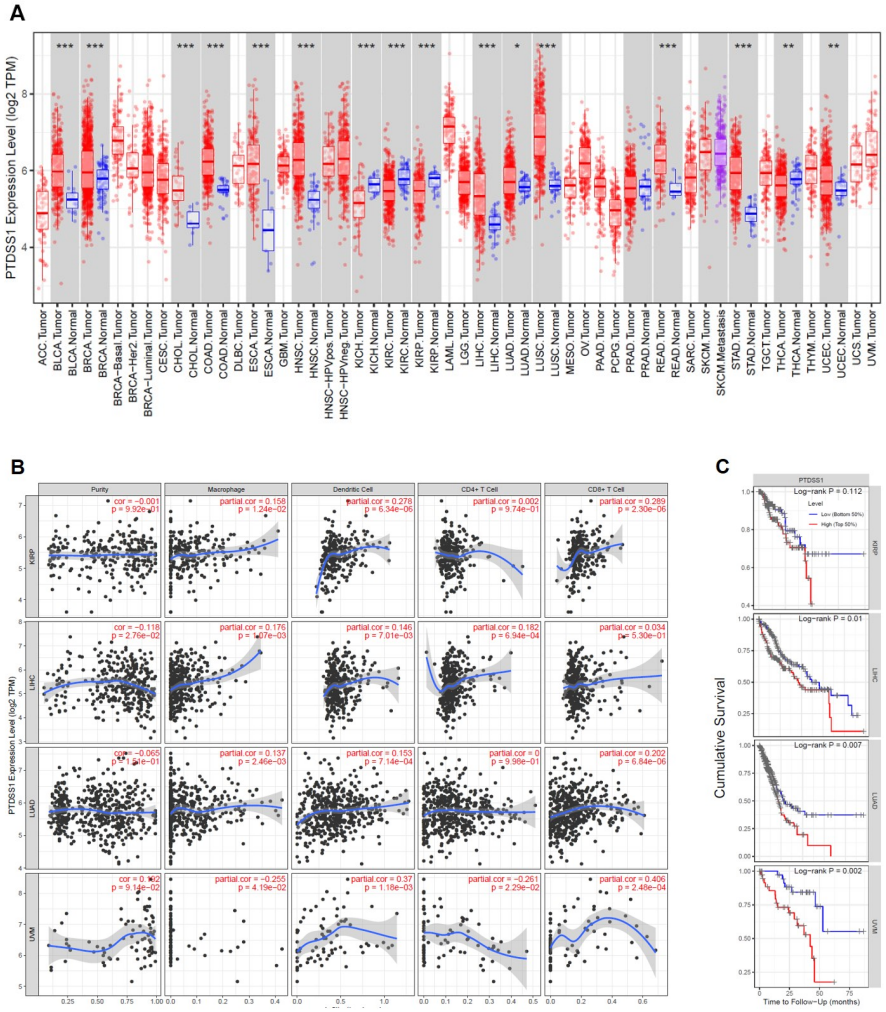

Figure 4. Profiling of PTDSS1 (PSS1) gene in TCGA data. (A) Differential expression of PSS1 between tumor and adjacent normal tissues from various types of cancer. Data were extracted from the Tumor Immune Estimation Resource web server (*P <0.05, **P <0.01, ***P <0.001 tumor vs adjacent normal tissue). (B) Correlation between PSS1 expression and tumor purity, the count on macrophage, dendritic cell, and $CD8^+$ T cells were presented. (C) Kaplan-Meier curves comparing the high and low expression of PSS1.

3.2 PSS1 highly expressing tumors are enriched in gene sets associated to immune suppression.

To further investigate correlation between PSS1 gene and immune landscape, the Gene Set Enrichment Analysis (GSEA) was executed. The lung adenocarcinoma patient transcriptome data were classified into the top 10% (n=65) and down 10% (n=65) of PSS1 expressing tumor (Fig. 13A). C2 (curated gene sets: chemical and genetic perturbation (CGP)) and C7 (immunologic signature gene sets) gene sets were used for analysis. The PSS1 highly expressing tumor was enriched with gene expression feature of regulatory T cells compared to conventional T cells (Enrichment score: 0.647, $P < 0.001$) and TGF-β treated $CD4^+$ T cells compared to untreated $CD4^+$ T cells (Enrichment score: 0.558, $P < 0.001$) (Fig. 13B). PSS1 lowly expressing tumor had positive enrichment with gene set of hallmarks of inflammatory response (Enrichment score: -1.446, $P < 0.001$) and hallmarks of TNF-α signaling pathway (Enrichment score: -1.555, $P < 0.001$) (Fig. 13C).

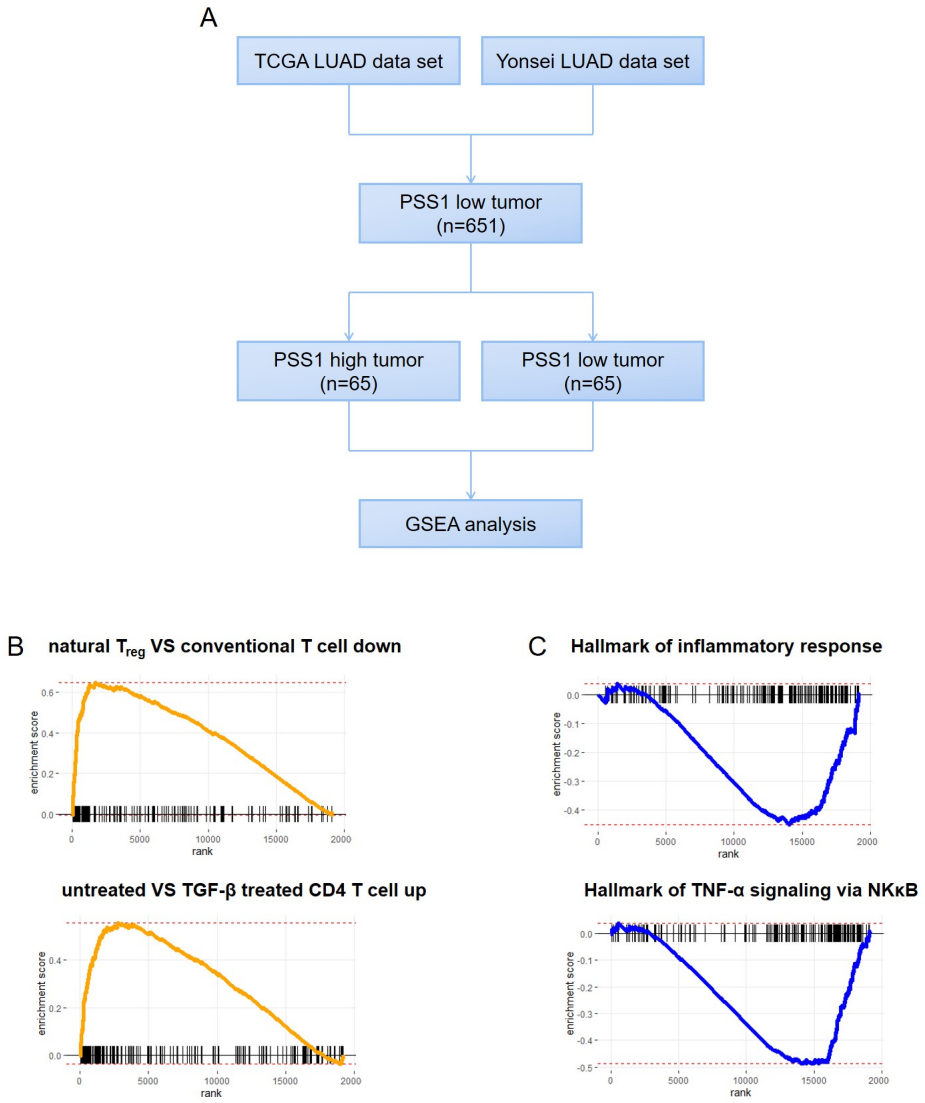

Figure 5. The Gene Set Enrichment Analysis (GSEA) of the transcriptome data from lung adenocarcinoma patients. (A) Transcriptome data of lung adenocarcinoma patients was prepared by pooling TCGA data and patient of yonsei university data. (B) PSS1 highly expressing tumors were enriched with gene features of immune suppression. (C) PSS1 lowly expressing tumors were enriched with gene features of inflammation.

3.3 Generation of PSS1 overexpression cell line

In order to address the function of PSS1 in cancer cells, we established PSS1 overexpressing cell line using CRISPR activation plasmid (Fig. 5A). Mock CRISPR activation plasmid was used as transfection control. TC1 mouse lung cancer cell line was utilized for PSS1 gene engineering. After transfection, gene expression level of PSS1 was quantified with RT-PCR. PSS1 mRNA transcription level was higher in PSS1 CRISPR activation plasmid transfected TC1 (PSAT TC1) compared to mock CRISPR activation plasmid transfected TC1 (mock TC1) (Fig. 6A). Translation level of PSS1 was determined by immunoblotting. PSS1 protein level was enhanced in PSAT TC1 cells compared to mock TC1 cells (Fig. 6B). Total amount of phosphatidylserine and phosphatidylcholine was profiled by thin layer chromatography (TLC). Total lipid was extracted from mock TC1 cells and PSAT TC1 cells and concentration of the total lipid extraction was quantified by sulfo-phospho-vanillin assay. Equivalent amount of lipid was loaded for TLC profiling. Signal intensity of phosphatidylserine and phosphatidylcholine was analyzed by ImageJ software. As shown in the TLC profiling, Phosphatidylserine per phosphatidylcholine ratio was higher in PSAT TC1 compared to mock TC1 (Fig. 6C).

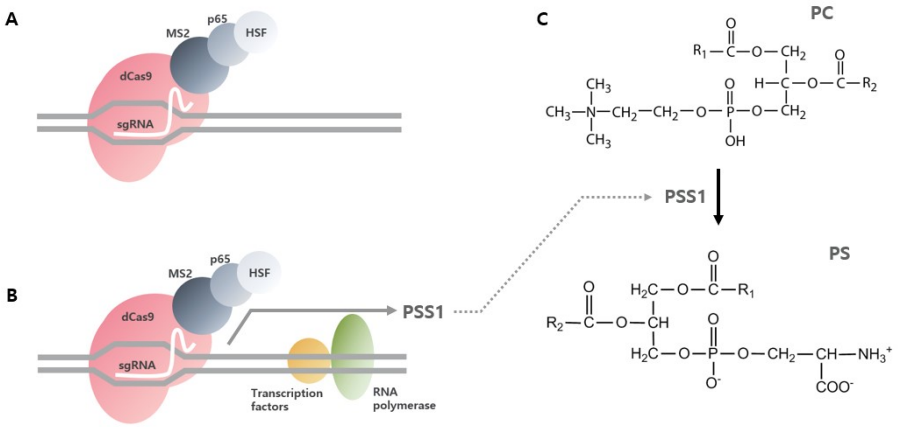

Figure 6. Design of CRISPR activation system in mamalian cell to enhance the epression of PSS1 at the level of transcription. (A) D10A- and N863A-deactivated Cas9 (dCas9) nuclease fused to upstream of PSS1 gene. The MS2-p65-HSF1 fusion protein binds to dCas9 and then function as transcriptional activators of PSS1 gene. (B) By applying the CRISPR activation system, mRNA transcription of PSS1 increases endogenously. (C) PSS1 catalyzes synthesis of phosphatidylserine from phosphatidylcholine.

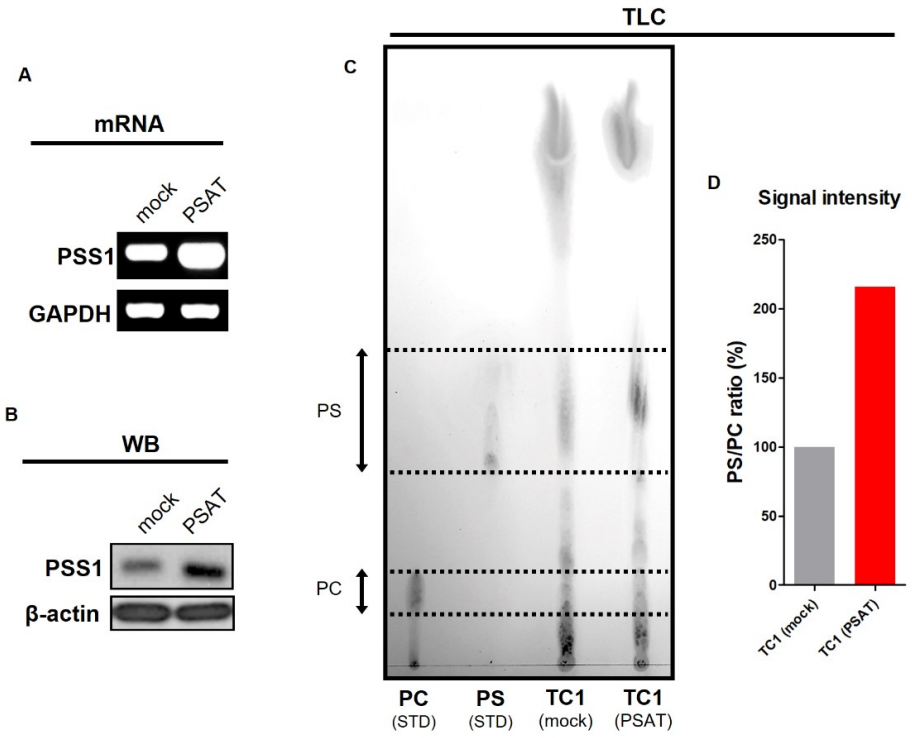

Figure 7. Transfection of PSS1 CRISPR activation vector with mouse lung adenocarcinoma cell line TC1 enhances the expression of PSS1. (A) mRNA expression level of PSS1 was addressed by conventional RT-PCR. GAPDH was used as loading control. (B) Immunoblot analysis of PSS1 expression in mock and PSAT TC1 cells. β-actin was used as loading control. (C) Total phospholipid was profiled by thin layer chromatography (TLC). Purified egg yolk Phosphatidylserine (PS) and egg yolk phosphatidylcholine (PC) run as molecular standards. (D) Signal intensity ratio of phosphatidylserine per phosphatidylcholine was determined by using ImageJ software. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

3.4 Increase of phosphatidylserine induces surface externalization of phosphatidylserine

We estimated that amount of phosphatidylserine affects the exposure level of phosphatidylserine. To measure the amount of externalized phosphatidylserine, mock and PSAT TC1 cells were analyzed by flow cytometry with FITC-labeled annexin V and propidium iodide (PI). To exclude the dead cells from the analyses, FITC fluorescence levels were determined in PI negative gate (Fig. 4A). In contrast to mock TC1 cells, PSAT TC1 cells exhibited higher level of surface phosphatidylserine (Fig. 6B).

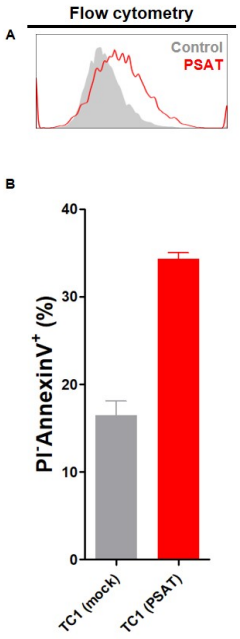

Figure 8. Overexpression of PSS1 in TC1 increases externalization of phosphatidylserine. (A) Geometrical mean fluorescence signal of Annexin V binding analyses defined expose level of phosphatidylserine on cell surface. (B) Bar graphs depicting the Annexin V^+ PI^- cancer cells.

3.5 Enhanced level of surface phosphatidylserine in PSAT TCl cells is not associated with apoptotic signal

Since phosphatidylserine externalization is signal of apoptosis, there is need to confirm that phosphatidylserine exposed on PSAT TC1 cell surface is not the apoptotic signal. Using flow cytometry, cell cycle distribution of mock and PSAT TC1 cells were determined by detecting the DNA contents of the cells. When the cell undergoes apoptosis, nuclears are condensed, apoptotic bodies are formed and cell shrinkage are occurred. Both mock TC1 and PSAT cells did not shown these apoptotic features (Fig. 7A-C).

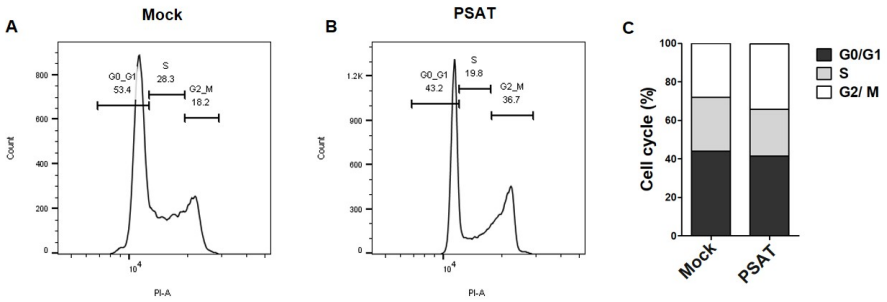

Figure 9. Flow cytometry analysis to investigate cell cycle distribution of mock and PSAT TC1 cells. (A) Representative flow cytometry profiling of the mock TC1 cell cycle distribution. (B) Representative flow cytometry profiling of the PSAT TC1 cell cycle distribution. (C) The percent of each cell cycle phase was presented by bar graph. Data represent the mean of three independent experiments.

3.6 Phosphatidylserine externalized on PSAT TC1 surface promotes efferocytosis

To confirm that macrophages recognize the phosphatidylserine exposed on cancer cells as a ‘eat me’ signals, mock TC1 and PSAT TC1 cells were stain with CFSE fluorescence and then co-cultured with bone marrow derived macrophages (BMDMs). PSAT TC1 cells were more engulfed by BMDMs, compared to mock TC1 cells (Fig. 10B). Furthermore, phosphatidylserine receptor, Tim3 expression was increased in BMDMs cultured with PSAT TC1 cells compared to mock TC1 cells (Fig. 10C).

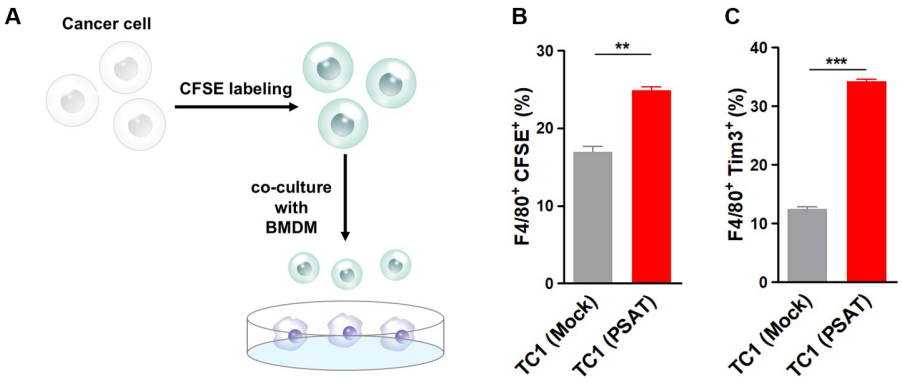

Figure 10. Macrophage recognition of phosphatidylserine exposed on cancer cell surface. (A) Cancer cells were stained with CFSE fluorescence and then co-cultured with bone marrow derived macrophage. (B) The engulfment level of cancer by macrophages were determined in F4/80, CFSE double positive cells. (C) Expression level of Tim3 on BMDM cell surface was determined in F4/80 positive gate.

3.7 Phosphatidylserine externalized on PSAT TC1 surface promotes M2 macrophage polarization

BMDMs were polarized for 4 days with mock TC1 and PSAT TC1, individually. After polarization, cells were collected and analyzed by flow cytometry. CD206 expression was analyzed as M2 macrophage marker, in F4/80 positive gate. As shown in Figure 10, macrophage cultured with PSAT TC1 showed higher M2 population compared to mock TC1.

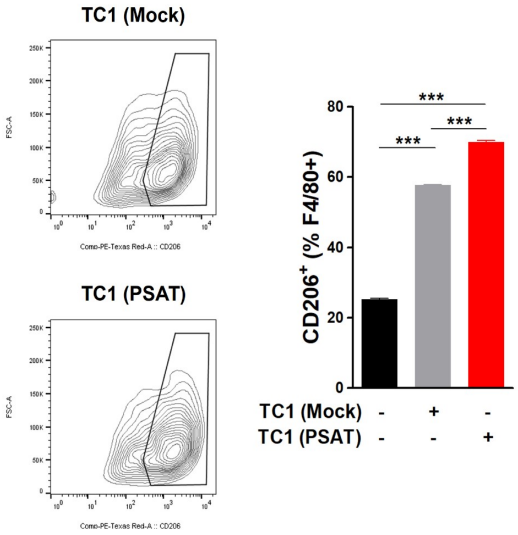

Figure 11. Macrophage populations was assessed by Flow cytometry after co-cultured with cancer cells. CD206 expression level was determined in F4/80 positive gate as a M2 macrophage marker.

3.8 Phosphatidylserine alter the cytokine production pattern of macrophage

To define functional change of macrophage polarized by the phosphatidylserine, pro-inflammatory and anti-inflammatory cytokine secretion were investigated. The level of IL-12 production was determined in F4/80 positive gate. IL-12 production was regulated by externalized phosphatidylserine (Fig. 11A). Cytokine secretion level of TNF-α, and TGF-β in cell-supernatant was also investigated by ELISA. TNF-α secretion was down regulated in macrophage treated with PSAT TC1 cells than treated with mock TC1 (Fig. 11B). The secretion of IL-10 and TGF- β was promoted by externalized phosphatidylserine on viable cancer cell surface (Fig. 11C, 11D).

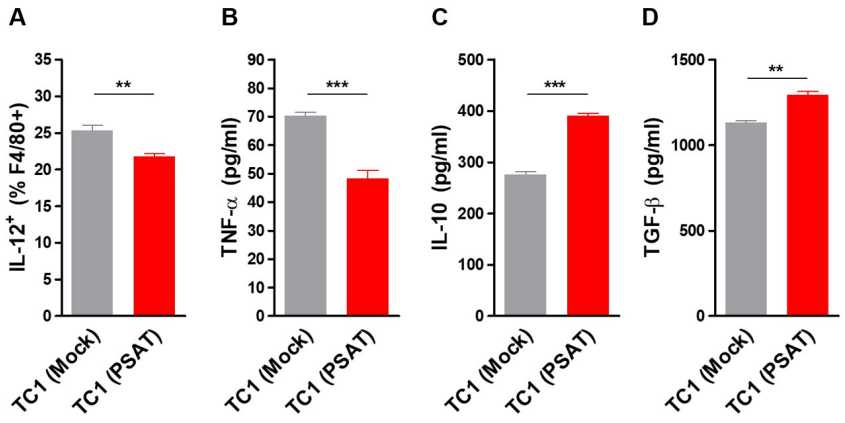

Figure 12. Cytokine produced by bone marrow derives macrophage after cultured with mock TC1 and PSAT TC1 was investigated. (A) IL-12 expression level was investigated by flow cytometry assay. IL-12 positive population was determined in F4/80 positive gate. Protein levels of TNF-α (B), IL-10 (C), TGF-β (D) in cell-supernatants were determined by ELISA.

3.9 Phosphatidylserine promotes BM precursors differentiate into TAM and MDSC

To investigate phosphatidylserine mediated cancer cell immune editing, exploration of myeloid cell differentiation induced by PSAT TC1 cells and mock TC1 cells was executed. Without any stimulation, BM precursor cells co-cultured with mock TC1 and PSAT TC1 after isolated from femur. After 4-day differentiation, myeloid population was determined by flow cytometry. As a result, BM precursor cells co-cultured with PSAT TC1 were more efficiently differentiated to macrophages compared to BM precursor cells cultured with mock TC1 cells (Fig. 12A). Next, M2 polarization of macrophage was investigated. Not only percent of M2 macrophage compared to total cell was higher in macrophage cultured with PSAT TC1 cancer cells, M2 type macrophage population was increased compared to macrophage population. (Fig. 12B) Next, MDSC differentiation induced by PSAT TC1 cells and mock TC1 cells were investigated. Granulocytic myeloid-derived suppressor cell (G-MDSC) population was determined in CD11b, Ly-6G double positive gate and monocytic myeloid-derived suppressor cell (M-MDSC) was determined in CD11b positive, Ly-6G negative gate (Fig. 12C). Both M-MDSC population and G-MDSC population were higher in bone marrow precursor cultured with PSAT TC1 cells compared to mock TC1 cells.

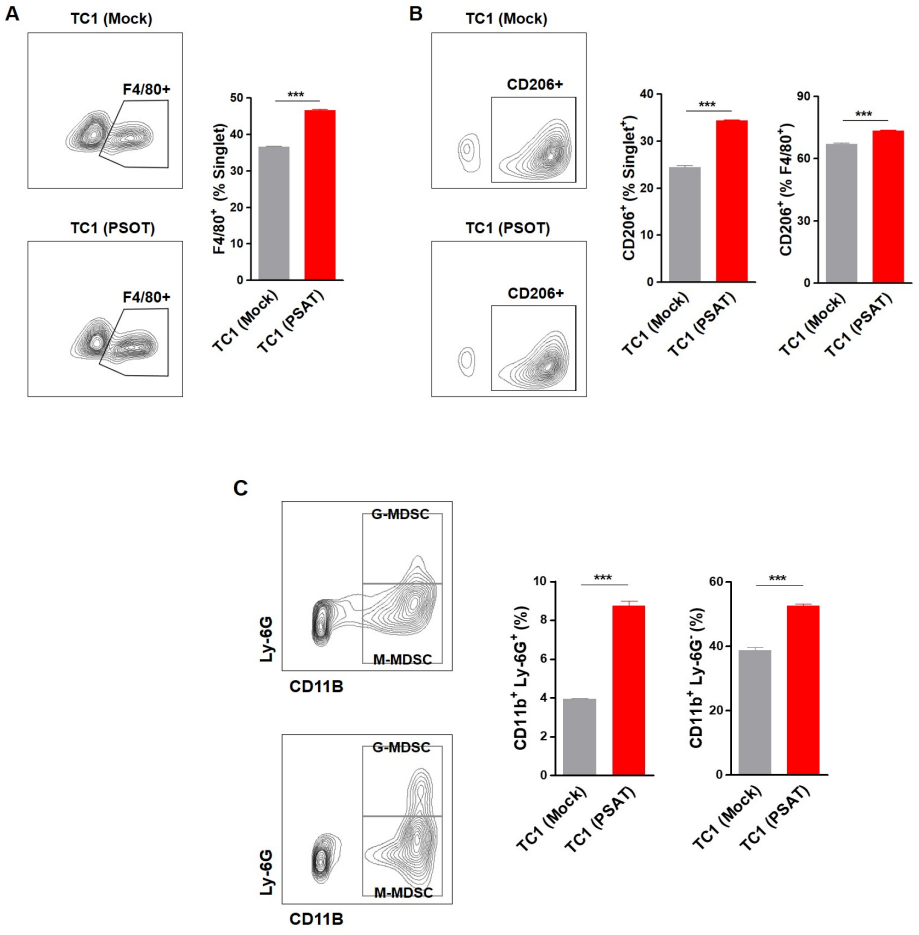

Figure 13. Myeloid cell differentiation was investigated by Flow cytometry. (A) Macrophage differentiation of myeloid cells were determined as F4/80 positive gate. (B) F4/80 and CD206 double positive cells are identified as M2 positive cells. M2 type macrophage differentiation compared to total myeloid cells and total macrophage were presented. (C) Monocytic MDSCs and granulocytic MDSCs population was analyzed by using CD11B and Ly-6G as surface marker. Abbreviations: G-MDSC, granulocytic myeloid-derived suppressor cell; M-MDSC, monocytic Myeloid-derived suppressor cell.

IV. DISCUSSION

Even though ICIs changed paradigm of cancer treatment with promising and durable treatment effect, the response rates still low [7,38,39]. Because of complexity of TME, the resistant mechanisms were not fully understood. TAMs are most pivotal and suppressive population in TME. Phosphatidylserine is well-preserved immune modulation mechanism, directly affecting macrophage. Tumor cells expose elevated level of phosphatidylserine on their surface. The profiling of PSS1 gene in cancer patient data of TCGA revealed that PSS1 gene expression was higher in tumors than in adjacent normal tissues. Besides, patients with tumor highly expressing PSS1 showed poor prognosis. High expression of PSS1 gene was associated to immune suppression. Higher PSS1 expression in tumor was associated to higher macrophage infiltration and lower $CD4^+$ T cell and $CD8^+$ T cell infiltration. Therefore, this study hypothesized that enhanced phosphatidylserine in cancer cell increase externalization level of phosphatidylserine and as a result, macrophage and BM precursors are induced to differentiate into TAMs and MDSCs, promoting tumor growth and immune evasion.

Previous studies indicate that not every phosphatidylserine exposed on cell surface are functionally equivalent [111,112]. Asp-to-Gly point mutation at amino acid position 409 in transmembrane protein 16F (TMEM16F or anoctamin 6), one of the Ca-dependent scramblase, constitutively active this enzyme, results in bidirectionally exchange of phospholipid. The mouse lymphoma cells bearing Asp-to-Gly mutation consistently externalize phosphatidylserine on their surface in normal condition. But these cells were not engulfed by mouse peritoneal macrophages and splenic dendritic cell [104]. Besides, the fatty acid composition, saturation, and oxidative status of phosphatidylserine were diverse. And oxidized phosphatidylserine is more efficient to induce phagocytosis by macrophages [113]. In the same vein, bridge proteins mediating phosphatidylserine recognition by macrophage, milk fat globule-EGF factor 8 protein

(MFG-E8), Gas6 has higher affinity to oxidized phosphatidylserine than non-oxidized phosphatidylserine [113]. Some viable monocyte and mature macrophage also externalize phosphatidylserine on their surface, but these phosphatidylserines do not induce phagocytosis [114,115]. To address the function of phosphatidylserine exposed by increasing PSS1 expression, gene engineering of PSS1 with CRISPR activation system was applied to TC1 mouse lung cancer cell line. The transfection of CRISPR activation system enhanced the phosphatidylserine synthesis. Increase of phosphatidylserine enhanced externalization of phosphatidylserine. Co-culture system revealed the phosphatidylserine externalized by PSS1 gene engineering promoted cancer cell engulfment by BMDMs.

Several studies of phosphatidylserine mediated immune suppression mechanism in oncology field were executed by inducing apoptosis in cancer cells [100,116,117]. After treatment platinum based chemotherapy to TC1 syngeneic mouse model, apoptotic cells were enriched in tumor. Annexin V was treated on this model to block phosphatidylserine on apoptotic cancer cells. As a result, $CD4^+$ and $CD8^+$ T cells in tumor microenvironment were increased by Annexin V treatment whether regulatory T cells were decreased [117]. In addition, cancer cells are highly express milk fat globule EGF factor 8 (MFG-E8), promoting phosphatidylserine recognition. Treatment of apoptotic cancer cells on macrophage increases M2 polarization compared to non-apoptotic cancer cells. And pre-treatment of anti-MFG-E8 antibody decreased macrophage polarization induced by apoptotic cancer cells [100]. Besides, differentiation of the macrophage and BM precursor induced by phosphatidylserine exposed on cancer cell surface has not been explored. Co-culture of mock TC1 and PSAT TC1 with BMDMs revealed that phosphatidylserine exposed on cancer cell surface promotes M2 polarization. Furthermore, the phosphatidylserine promoted IL-12 and TNF-α secretion and regulated IL-10 and TGF-β secretion. Co-culture of mock TC1 and PSAT TC1 with BM precursors revealed that the phosphatidylserine exposed on cancer cell surface promotes BM precursors into TAMs, M-MDSCs and G-MDSCs.

After several studies demonstrated that viable cancer cell consistently expressing phosphatidylserine on their surface, various attempts followed to utilize phosphatidylserine as a marker of cancer [103,104,106]. Phosphatidylserine targeting antibody (Bavituximab), phospholipid Saposin C dioleoylphosphatidylserine (SapC–DOPS), lysosomal protein, PS-binding 14-mer peptide (PSBP-6), peptide-peptoid hybrid PPS1, and hexapeptide (E3) have been explored for cancer imaging or cancer cell specific cytotoxic effect. Bavituximab targets beta-2-glycoprotein Ⅰ which establish complex with phosphatidylserine. Using Bavituximab enables engagement of Fc-γ receptors, tumor specifically. As a result, M1-phenotype macrophage polarization was promoted and an increase in pro-inflammatory cytokines [118]. Using liposome containing Sposin C, small, non-enzymatic glycoprotein that induce degradation of fatty acid to ceramide, apoptosis inducer, has strong affinity with phosphatidylserine, tumor cells were selectively targeted [119-123]. However, there is no biomarker for phosphatidylserine targeting drugs. Because the mechanism of PSS1 and phosphatidylserine mediated immune suppression was defined in this study, PSS1 can be a biomarker for phosphatidylserine-targeting drugs. Furthermore, the existing drugs utilize phosphatidylserine as target molecule to specifically target tumor cells. However, PSS1 and phosphatidylserine itself can be a novel target for cancer treatment.

V. CONCLUSION

Cancer cells externalized phosphatidylserine by increasing PSS1 expression. Enhanced phosphatidylserine on cancer cell surface promoted TAM and MDSC differentiation.

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APPENDICES

ABSTRACT (IN KOREAN)

포스파티딜세린 합성 효소 1 발현 증가는 종양 세포 표면에 포스파티딜세린을 외부화시킴으로써 종양 관련 대식세포의 분화를 유도한다.

< 지도교수 조병철 >

연세대학교 대학원 의과학과

김도희

면역 관문 억제제의 개발로 종양의 면역 치료는 새로운 국면을 맞았으나 면역 관문 억제 치료에 반응을 보이는 비소세포폐암 환자 군의 비율은 7-27%에 그쳤다. 이는 면역 관문 억제제에 대한 종양의 내성 기전이 존재함을 암시한다. 이러한 내성 기전은 종양미세환경의 복잡성과 이질성 때문에 규명되지 않은 부분이 많다. 종양미세환경에서 가장 풍부하게 존재하면서 중심이 되는 역할을 하고 있는 요소는 종양 관련 대식 세포로 골수 전구세포로부터 항 염증 표현형인 M2와 유사한 표현형을 지니고 있다. 그러나 종양이 대식세포를 억제적인 표현형으로 전환시키는 기전에 대해서는 규명되지 않은 바가 많다. 세포자연사 시 세포 표면에 노출되는 포스파티딜세린은 M2 분화를 촉진한다. 한편, 종양세포는 세포자연사에 돌입하지 않았음에도 포스파티딜세린을 표면에 많이 노출시키고 있는 것으로

밝혀졌다. 그러나 종양세포가 어떻게 포스파티딜세린을 표면에 노출시키는지에 대해서는 밝혀진 바가 없다. 또한 모든 포스파티딜세린이 기능적으로 동등하게 작용하지도 않는다. 종양 환자의 RNA 발현을 분석한 결과 포스파티딜세린 합성 효소의 전령 RNA 발현이 종양 세포에서 증가하여 있었고 포스파티딜세린 합성 효소의 발현이 높은 종양은 대식세포의 침투도가 높은 한편 $CD8^+$의 침투도가 낮았다. 따라서 본 연구는 종양세포가 포스파티딜세린의 합성을 증가시킴으로써 포스파티딜세린의 표면 노출을 증가시키고 노출된 포스파티딜세린이 대식 세포와 골수 전구 세포의 종양 관련 대식세포로의 분화를 유도할 것으로 가설했다. 이를 조사하기 위하여 포스파티딜세린 합성 효소 유전자를 과발현시킨 종양 세포주를 제작하여 포스파티딜세린 합성 효소의 과발현이 대식 세포와 골수 전구 세포의 분화에 미치는 영향을 조사하였다. 포스파티딜세린 합성 효소 발현 증가에 의해 노출된 포스파티딜세린은 마크로파지의 종양 관련 대식세포로의 분화를 촉진했을 뿐만 아니라 골수 전구 세포가 종양 관련 대식 세포와 골수 유래 억제 세포로 분화하도록 유도하였다. 따라서 종양 세포는 종양 관련 대식 세포와 골수 유래 억제 세포의 분화를 촉진하기 위해 포스파티딜세린 합성 효소 발현을 증가시킴으로써 포스파티딜세린의 표면 노출을 증가시킨다.

핵심되는 말 : 대식세포, 면역치료, 포스파티딜세린, 폐암, 종양미세환경