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Hypothesis

MASKING ANTI-PHAGOCYTIC SIGNAL OF TUMOR BY PRO-PHAGOCYTIC SIGNAL-A KEY TO IMMUREMENT OF CANCER CELL

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ABSTRACT

Immune surveillance is a mechanism where cells and tissues are watched constantly by ever alerted immune system. Most incipient cancer cells are recognized and eliminated by the immune surveillance mechanism, but still tumors have the ability to evade immune surveillance and immunological killing. One greater arm that tumor use to evade immune surveillance, is by expressing anti-phagocytic signal (CD47). Here we present a provocative hypothesis where cancer cells are removed alive by phagocytic cell (DC). That in turn will elicit effective and higher immunogenic condition. All this could be possible by addition pro-phagocytic signal (PtdSer) over cancer cell surface (Breast Cancer), that mask the presence of anti-phagocytic signal (CD47). In other words, adding eat me signal (PtdSer) over the breast cancer cell surface that mask the presence of don't eat me signal or anti-phagocytic signal present in breast cancer cell surface. This could be possible by using bi-specific antibody, conjugated to PEG-modified liposomes, which carry (PtdSer) pro-phagocytic signal (or) eat me signal, which target both CD47 and EGFRVIII on breast carcinoma. The simultaneous masking of anti-phagocytic signal, and adding of pro–phagocytic signal over cancer cell, will enhance the phagocytic clearance of live tumor cell and elicit immunological killing.

Keywords: Phagocytosis, CD47, EGFRVIII, Phagocytic cells, PtdSer, Immunological killing

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INTRODUCTION

Tumor immune surveillance is the leading mechanism, for defense against cancer. The ever alerted immune system recognizes cancerous and/or precancerous cells on the basis of expression of tumor specific antigens (or) molecules, induced by cellular stress. To a large extent, nascent tumors are identified and eliminated by the immune system via phagocytosis, before they cause harm [1]. Despite tumor immune surveillance, solid tumors do grow and avoid recognition, and complete clearance from alerted immune system, via macrophage-mediated phagocytosis thereby evades immune-mediated eradication. One of the important mechanism, by which cancer cells evade immune destruction (macrophage-mediated phagocytosis) is by enhancing increased expression of CD47 (don't eat me signal or antiphagocytic signal) [2].

CD47 is a cell surface transmembrane glycoprotein, in the immunoglobulin superfamily, involved in T-cell and dendritic cell activation [3], cell migration [4] and axon development [5]. In addition, it acts as a ligand for a signal regulatory protein–α (SIRP-α) expressed on macrophages and dendritic cells [6]. The interaction between CD47 and SIRPα initiates signaling events which lead to inhibition of phagocytosis of the viable host cells [7, 21]. Normal cells by the expression of CD47 protect themselves from phagocytosis and also avert phagocytic mediated cell death. [8, 9]. For example, CD47 is highly expressed on tumor cells, such as AML (Acute myeloid leukemia) [10], CML (Chronic myeloid leukemia) [2], NHL (Non-Hodgkins lymphoma) [11], bladder cancer and various solid tumors [12, 13] compared with its low expression in normal cells [7]. In spite of the expression of CD47 in tumor and normal cells, immunotherapy using anti-CD47 antibody selectively eliminates the tumor cells and spare the normal cells [10, 11, 14]. Thus, anti-CD47 antibody blocks a negative phagocytic signal, however a positive phagocytic signal is still needed for phagocytosis. The selective phagocytosis of tumor cell is determined by expression of a pro-phagocytic signal(s) on tumor cells that is absent on normal cells.

Human hematological malignancies and solid tumors express the pro-phagocytic signal calreticulin (CRT) on the cell surface, while their normal counterpart does not [15]. (fig. 1 and 2). Anti–CD47 antibody targeting of tumor cell depends on both the blockade of anti-phagocytic CD47 signals and exposure of pro-phagocytic CRT signals. In addition to calreticulin, the dying cell possesses other pro–phagocytic ligand such as phosphatidylserine [14]. All these data present reasonable grounds to suggest that phagocytic removal of apoptotic cell requires ligand–receptor interaction of pro-phagocytic signal and inhibition of anti-phagocytic signal brought via CD47-SIRPα.

Hypothesis

Here, we present a provocative hypothesis to phagocytose live whole breast cancer cell. According to our model, scavenger cell can effectively and specifically remove live immunogenic breast cancer cell, provided addition of pro-phagocytic signal (PtdSer) on to the surface of breast cancer cell. In other words, labeling PtdSer over the anti-phagocytic signal would mask the anti-phagocytic signal in tumor cell and simultaneously add weightage to pro-phagocytic signal on the cancer cell. This could be a novel approach to immurement of the whole breast cancer cell by phagocytosis and in turn lead to induction of effective anti-tumor T cell response.

Evaluation of hypothesis

Evasion of immune destruction

Highly immunogenic tumor evades immune destruction by diverse mechanisms. One such key mechanism is immune tolerance which is avoiding induction of effector cells and avoiding the encounter with host effector cells by secreting anti-inflammatory and immune-suppressive factors. The tolerance that naturally exists to prevent autoimmune disease also participates in favor of the cancer cell to avoid rising of the immune response against their antigen. In general, tolerance to any given antigen is possible by two broad mechanisms, i.e. deletion mechanism and the non-deletion mechanism [16].

Deletion mechanism involves complete removal of antigen-reactive cells. One such antigen reactive cell is T-cell clone, which is generated against a tumor antigen. Genetic and epigenetic changes in the genome of normal tissue, give rise to tumor and tumor antigen i. e the antigen expressed is its own origin-normal self-antigen. To this antigen, a deletional tolerance of T-cell clone exists either

centrally or peripherally [16]. This tumor antigen-specific reactive T-cell is killed or inhibited by immune suppressive cytokines such as interleukin (IL-10) and transforming growth factor beta (TGF-β) secreted by a tumor cell, and expression of apoptotic inducing factor fas ligand, resulting in immune evasion [20, 21]. Furthermore, T-cells and NK cells that express receptors for ligand RCAS1 (tumor associated antigen) expressed by tumor are eliminated by apoptosis [22, 23].

Non deletion mechanism involves failure in recognition of antigen specific T-cell, due to insufficient activation stimuli of T-cell and dysfunction of TCR signaling pathway. This is brought by decreased expression of TCR zeta chain and loss of Syk tyrosine kinase in T-cell infiltrating tumors [24, 25]. Localization of tumor is also an important factor for immunological tolerance as certain tumors are not accessible to circulating T cells, by which it hides its presence [26]. The availability of regulatory T cell CD4+ CD25+ within the tumor plays a key role in tumor escape by suppressing T-cell response against tumor [27-29]. Antibody dependent cell mediated cytotoxicity (ADCC) is evaded by cancer cells by expressing self MHC class I molecule, which interacts with killer cell inhibitory receptors (KIRs) expressed on NK cells. The interaction between Class I MHC in tumor and KIRs in NK cell, leads to activating NK cell's KIRs. The activated KIRs recruit and activates SHP-1 (Src-homology-2-domain-containing protein tyrosine phosphatase 1) and or SHP-2 (Src-homology-2-domain-containing protein tyrosine phosphatase 2) that down regulate FC receptor signaling i.e. CD16, a membrane receptor for the carboxyl–terminal end of the IgG molecule, known as FC region. As a result, NK cell unable to attach to the antibody generated against the antigen expressed by the tumor cells, which make NK cells not to secrete substances such as lytic enzyme, TNF, perforin and granzyme, which are involved in target cell destruction.

There exists another prominent inhibitory membrane receptor SIRPα [30] in phagocytic cell such as dendritic cell, macrophages and granulocytes. SIRPα works in similar fashion that of the KIRs to avoid antibody mediated tumor cell destruction **[**8, 83]. Upon engagement of SIRPα with CD47 (anti-phagocytic signal) in tumor, results in intercellular signaling in bi-directional manner by forming homo-dimer [31, 32] of CD47 and SIRPα. The intercellular signaling generated in macrophages that contain SIRPα, leads to phosphorylation of the tyrosine residues on SIRPα's immune receptor tyrosine based inhibitory motif (ITIM) in macrophages. This in turn activates, Src homology domain containing protein, tyrosine phosphatases SHP-1 and SHP-2. Activation of SHP-1 and SHP-2 in macrophage can in turn dephosphorylate specific protein substrates and thereby regulate cellular function in a negative fashion. One such example is the deactivation of myosin–II and the contractile cytoskeletal activity, which is involved in pulling a target into macrophage [33] thus phagocytosis process is hindered. In contrast, the SIRPα act as a ligand to CD47 in tumor cell and or normal cell (neurons) and promote activation of CDC4, a member of Rho family of small GTP binding protein [34]. However, it is unclear with the molecular components that generate downstream signaling and its outcome.

CD47 and EGFRVIII–A good scaffold for PtdSer

It is well proposed that CD47 expressed by the tumor cell act as a protective mechanism against phagocytosis and other immune effector functions. CD47 not only helps the tumor to prevent phagocytosis but also aid the tumor in its growth, dissemination, metastasis and poor clinical outcome [14, 35].

It has been shown that the expression level of CD47 progressively increases with tumor progression. The cancer genome anatomy project has shown that CD47 expression in renal and breast cancer is considerably increased. Unfortunately, the CD47 is not tumor specific, it is mild and ubiquitously expressed in a majority of normal tissues [2, 36] to protect the normal cell from phagocytosis.

Thus, modulating the CD47 and or its interaction with SIRPα, not only helps in destruction of tumor by phagocytosis mediated cell death, but also enhances the clinical outcome. The very common strategy that widely exists in cancer immunotherapy is to generate monoclonal antibody against CD47 [37] and or recombinant SIRPα [38] to block "don't eat me" signal. As CD47 is expressed in normal cell, the blocking might possibly generate an adverse effect. In the case of erythrocytes, which require CD47 expression for its survival, [39] but the blocking of CD47 might cause severe side effect, including anemia. But, fortuitously it has been shown that blocking [10, 14, 15] the interaction of CD47 with SIRPα, with the help of anti–CD47 antibody, rules out tumor cells but not the normal cells. The possible explanation is the presence of pro-phagocytic signal calreticulin (CRT) in tumor cell and not in normal cell [15] shown in fig. 1 and 2. The positive phagocytic signal dominates the negative phagocytic signal, when CD47 is blocked by anti CD47 antibody. Moreover, it was found that anti CD47 antibody is large in size, which can impede their penetration into tumor [40] i.e. they can be poorly internalized. This drawback of low penetration favors the CD47 as a scaffold for bispecific antibody (bsAbs). Bispecific antibodies (bsAbs) have the ability to bind simultaneously to two different targets.

In our hypothesis**,** CD47 act as a target along with tumor specific mutant variant epidermal growth factor receptor (EGFRVIII) as a co-target. EGFR lacks extracellular domain, as a result the ligand EGF (Epidermal growth factor) binding is averted. In spite of lacking of EGF binding, the EGF receptor variant 3 constitutively activates downstream signaling, by undergoing auto phosphorylation in a ligand independent manner [41-43]. The internalization and degradation of EGFRVIII are slower than EGFR that add advantage to retain the bispecific antibody on cell surface. Moreover breast cancer cell expresses EGFRVIII and helps in initiation, promotion and progression of breast cancer and also it brings resistance to chemotherapy, radiotherapy and EGFR targeting drug like cetuximab [21, 49]. Thus, EGFRVIII and CD47 of breast tumor that lacks receptor internalization, pave way to use CD47 and EGFRVIII as a good scaffold for the binding of bispecific antibody. One arm of bispecific antibody block CD47 and the other arm block tumor specific antigen EGFRVIII in breast cancer. The use of single antibody molecule in bispecific format could reduce the potential off target toxicity [44] generated by CD47, as they are expressed in both normal and tumor cells. Moreover, it leads to specific killing of tumor cell. The usage of bispecific antibody further prevents the internalization property of CD47, through binding to EGFRVIII in breast cancer and makes CD47 and EGFRVIII a good scaffold.

Masking "don't eat me" signal by "eat me signal"

The mere presence of prominent eat me signal such as PtdSer (Phosphatidylserine) and/or CRT (Calreticulin) on the surface of tumor cell and the normal cell is not sufficient for engulfment by a phagocytic cell. Their existence is dominated by the presence of don't eat me signal (CD47) [45, 46]. Moreover the natural existence of PtdSer ((Phosphatidylserine) on the live cell is very low<0.9 picomoles/million cells [46], which is not sufficient to engulf a non-apoptotic cell.

In contrast, upon induction by apoptosis by anti-fas or campto-thecin, the PtdSer on the outer leaflet of membrane goes up to>240 picomoles/million cells [46]. These differences in the amount of eat me signal between live and apoptotic cell make the phagocyte to recognize the apoptotic cell. Earlier study elucidated that PtdSer containing liposomes can be used to add sufficient signal on the cell to elicit phagocytic recognition [47]. It has been proven that PtdSer externalization is essential for phagocytosis, but the PtdSer alone is not enough to engulf PtdSer externalized apoptotic cell/viable cell under normal condition [64-66] as well as in oxidative condition [70]. Some chemotherapeutic drugs like doxorubicin, cisplatin fails to induce apoptotic cell death and phagocytosis in oxidative stress condition [71] primarily by inducing the expression of phagocytosis inhibiting factor (anti-phagocytic signal) [70]. Therefore, it is important to make a tumor cell to get engulfed in oxidative as well as non-oxidative environment. This could be possible by bringing additional changes to cell surface that could promote phagocytosis in both oxidative as well as non-oxidative environment. One such additional changes on the cell surface along with adding of pro-phagocytic signal is masking the presence of CD47 (don't eat me) on the tumor cell surface. This dual change of tumor cell surface can be brought simultaneous for efficient and complete clearance of live tumor cell by using bispecific antibody conjugated with PtdSer

containing PEG-modified liposomes, targeting CD47 and tumor specific EGFRVIII [48, 49]. Both CD47 and EGFRVIII are non-internalized receptor [50]. Moreover, the internalization of PtdSer on tumor cell surface is impeded, as tumor cell that rely on glycolysis rather than mitochondrial respiration [51] As a result low ATP is generated in tumor cells compared to the normal cells.

Increased ATP level is much essential for internalization of exogenously added PtdSer. Once PtdSer are labeled over the tumor cell surface by encapsulated PEG liposomes, the well protected PtdSer liposomes surface must transform to one that is reactive. This transformation can be controlled by selecting PEG modified lipids that desorb from the liposome, thus exposing PtdSer on membrane surface [52, 53]. The dual modification i.e. adding of pro phagocytic signal PtdSer and masking don't eat me by adding eat me signal PtdSer, will allow efficient tumor specific phagocytosis. As don't eat me signal (CD47) that act as phagocytic inhibitory signal is blocked by eat me signal (/PtdSer) and also the eat me signal is enriched on tumor cell surface shown in fig. 1. Meanwhile, the natural existence of another pro-phagocytic signal like calreticulin only in tumor cell surface [15] might add advantage to a selective and enhanced uptake of surface altered tumor cell by phagocytic cell shown in fig. 1.

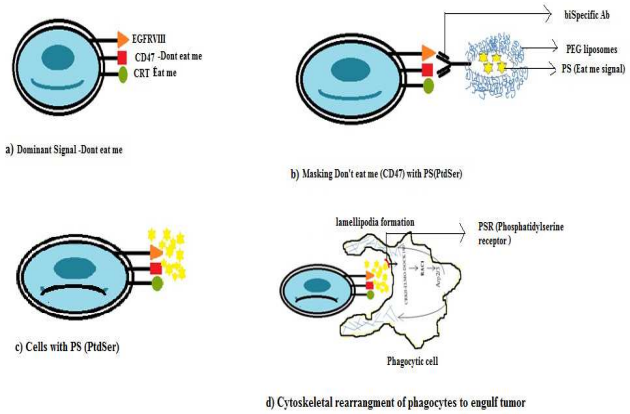

Fig. 1: Phagocytosis of breast cancer cell-masking of anti-phagocytic signal (CD47), and adding of pro-phagocytic signal (PtdSer) over breast cancer cell

(a). CD47 (antiphagocytic signal), and Calreticulin (CRT-pro phagocytic signal) both present on tumor cell surface, (b). bispecific antibody conjugated with PEG liposomes are targeting CD47 and EGFR VIII, (c). Desorbing PEG liposomes lead to adding of pro-phagocytic signal (PtdSer) over CD47, which masks CD47 presence in tumor cell surface, (d). Phagocytic cell undergoes cytoskeletal rearrangement after binding with PtdSer and CRT to engulf CD47 masked tumor cell

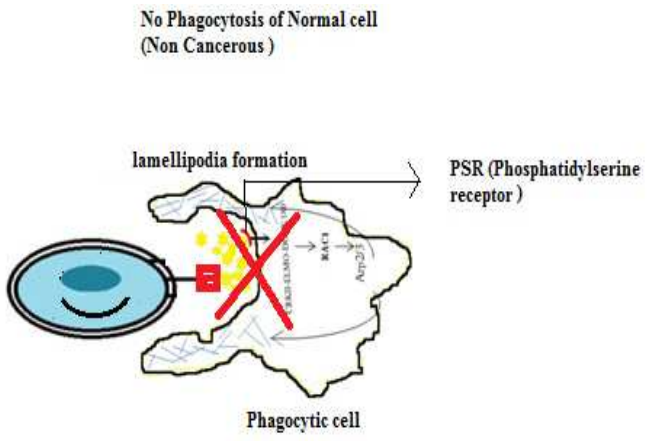

Fig. 2: Normal cell having only anti-phagocytic signal (CD47) and devoid of pro-phagocytic signal (CRT) and mutant epidermal growth factor receptor (EGFRVIII) are failed to recognize and engulfed by phagocytic cell

Immurement of immunogenic cancer

Once the PtdSer (Phosphatidylserine) is displayed on cell surface, it gets directly bound by any of the phagocytic cell surface receptor such as PSR (Phosphatidylserine receptor), BAI1 (Brain specific angiogenesis inhibitor 1), Stabilin-2 (also known as hyaluronic acid receptor for endocytosis or HARE), RAGE (receptor for advanced glycation end product) [54, 55]. The bonding between the/PtdSer and its cognate receptor activates signaling cascade employing CRKIII-DOCK180–ELMO. CRKIII-DOCK180–ELMO activates Arp2/3 complex; which in-turn activates RAC1 (Small GTPase protein) leading to re-organization of the cytoskeleton [56] and actin polymerization, neurite growth, membrane ruffling (macropinocytes) which are essential for engulfment [57-59]. This cytoskeletal rearrangement is necessary for internalization of apoptotic bodies, corpse [60] and also whole live tumor cell [61-63] fig. 1.

Tumor cells by itself have poor antigen presentation, which is not sufficient to elicit immune response. Tumor antigens captured via phagocytosis are processed and presented by professional antigen presenting cells, especially dendritic cells [67] to activate naive T-cells and initiate primary immune response [68, 69]. Cross presentation is crucial for stimulation of CD8+T-lymphocytes. Among cellular antigens, apoptotic cells are commonly considered as the best for cross presentation by dendritic cells (DCs) [72]. Surprisingly, this notion was altered, when HIV infected live CD4+T lymphocytes are engulfed by human monocyte derived DCs, which elicit effective cross-presentation of HIV antigen from live infected CD4+ T lymphocytes. This is as effective as cross presentation from apoptotic cells [73]. Immature pDCs capture the live influenza-exposed cells and subsequently gets matured and cross present the viral antigen very efficiently to specific CD8+ T cells [74]. Then, whole killed tumor cell is used as a source of tumor antigen for treating tumors, by pulsing killed tumor cell with mature dendritic cell *in vitro*. The killed tumor cell is efficiently processed and cross present the tumor antigen by dendritic cell and activate tumor specific CTLs as well as CD4 T-helper cells [75-77].

The usage of killed tumor cell sets a drawback, where the dendritic cell generated *in vitro* fails to migrate to the lymph nodes from the tissue of vaccinated site. This could be averted, when the live whole tumor cells are brought into usage as an alternative approach to the usage of killed tumor cell, as the live whole tumor cells are highly immunogenic. *In vivo* DCs induces CD8+T cell priming by cross-presentation of antigen from live tumor cells [78], which are 20 fold more immunogenic than apoptotic cells [79]. Cell to cell contact which is similar to the nibbling process is mediated by dendritic cell with its scavenger receptor (SR-A) as an essential mechanism to give rise to tumor antigen cross-presentation [80]. Endogenous spleen DCs fails to internalize and cross-present the cellular material from live normal B lymphocytes or splenocytes, but it readily uptake the material from apoptotic cells. DCs take up live tumor cells, but not the live normal cells. This difference could be due to the presence of eat me signal such as calreticulin (CRT) or oxidized low density lipoprotein in tumor cell, that could overpass don't eat me signal (CD47) normally expressed by steady state cells [81].

CONCLUSION

Commonly used treatment modalities in cancer are Surgery, chemotherapy and radiation therapy, which significantly reduce the tumor mass and improve the prognosis of patients. In spite of these effective treatments, small population of precursor tumor cells and/or cancer stem cells still resistant to chemo/radiation. They often survive and give rise to new population of tumor cells, which are much more aggressive and highly resistant to existing standard treatment and consequently lead to tumor relapse, thereby increase the cancer mortality. To overcome this problem and destroy the residual tumor cells that exist after therapy, a novel immunotherapy approach can be used along with the conventional treatment. One such strategy is to modulate the tumor cell and weakening them by masking anti phagocytic signal (CD47) in tumor cell with a pro phagocytic signal (PtdSer) with the aid of bispecific antibody conjugated with liposomes targeting the tumor cell surface protein CD47 and EGFRVIII. Thereby the residual tumor cells will be encountered and immured–by antigen presenting cells such as

dendritic cells and elicit T-cell mediated immunity that could completely remove every tumor cell from the host. This strategy will be mimicking the way of removing the naturally arising incipient cancer cell from the host, to an immunogenic tumor cells. Thus masking anti phagocytic signal CD47 in tumor by pro-phagocytic signal will be a strong key to immurement of immunogenic tumor cell.

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CONFLICT OF INTERESTS

The authors declared that they don't have any conflict of interest

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