Platelets and Checkpoint Inhibitors: Another Arm of the Immune System

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Immunotherapy for cancers has been evolving for the past decade. Here we discuss an approach utilizing platelets along with check point inhibitors for local excision cleansing.

PLATELETS AND CHECKPOINT INHIBITORS: ANOTHER ARM OF THE IMMUNE SYSTEM

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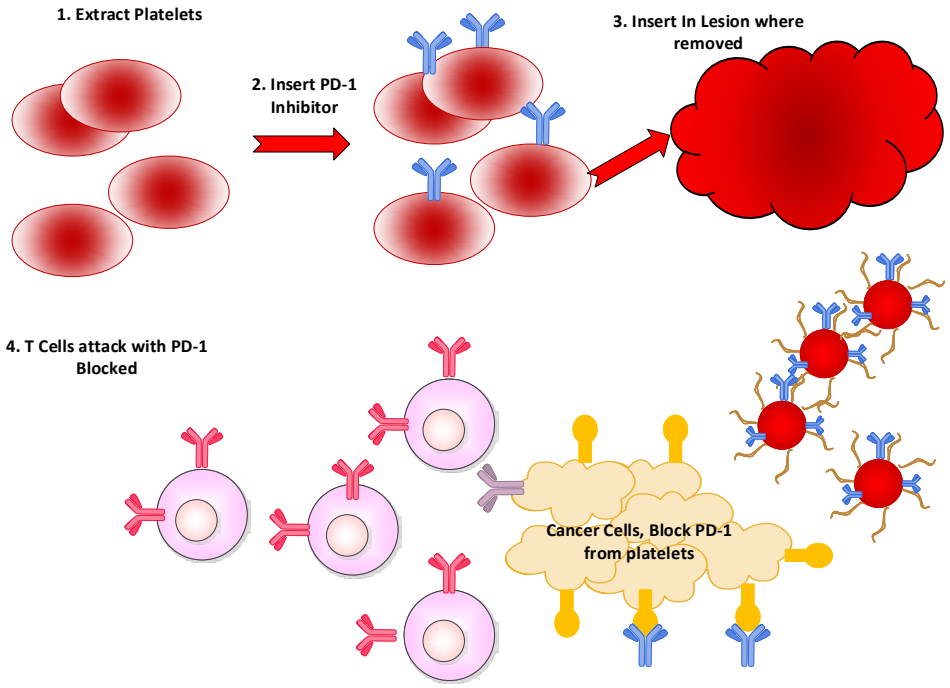
1 INTRODUCTION

Various elements of the immune system have been used to attack cancers. T cells, Dendritic cells, antibodies, NK cells, and now the humble platelet or thrombocyte. The platelet is a remnant of the megakaryocyte which can eject thousands of these small nucleus free cells, albeit with many mitochondria. Now when these cells see a break in the blood stream, say a cut or some other way they are exposed to activating cells not the walls of the blood system, the begin aggregating and forming a clot by sticking to these non-normal, for them, cells. They have the ability to see what is not supposed to be there, attach themselves and ten produce a plug.

When surgical excision of a tumor occurs, there is frequently a few cells left behind, even in a wide margin excision. The idea is to now treat that area with immune therapy but in a local manner, not systemically. This means attacking the tumor cells with a PD-1 blocker. The vehicle for that is a modified platelet delivery system. Now the idea is to modify platelets so that when they are activated they release anti PD-1 or similar checkpoint inhibitors so that T cells can then attack and kill remaining cells.

From Nature we have the following Figure[1]:



As Nature notes[2]:

[1] http://www.nature.com/articles/s41551-017-0031

[2] http://www.nature.com/articles/s41551-017-0031/figures/2

Platelets are modified by covalent attachment of anti-PD-L1 to surface proteins through a bifunctional linker. The engineered platelets are deployed to the surgical wound site, become activated, and produce both inflammatory mediators and platelet-derived microparticles (PMPs) with anti-PD-L1 on their surfaces. The release of PMPs and inflammatory mediators results in the activation of CD8+ T cells and hence antitumor activity mediated by the interaction between T-cell receptors (TCR) and the major histocompatibility complex (MHC).

From the recent paper by Wang et al the authors state:

Cancer recurrence after surgical resection remains a significant challenge in cancer therapy. Platelets, which accumulate in wound sites and interact with circulating tumour cells (CTCs), can however trigger inflammation and repair processes in the remaining tumour microenvironment. Inspired by this intrinsic ability of platelets and the clinical success of immune checkpoint inhibitors, here we show that conjugating anti-PDL1 (engineered monoclonal antibodies against programmed-death ligand 1) to the surface of platelets can reduce post-surgical tumour recurrence and metastasis.

Using mice bearing partially removed primary melanomas or triple-negative breast carcinomas (4T1), we found that anti-PDL1 was effectively released on platelet activation by platelet-derived microparticles, and that the administration of platelet-bound anti-PDL1 significantly prolonged overall mouse survival after surgery by reducing the risk of cancer regrowth and metastatic spread. Our findings suggest that engineered platelets can facilitate the delivery of the immunotherapeutic anti-PDL1 to the surgical bed and target CTCs in the bloodstream, thereby potentially improving the objective response rate.

2 CHECK POINT INHIBITORS

There are a multiplicity of ligands and receptors which can enhance the process or inhibit the process. The inhibitory one are checkpoints and therapy addressing these inhibitory functions are termed checkpoint blockade. We briefly depict some of the current and somewhat well-known ones. A warning should be noted. It seems to be common place amongst immune therapies that as one barrier is climbed others soon appear. Thus, this may very well be merely a first step in an ever continuing understanding of the complexities of the immune systems.

2.1 CTLA-4

CTLA-4 is a checkpoint inhibitor. It has the potential to inhibit the actions of the immune cells to the cell expression this. As Topalian et al state:

The conventional wisdom underlying our vision of how CTLA-4 blockade mediates tumor regression is that it systemically activates T cells that are encountering antigens.

CTLA-4 represents the paradigm for regulatory feedback inhibition. Its engagement down-modulates the amplitude of T cell responses, largely by inhibiting co-stimulation by CD28, with which it shares the ligands CD80 and CD86. As a ‘‘master T cell co-stimulator,’’ CD28 engagement amplifies TCR signaling when the T cell receptor (TCR) is also engaged by cognate peptide-major histocompatibility complex (MHC).

However, CTLA-4 has a much higher affinity for both CD80 and CD86 compared with CD28, so its expression on activated T cells dampens CD28 co-stimulation by out-competing CD28 binding and, possibly, also via depletion of CD80 and CD86 via ‘‘trans-endocytosis’’. Because CD80 and CD86 are expressed on antigen-presenting cells (APCs; e.g., dendritic cells and monocytes) but not on non-hematologic tumor cells, CTLA-4’s suppression of anti-tumor immunity has been viewed to reside primarily in secondary lymphoid organs where T cell activation occurs rather than within the tumor microenvironment (TME).

Furthermore, CTLA-4 is predominantly expressed on CD4+ ‘‘helper’’ and not CD8+ ‘‘killer’’ T cells. Therefore, heightened CD8 responses in anti-CTLA-4-treated patients likely occur indirectly through increased activation of CD4+ cells. Of note, a few studies suggest that CTLA-4 can act as a direct inhibitory receptor of CD8 T cells, although this role in down-modulating anti-tumor CD8 T cell responses remains to be directly demonstrated. The specific signaling pathways by which CTLA-4 inhibits T cell activation are still under investigation, although activation of the phosphatases SHP2 and PP2A appears to be important in counteracting both tyrosine and serine/threonine kinase signals induced by TCR and CD28.

CTLA-4 engagement also interferes with the ‘‘TCR stop signal,’’ which maintains the immunological synapse long enough for extended or serial interactions between TCR and its peptide-MHC ligand. Naive and resting memory T cells express CD28, but not CTLA-4, on the cell surface, allowing costimulation to dominate upon antigen recognition.

2.2 PD-1

In a similar manner to CTLA-4, PD-1 is also an inhibitor. As Topalian et al state:

The PD-1 system of immune modulation bears similarities to CTLA-4 as well as key distinctions. Similar to CTLA-4, PD-1 is absent on resting naive and memory T cells and is expressed upon TCR engagement. However, in contrast to CTLA-4, PD-1 expression on the surface of activated T cells requires transcriptional activation and is therefore delayed.

Also in contrast to CTLA-4, PD-1 contains a conventional immunoreceptor tyrosine inhibitory motif (ITIM) as well as an immunoreceptor tyrosine switch motif (ITSM). PD-1's ITIM and ITSM bind the inhibitory phosphatase SHP-2. PD-1 engagement can also activate the inhibitory phosphatase PP2A. PD-1 engagement directly inhibits TCR-mediated effector functions and increases T cell migration within tissues, thereby limiting the time that a T cell has to survey the surface of interacting cells for the presence of cognate peptide-MHC complexes.

Therefore, T cells may ''pass over'' target cells expressing lower levels of peptide-MHC complexes. In contrast to CTLA-4, PD-1 blockade is viewed to work predominantly within the TME, where its ligands are commonly overexpressed by tumor cells as well as infiltrating leukocytes. This mechanism is thought to reflect its important physiologic role in restraining collateral tissue damage during T cell responses to infection. In addition, tumor-infiltrating lymphocytes (TILs) commonly express heightened levels of PD-1 and are thought to be ''exhausted'' because of chronic stimulation by tumor antigens, analogous to the exhausted phenotype seen in murine models of chronic viral infection, which is partially reversible by PD-1 pathway blockade.

Importantly, the phenotypes of murine knockouts of PD-1 and its two known ligands are very mild, consisting of late-onset organ-specific inflammation, particularly when crossed to autoimmune- prone mouse strains. This contrasts sharply with the Ctla-4 knockout phenotype and highlights the importance of the PD-1 pathway in restricting peripheral tissue inflammation. Furthermore, it is consistent with clinical observations that autoimmune side effects of anti-PD-1 drugs are generally milder and less frequent than with anti- CTLA-4. Despite the conventional wisdom that CTLA-4 acts early in T cell activation in secondary lymphoid tissues whereas PD-1 inhibits execution of effector T cell responses in tissue and tumors, this distinction is not absolute.

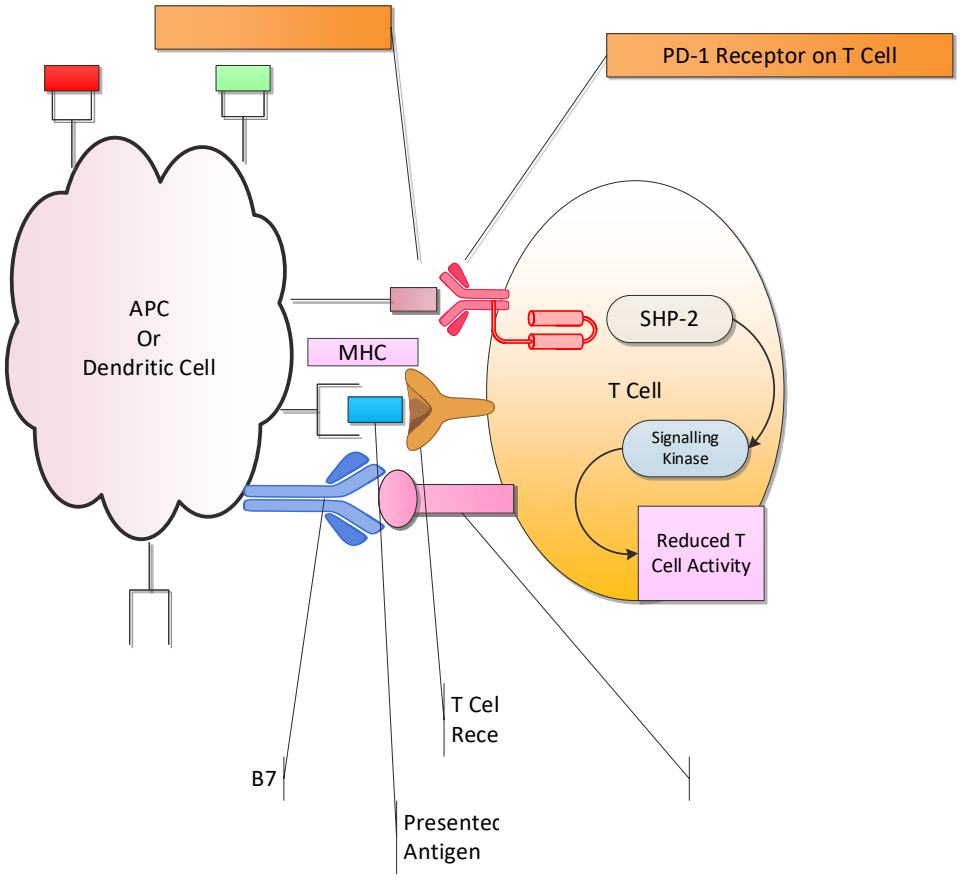
Beyond its role in dampening activation of effector T cells, CTLA-4 plays a major role in driving the suppressive function of T regulatory (Treg) cells. Tregs, which broadly inhibit effector T cell responses, are typically concentrated in tumor tissues and are thought to locally inhibit anti-tumor immunity.

Therefore, CTLA-4 blockade may affect intratumoral immune responses by inactivating tumor-infiltrating Treg cells. Recent evidence has demonstrated anti-tumor effects from CTLA-4 blockade even when S1P inhibitors block lymphocyte egress from lymph nodes, indicating that

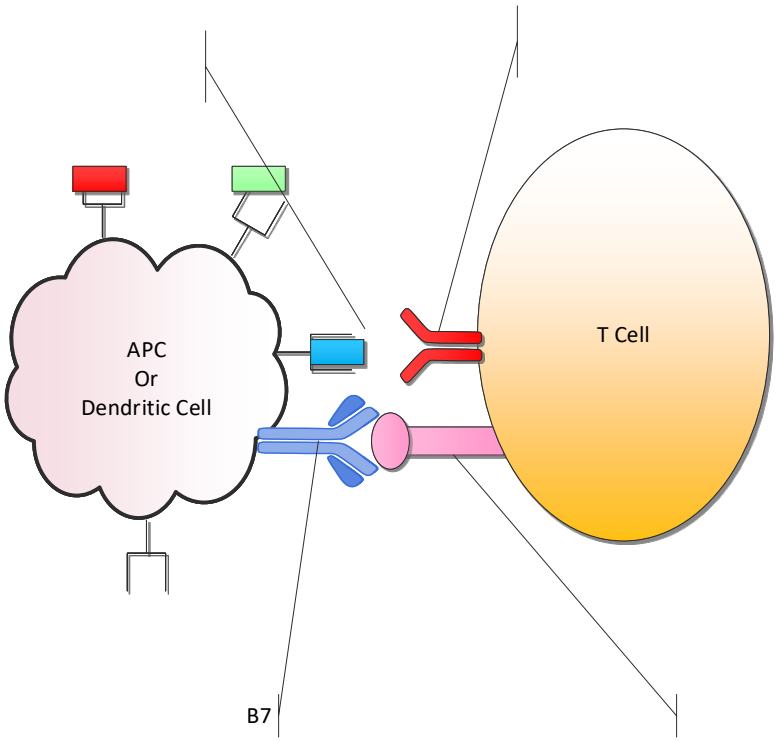
this checkpoint exerts at least some effects directly in the TME as opposed to secondary lymphoid tissues.

Conversely, PD-1 has been shown to play a role in early fate decisions of T cells recognizing antigens presented in the lymph node. In particular, PD-1 engagement limits the initial ‘‘burst size’’ of T cells upon antigen exposure and can partially convert T cell tolerance induction to effector differentiation.

The authors present a graphic regarding how this blocking or checkpoint functions. We depict this below. Let us briefly review the issue of checkpoint inhibitors.

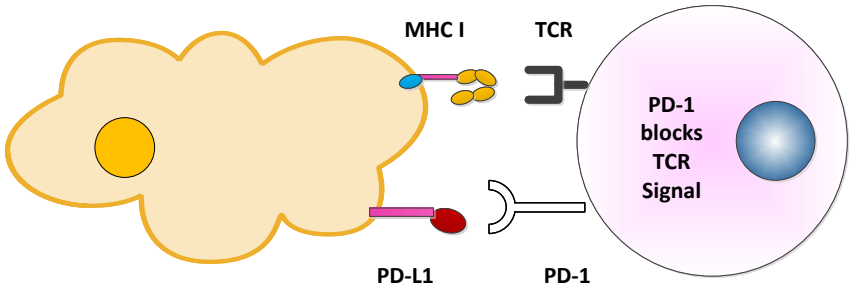


The process can be simplified as below:



CD28 (Cluster of Differentiation 28) is one of the proteins expressed
T cell activation and survival. T cell stimulation through CD28 in addit
signal for the production of various interleukins (IL-6 in particular). C
proteins. When activated by Toll-like receptor ligands, the CD80 expr
The CD86 expression on antigen presenting cells is constitutive (expr
the only B7 receptor constitutively expressed on naive T cells. Associa
complex without CD28:B7 interaction results in a T cell that is anergic

The authors present a graphic regarding how this blocking or checkpoint functions. We depict this below.



Oncogenic pathway in tumor cell uses AKT for gene amplification allowing PD-L1 expression

As Freeman states:

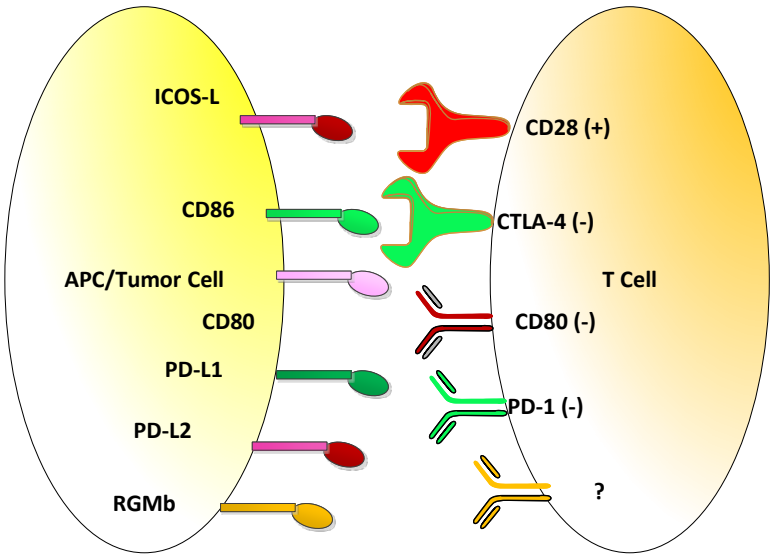
T cell activation requires a TCR mediated signal, but the strength, course, and duration are directed by costimulatory molecules and cytokines from the antigen-presenting cell (APC). An unexpected finding was that some molecular pairs attenuate the strength of the TCR signal, a process termed coinhibition. The threshold for the initiation of an immune response is set very high, with a requirement for both antigen recognition and costimulatory signals from innate immune recognition of ‘‘danger’’ signals.

Paradoxically, T cell activation also induces expression of coinhibitory receptors such as programmed death-1 (PD-1). Cytokines produced after T cell activation such as INF- and IL-4 up-regulate PD-1 ligands, establishing a feedback loop that attenuates immune responses and limits the extent of immune-mediated tissue damage unless overridden by strong costimulatory signals. PD-1 is a CD28 family member expressed on activated T cells, B cells, and myeloid cells. In proximity to the TCR signaling complex, PD-1 delivers a coinhibitory signal upon binding to either of its two ligands, PD-L1 or PD-L2.

Engagement of ligand results in tyrosine phosphorylation of the PD-1 cytoplasmic domain and recruitment of phosphatases, particularly SHP2. This results in dephosphorylation of TCR proximal signaling molecules including ZAP70, PKC, and CD3, leading to attenuation of the TCR/CD28 signal.

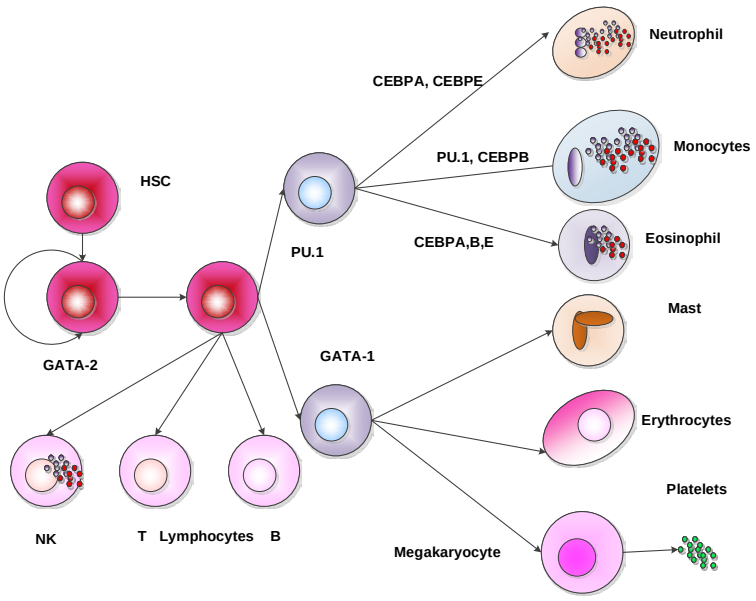
The role of the PD-1 pathway in peripheral T cell tolerance and its role in immune evasion by tumors and chronic infections make the PD-1 pathway a promising therapeutic target.

There are potentially many such check points. Some of them are graphically shown below.



3 PLATELETS

Now we briefly review the issue of platelets. The diagram below is the now classic description of the blood stem cell development. Platelets derive from Megakaryocytes. Their primary function is to provide barriers to the blood stream when there is some form of compromise such as a cut. Normally platelets just flow in the blood stream and do nothing, unless there is some aberrant factor where a clot may result as may happen in a clot in a stroke or heart attack.



The platelet is a small cell like structure with multiple mitochondria along with several other elements all of which allow the platelet to perform its function.

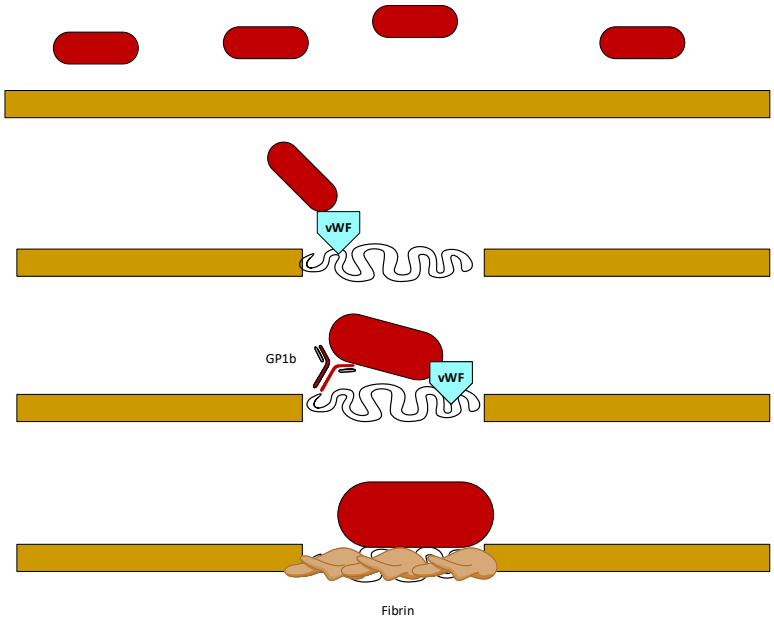
Now we can describe how the platelet is activated to do what it is supposed to do. Below we show the basic processes which occur. Normal blood flow allows the platelets to just move through the blood stream.

Then if a break or cut occurs and the platelets are exposed to the subendothelial collagen they get activated and adhere via a von Willenbrand Factor connection.

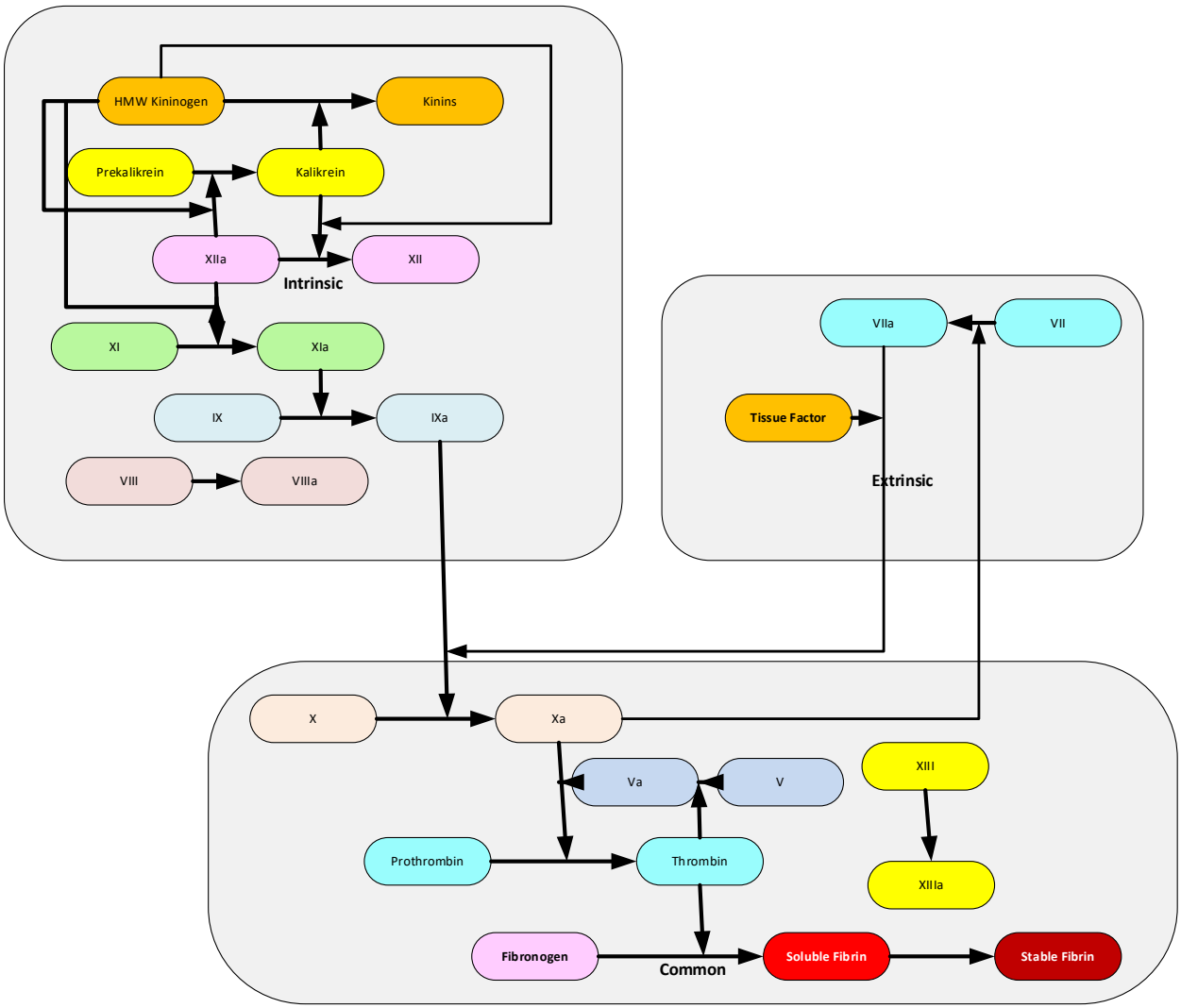
Once this attachment occurs several other steps make the various attachments and allow the platelet to expand.

Finally via the process of the pathways the process produces a clot that seals the open lesion.

Fundamentally all of this involves the adhesion of the platelets to the surfaces outside the blood stream.



Now the formation of the clot is a result of the interaction of a set of proteins in the blood stream. The flow of these proteins and their different activations result in a fibrin clot. We show below the three pathways that effect; extrinsic, intrinsic and common.



As noted in Hoffman et al:

If vascular injury occurs, a measured response is triggered in that the extent of damage regulates platelet and fibrin deposition.

Activated platelets provide the membrane surfaces upon which coagulation enzymes can be anchored, assembled, and expressed. Therefore, the activated platelet membrane, provides both an initiating and limiting component to the extent of a coagulation reaction.

More vascular damage produces more anchored activated platelets, and more membrane allows the assembly of more coagulation enzymes, which ultimately results in increased fibrin formation. When the vascular system is perturbed, the initial stages of the hemostatic response are triggered.

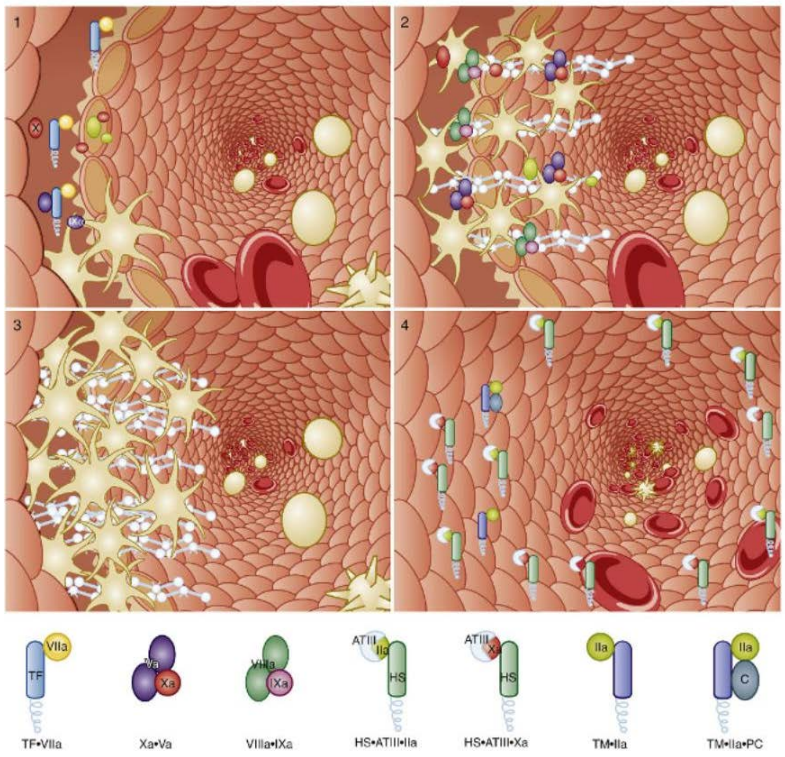
The initial principal player is the extrinsic tenase complex (tissue factor; factor VIIa), which is composed of a cell membrane; tissue factor exposed by vascular damage or cytokine stimulation; Ca2; and the serine protease plasma factor Vila, which is already present in its active form at 1% to 2% of the factor VII zymogen concentration.*

Before binding to tissue factor, the plasma serine protease factor Vila is essentially inert from the catalytic perspective and thus impervious to the abundant protease inhibitors in plasma.289 Factor VII also competes with factor Vila for tissue factor binding, thus serving as a negative regulator that buffers the overall reaction.

Factor VII activating protease (FSAP) has also been shown to activate factor VII in the absence of tissue factor. The physiologic function of FSAP still is unclear but most recently has been suggested to be involved in inflammation.

The extrinsic tenase complex (tissue factor-factor Vila) activates low levels of the zymogens factor X and factor IX to their respective serine protease enzymes factor Xa (~10 pM) and factor IXa (=1 pM).296,297 Factor X is the more efficient and abundant substrate.

A simplistic view from Hoffman et al is shown below:



As Mahmoudi and Farokhzad note:

Platelet activation has been shown to result in the release of an estimated 300 proteins and biomolecules, among which are key pro-inflammatory molecules including CD154 (also known as CD40 ligand), which is a potent molecule involved in lymphocyte activation11. Capitalizing on the functions of platelets both in haemostasis and inflammation, Gu and colleagues show that the engineered anti-PD-L1 immunoplatelet conjugates accumulate and activate at the site of surgery, inducing T-cell activation through the synergistic action of PMP-bound anti-PD-L1 and a myriad of released pro-inflammatory mediators. The authors also demonstrate the value of their technology over conventional anti-PD-L1 therapy.

Now Hoffman et al discuss how these propagate:

When a sufficient stimulus is provided to overcome the antagonist-inhibitor threshold, the accumulating mass of activated platelets will support increasing intrinsic tenase and prothrombinase complex formation on their surfaces through specific platelet receptors, and the local inhibitor concentrations are overwhelmed.

These platelet-bound catalysts execute the propagation phase of the reaction, during which massive amounts of thrombin are produced."' This phase of thrombin generation continues, independent of the initially presented tissue factor, as long as there is a continuous supply of blood to deliver new plasma procoagulant reactants, platelets, and fibrinogen to the site of perforation in the vascular endothelium.

Important to the formation of the prothrombinase complex is the generation of factor Xa. Factor Xa is a unique regulatory enzyme in that it is formed through both the intrinsic tenase and the extrinsic tenase complexes. Under normal conditions, the concentration of factor Xa is the rate-limiting component of the prothrombinase complex.

The other components of the complex, platelets (membrane surface binding sites), and the cofactor (factor Va) are activated rapidly to produce a surplus that is ready for action.'1" The coagulation mechanism can become sensitive to factor V or platelets when confronted with congenital deficiencies, thrombocytopenia, platelet pathology, or pharmacologic interventions. The initial factor Xa is generated via the tissue factor-factor Vila complex during the initiation phase. Additional factor Xa is then generated by the intrinsic tenase complex (factor IXa-factor VIIIa-membrane-Ca2"). Initially, the concentration of the factor Vila -tissue factor complex is higher than the concentration of the factor Villa-factor IXa complex, which requires activation and assembly.

As time progresses, the contribution of the intrinsic tenase complex to factor Xa generation exceeds that of the extrinsic tenase. " The intrinsic tenase complex is kinetically more efficient and activates factor X at a 50- to 100-fold higher rate than the extrinsic tenase complex. The burst of factor Xa that is generated overcomes the levels of factor Xa inhibitors, such as TFPI, and achieves maximal prothrombinase activity and propagation of the procoagulant response.

The bulk of thrombin (.95%) is formed during the propagation phase after fibrin clot formation.' Without the intrinsic tenase complex being formed, as occurs in hemophilia A or B, factor Xa is not generated in levels sufficient to produce the propagation phase of thrombin generation.

Thus the controls related to plug formation perform the function of creating a plug carrying the essential elements necessary for check point blockade.

4 OBSERVATIONS

The principle of checkpoint blockade has become an essential paradigm in the treatment of cancers via immunotherapy. Namely, these checkpoint molecules can prevent the immune system from attacking the cancer cell. However if one were to systemically introduce blockers to these checkpoints, using say a monoclonal anti-body, Mab, then one could allow the immune system to do what it is supposed to do. This is a systemic approach however, since it blocks all PD-1 or similar check points. In this example use if made of platelets, which can be used to seek out specific spots, namely where surgery on a tumor may have been performed, and use that approach to block the checkpoints using locally provided Mabs. It is an intriguing application that deserves some examination.

As Mahmoudi and Farokhzad state:

Striking a balance between T-cell activation and inhibition is crucial for the proper functioning of the immune system. Among the expanding list of molecules referred to as immune checkpoints and involved in the inhibition of T-cell function, the two most validated to date are the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), expressed on activated T cells, and the programmed cell death protein-1 (PD-1), which binds to its ligands (the PD-L1 and PD-L2 proteins expressed on many cell types) and interrupts signalling mediated by the T-cell antigen receptor.

The ability to modulate the activity of these checkpoints has given rise to the field of immune-checkpoint therapy, now considered a pillar of cancer therapy. On the basis of the most validated immune checkpoints identified to date (CTLA-4, PD-1 and PD-L1), four antibodies have been approved for clinical use: ipilimumab, nivolumab, pembrolizumab and atezolizumab. Durable patient responses have been documented, and in the case of ipilimumab, which has the longest clinical history, survival of 10 years or more has been reported in a subset of patients with melanoma.

As they note, currently there are a limited number of Mabs which have been approved but their existence and use has become a substantial paradigm shift. Yet one of the major concerns is still the systematic impact. Some patients can respond reasonably while other have adverse responses. Perhaps it is the systematic application

Although the clinical success of these drugs has transformed immune checkpoint inhibitors into a powerful class of therapeutics (for example, global sales of nivolumab are projected to reach ~US$3 billion in 2020)5, the exaggerated T-cell activation that they trigger leads to a broad range of adverse effects.

The latter remark is often the most compelling. Mabs, albeit somewhat straightforwardly designed and produced still have to bear the substantial clinical costs associated with safety and efficacy.

The above authors also note:

Gu (see Wang et al) and colleagues' conjugation strategy for the targeted delivery of immune checkpoint inhibitors to the surgical wound may pave the way for safer and more effective checkpoint therapy in an adjuvant setting while ameliorating some of the adverse effects of systemic administration.

Although the system is complex and the scalability, regulatory and manufacturing hurdles are considerable, it is tantalizing to imagine that the efficacy of the authors' approach may be further improved by attachment of anti-CTLA-4, which has a mechanism of action distinct from, but synergistic with, that of PD-L1. In fact, clinical experience to date has shown that combination therapy with anti-CTLA-4 and anti-PD-1 confers additional clinical benefits. But the application of such therapy is limited by a higher incidence of toxicities and their broader spectrum. Gu and co-authors' approach could thus facilitate such combination therapy and mitigate its adverse effects.

This again addresses the focusing of the Wang/Gu approach to specific locations. However as we know the concerns that some of these address have a significant potential for rapid metastasis. That presents a challenge if we see only local application.

Additionally, the authors' conjugate approach could be combined with recently developed nanotechnologies that make use of platelet biology, such as nanoparticle-coating platelet membranes isolated from human blood that enhance the nanoparticles' circulation time and their ability to target injured vasculature.

The combination of both methods might eventually lead to the in situ synthesis of anti-PD-L1-conjugated platelet-membrane-coated nanoparticles that can simultaneously carry other agonists to augment T-cell responses. To accelerate the clinical translation of such strategies, proper regulatory standards must be established to overcome any issues associated with the development of biologically modified platelets and to ensure robust and reproducible conjugation chemistry in combination with analytical methods to support the manufacturing of the final biologic product.

As Wang et al note:

Cell-based systems have recently emerged as biological drug carriers; examples include erythrocytes, bacterial ghosts, and genetically engineered stem and immune cells. Among them, platelets are anucleated cellular fragments released from megakaryocytes and are best known for their function in hemostasis. The average life span of circulating platelets is 8 to 9 days, which could greatly improve the pharmacokinetics of intravenously injected therapeutics.

Moreover, transfused platelets could migrate to the site of surgical wounds33, where residual tumours may survive after surgery. Emerging evidence has shown that platelets also have the capability to recognize and interact with CTCs, which are shed from the primary tumour into the bloodstream and can lead to metastases. Along with their intrinsic tendencies to accumulate at wounds and to interact with CTCs, platelets are also considered immune 'cells' that initiate and

enhance many inflammatory conditions. Platelet-derived chemokines recruit and awaken T cells as well as other immune cells.

As the major source of soluble CD40L (sCD40L), platelets can boost the T-cell immune response and are necessary for inducing dendritic cell maturation and B-cell isotype switching for production of immunoglobulin G (IgG). It has also been reported that PDL1 and PDL2 are upregulated in response to inflammation, which results in PDL1-positive tumours, making the tumour more sensitive to anti-PD therapy and potentially improving the objective response rate. In this work, inspired by the intrinsic properties of platelets, we conjugated anti-PDL1 (antibodies against PDL1; hereafter, aPDL1) to the surface of platelets for use as a preventative treatment for post-surgical tumour recurrence.

With the help of platelets, aPDL1 can be targeted to the cancer cells after surgery, while reducing off-target effects.

It is the above observation which has substantial merit. Many of the Mab check point inhibitors systematically engage all cells in the body, often with substantial effects.

We found that the binding of aPDL1 to non-activated platelets was highly stable, while release of aPDL1 could be significantly promoted on the activation of platelets. We reasoned that the aPDL1 release may result from the platelet-derived microparticles (PMPs), which are generated from the plasma membrane of activated platelets43. Such structural alterations can facilitate aPDL1 binding to tumour cells and APCs.

By intravenous injection of aPDL1-conjugated platelets (P–aPDL1) into mice with B16 melanomas and triplenegative mammary carcinomas that had been previously resected (~1% remaining), we showed that platelets can facilitate aPDL1 transport to residual microtumours at the surgical site, and to CTCs in the blood.

The issue of micro-tumors has always been of significant interest. Melanomas are notorious for escaping un-noticed. Often with lymph node resection, small clusters of melanoma cells can be seen, even where they are least expected.

A T-cell-inflamed tumour microenvironment was created by the platelets on activation, leading to increased PDL1 expression at the tumour site. aPDL1 was effectively released after platelet activation, thereby blocking PDL1 on tumour and APCs. Our results show that platelets have promise as a means of targeted, controlled delivery of aPDL1 for the prevention of cancer recurrence post-surgery.

The post-surgical results are especially favorable and this is for a quasi-system approach. Notwithstanding this approach could be used in a balanced multiprotocol effort as well as a post-surgical adjuvant approach.

5 APPENDIX: PREPARATION OF PLATELETS

The following is the protocol suggested by Wang et al.

Preparation of aPDL1 conjugated platelets.

1. *Murine platelets were isolated. In brief, whole blood was collected from the C57BL/6 or BALB/c mice (non-terminal collection from the orbital sinus or saphenous vein; 20 mice were used) into a plastic syringe containing 1.0 ml citrate-phosphatedextrose (16 mM citric acid, 90 mM sodium citrate, 16 mM NaH2PO4, 142 mM dextrose, pH 7.4) and centrifuged at 100 g for 20 min at room temperature.*

2. *The platelet-rich plasma (PRP) was transferred to a separate tube using a transfer pipette (wide orifice), and PGE1 was added to each tube at a final concentration of 1 μ M. (Note that if the PRP has a reddish color, discard these samples.)*

3. *Platelets were isolated from the PRP via centrifugation at 800 g for 10 min.*

4. *The plasma was discarded, and the platelets were resuspended carefully and slowly in Tyrode's buffer (134 mM NaCl, 12 mM NaHCO3, 2.9 mM KCl, 0.34 mM Na2HPO4, 1 mM MgCl2, 10 mM HEPES, pH 7.4) or PBS with PGE1 added at 1 μ M. Note that this buffer was released slowly along the tube wall, while minimizing agitation. Each in vivo injection required 500–600 μ l whole blood.*

5. *The surface of the platelets was functionalized with aPDL1 in three steps.*

   i. *First, 100 μ l of platelets (1 × 108) was resuspended in 400 μ l of PBS (pH 8), including PGE1 (1 μ M), and incubated with 0.1 mg ml−1 Traut's Reagent (2-iminothiolane; Pierce) for 30 min at room temperature.*

   ii. *After 30 min of reaction, the excess Traut's Reagent was removed by centrifugation at 800 g for 10 min and washed with Tyrode's buffer (with PGE1 added at 1 μ M) three times (without resuspension to avoid unnecessary platelet activation). In the meantime, aPDL1 was mixed with sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1- carboxylate (Sulfo-SMCC, Pierce) in PBS (pH 7.4) at a molar ratio of 1:1.2 for 2 h at 4 °C. The excess Sulfo-SMCC was removed using a centrifugal filter device (molecular weight cut-off = 10 kDa) to purify the SMCC activated antibody.*

   iii. *Lastly, platelets and antibodies were mixed in Tyrode's buffer (+ PGE1 at 1 μ M). After 2 h at room temperature, the excess antibodies were removed by centrifugation at 800 g for 10 min. The precipitate fraction was retained and washed with Tyrode's buffer (+ PGE1 at 1 μ M) twice.*

1. *Platelet recovery was higher than 80% after conjugation based on the platelet count analysis. The obtained aPDL1–platelets were stored in Tyrode's buffer (+ PGE1 at 1 μ*

M) at room temperature prior to use in experiments. Unconjugated platelets were not separated from conjugated platelets. The amount of aPDL1 conjugated to the platelets was measured via ELISA (rat IgG total ELISA kit, eBioscience, cat. no. 88-50490-22). Freshly isolated platelets were used within 6 h. The platelet-activation marker, CD62P, was used for evaluating platelet activation. All platelet manipulations were performed at room temperature. To study the conjugation efficiency, various amounts of aPDL1 were added to the platelets for conjugation. Unconjugated aPDL1 was removed with the supernatant after centrifugation at 800 g for 10 min. The P–aPDL1 pellet was then washed twice with Tyrode's buffer (+ PGE1 at 1 μ M), using centrifugation at 800 g for 10 min.

2. *After that, the P–aPDL1 was resuspended in 100 μ l deionized water and ultrasonicated to lyse the platelets and release the aPDL1. The amount of aPDL1 conjugated to the platelets was measured using ELISA. The efficiency of aPDL1 conjugation to platelets (conjugated aPDL1 as a percentage of total aPDL1 added) was about 75% when 0.2 pg aPDL1 per platelet was added.*

3. *To assess the stability of the platelets after decoration with aPDL1, platelet counts were determined at 0 h and 24 h.*

4. *To study the stability of aPDL1 on platelets over time, the platelets were stored in Tyrode's buffer (+ PGE1 at 1 μ M) at room temperature. At different times, 50 μ l P–aPDL1 was extracted.*

5. *Released aPDL1 in the supernatant was removed by centrifugation at 800 g for 10 min. The conjugated platelets were suspended in 100 μ l deionized water and ultrasonicated to release the aPDL1. The amount of aPDL1 conjugated to the platelets was measured via ELISA. To activate the platelets, 0.5 U thrombin ml−1 were added to the platelet suspension. PGE1 was removed prior to platelet activation.*

6. *Platelets were activated for 30 min at 37 °C. PMPs were prepared from platelets as previously described53. Before the experiments, platelet concentrates were activated by thrombin (2 U ml−1) for 30 min and centrifuged at 800 g for 10 min.*

7. *The PMP-enriched supernatants were then collected and examined via antibodies for mouse aIIbb3 and CD62P (P-selectin) combined with flow cytometry. Next, aPDL1 was conjugated to the PMPs as described above. The conjugation efficiency and stability were examined using ELISA.*

6 REFERENCES

1. Chen and Mellman, Elements of cancer immunity and the cancer–immune set point, 19 January 2017, Vol 541, Nature, p 321
2. Gupta and Massagué, Cancer Metastasis: Building a Framework, Cell 127, November 17, 2006
3. Hoffman et al, Hematology, 6th Ed, Elsevier (new York) 2013.
4. Mahmoudi and O. Farokhzad, Wound-bound checkpoint blockade, Nature Biomedical Engineering 1, 0031 (2017)
5. Topalian et al, Immune Checkpoint Blockade: A Common Denominator Approach to Cancer Therapy, Cancer Cell 27, April 13, 2015
6. Wang et al, In situ activation of platelets with checkpoint inhibitors for post-surgical cancer immunotherapy, Nature Biomedical Engineering 1, 0011 (2017)