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Selective Costimulation Blockade With Antagonist Anti-CD28 Therapeutics in Transplantation

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Author Contributions

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Abstract

Nephrotoxicity of calcineurin inhibitors and uncontrolled effector function of alloreactive T lymphocytes are main drivers of transplant dysfunctions. T lymphocytes either directly damage tissues or indirectly promote inflammation and antibody responses. Beside inhibitors of calcium-dependent pathways and anti-metabolites, modulators of T cell co-stimulation are elected pharmacological tools to enable interference with immune-mediated transplant dysfunctions. CD28 and CTLA-4 are major co-stimulatory and co-inhibitory cell surface signaling molecules interacting with CD80/86, known to be critically important for immune response of committed T cells and regulation. Initial "bench to beside" translation, two decades ago, resulted in the development of belatacept (CTLA4-Ig), a biologic inhibiting interaction of both CD28 and CTLA-4 with CD80/86. Despite proven effectiveness in inhibiting allo-immune responses, clinical use of belatacept in kidney transplantation revealed a substantially high incidence of acute, cell-mediated rejection. The etiology of « belatacept-resistant graft rejection » was allocated to elevated pretransplant frequencies of CD28+ memory T cells. Owing to different requirement in CD28 costimulatory and CTLA-4 co-inhibitory signals to control naïve and memory T cells, selective antagonists of CD28-CD80/86 interactions have been developed on the rationale that preservation of CTLA4-mediated regulatory mechanisms would result in a better control of alloreactivity and would represent a "Treg-compatible" immunosuppression. After the successful testing of selective CD28 antagonists in First In Human studies, this review delineates how this shift in paradigm performed in preclinical transplantation models and evaluates its clinical potential.

Introduction

T cell activation results from the integration of three types of signals: TCR signaling triggered by antigen-presenting cells harboring peptide antigen/HLA complexes (signal 1). This is either reinforced or dampened by engagement of co-stimulatory/co-inhibitory molecules (signal 2), and cytokines (signal 3). Signal integration leads to T cell differentiation into pathogenic effector T cells (Teff), anti-inflammatory regulatory T cells (Treg), or memory T cells.

Whereas >50 T cell costimulatory/co-inhibitory molecules have been identified so far, two "checkpoint systems" only are currently in clinical use to dampen or enhance immune responses. The programmed death 1 (PD-1) receptor: PD-Ligand (PD-L) pathway is a major receptor-ligand network that functions primarily to provide a coinhibitory signal. This pathway has emerged as a potent therapeutic target for enhancing the immune response in solid tumors and lymphoma.[1] The second system, in fact the first identified, is composed of the interaction of CD28 and CTLA-4 on T cells with CD80/86 on antigen presenting cells (APC) acting as a rheostat to turn T cells on and off. Signal 1 plus CD28-mediated co-stimulation results in T cell activation, proliferation, synthesis of anti-apoptotic genes and pro-inflammatory responses. CTLA-4 upregulated on naïve T cells shortly after activation or constitutively expressed on Treg cells prevents CD28-mediated signals by inducing down-regulation of CD80 on antigen presenting cells (APCs) by trans-endocytosis, thereby altering the level of CD28 engagement[2] and, in a cell intrinsic manner, by directly recruiting phosphatases opposing TCR and CD28-mediated signals raft formation.[3,4]

The first biologics used to interfere with CD28-mediated signals were recombinant soluble domains of CTLA-4 fused with an immunoglobulin Fc domain (CTLA4-Ig). The corresponding drugs, abatacept and belatacept are in clinical use to dampen T cell responses in rheumatoid arthritis, juvenile arthritis and kidney transplantation.[5-7] CTLA4-Ig molecules

dock onto CD80 (and CD86) and therefore inhibit binding of CD28 and T cell activation. Since CD80 binds to CD28 and to CTLA-4 using similar interaction domains, CTLA4-Ig also inhibits binding of CD80 to CTLA-4 and also presumably to PD-L1[8] (illustrated Figure 1).

We and others have shown that preventing engagement of CTLA-4 with CD80 is detrimental for full control of alloreactive T cell activation, particularly for Tfh, effector memory T cells, TH17 cells[9] and Treg cells[10-14] which are tightly controlled by CTLA-4. From these observations, it has been proposed[15, 16] that selectively targeting CD28 might share the benefit of CTLA4-Ig (blockade of CD28-mediated signals) without perturbing the co-inhibitory CD80/86-CTLA4 axis and might therefore present a superior immunosuppressive index as compared to CTLA4-Ig by preserving Treg functions. We previously reviewed available preclinical experience in autoimmunity models.[17] In this manuscript, we report on accumulated experience with selective CD28 antagonists in preclinical transplantation models (summarized in Table 1) and their clinical potential.

Monovalent binders are required for antagonist activity towards CD28

Antagonizing CD28 with antibodies has been challenging since CD28 as well as IgG antibodies are homodimeric molecules. Anti-CD28 antibodies interactions with CD28 therefore induce a clustering of CD28 molecules which results in the phosphorylation of PI3K, a molecular signal also induced by engagement of CD80/86.[18] This occurs independently of the binding epitope so that a given anti-CD28 antibody can be antagonist of CD80/86 (if it binds to the MYPPPY domain recognized by CD80/86) while still presenting agonistic properties. To date all antibodies directed against CD28 were found to activate the receptor instead of only blocking access to its ligand. An exception is the anti-rat JJ319 mAb which in vivo rapidly induces internalization of CD28 and presents functional antagonist properties.[19] Importantly, antagonist antibodies must not bind to the laterally exposed C''D loop of CD28, which then results in a "superagonistic" activity (such as for the TGN1412

antibody[20]), i.e. a non-physiological engagement of CD28 resulting in polyclonal T cell activation. This was unfortunately illustrated in the well-known TGN1412 trial where all the 6 volunteers experienced life-threatening cytokine storm, required dialysis and ICU admissions.[21]

To develop "antagonist-only" anti-CD28 antibodies, mutations in the Fc domain have been introduced to prevent cross-linking of CD28 through Fc/FcγR interaction. However, while this strategy was efficient in vivo in rodents,[22,23] "Fc-silenced" anti-CD28 mAb still co-stimulated human T cell proliferation and cytokine release in vitro.[24] Another strategy was use of monovalent anti-CD28 antibody fragment (i.e. Fab or scFv) presenting an "antagonist-only" action on T cells since presenting no cross-linking activity (Figure 1).[15,18] To increase the otherwise limited in vivo half-life of monovalent antibody fragments, molecular fusions with alpha-1-antitrypsin[15] or chemical conjugation with a polyethylene glycol moiety have been proposed.[16,25] To our knowledge, two "antagonist-only" anti-CD28 Fab'-PEG antibodies entered clinical development: FR104 (OSE Immunotherapeutics, formerly developed by Effimune) and lulizumab pegol (BMS-931699; Bristol Myers Squibb).

Models of kidney and heart transplantation in rodents

In the rat species, a single week treatment with the JJ319 mAb induces CD28 internalization in vivo for up to 12 days[26] and thereby blocks T cell costimulation.[27] The treatment induces indefinite graft acceptance in the Lewis 1W to 1A kidney transplant model. The mechanism of action involves an initial control of alloreactive T cell activation due to CD28 unavailability, relayed by tolerance induction supported by an increase of Treg cells, by IDO-dependent mechanisms and by the suppressive activity of peripheral MDSC.[28] In contrast with kidney transplantation, it was not possible to induce tolerance to a heart transplant in the same Lewis 1W to 1A rat model with a JJ319 monotherapy. Rather, tolerance

induction required combination with high doses cyclosporin A (10 mg/Kg for 3 days) and relied on presence of Lag-3-positive Treg cells.[29]

Similar experiments have been conducted in a murine cardiac transplant model, using a non-activating single-chain Fv-based monovalent antibody.[30] Blockade of CD28 promoted allograft survival, and significantly attenuated chronic rejection when combined with transient CD154 blockade or calcineurin inhibitors (CNI). Accepted graft in treated animals contained increased proportion of regulatory T cells and regulatory dendritic cell genes. Co-administration of a blocking anti-CTLA4 antibody abrogated induction of regulatory cells and led to eventual rejection in all animals.

These experiments in rodents revealed a dissociation between mechanisms of allograft rejection blockade and of tolerance induction. Initial blockade of CD28-mediated costimulation prevented acute rejection. Then, after re-expression of CD28, in a model-dependent manner, Treg and MDSC were able to relay immunosuppression and to abrogate alloreactivity. Induction of regulatory cells was possibly directly due to the treatment with the JJ319 anti-CD28 or, alternatively, regulatory cells might respond and expand in response to the graft immune burden in a context where effector T cell responses are blunted. Since tolerance induction in this rat transplant model could also be induced by other agents modifying alloreactive effector responses (such as anti-donor Class-II antibodies[31,32]), and since infusion of the JJ319 mAb does not result in Treg expansion in control, non-grafted animals, it is likely that increase of regulatory cells (Treg and MDSC) occurs spontaneously when effector T cells are selectively inhibited).

This would also support the concept that regulatory T cell response can occur or is facilitated when CD28 signals are inhibited. The concept that selective CD28 antagonists might constitute a "Treg-permissive immunomodulation" has been further supported by experiments in primates and humanized mice (see below).

Models of kidney and heart transplantation in non-human primates

Baboon is a better non-human primate species than macaques to study CD28 biology at the preclinical level because contrary to macaques, and like in man, all baboon CD4-positive T lymphocytes express CD28, including in their effector memory cells compartment, a lymphocyte subtype that is the most prone to releasing cytokines after reactivation.[33] Like in human, kidney transplant rejection in baboons can hardly be controlled by monotherapies. Administration of the selective CD28 antagonist FR104 (given weekly) or of clinical doses or either rapamycin or mycophenolate mofetil failed to prolong survival more than a few weeks.[34] Whereas clinical doses of tacrolimus also failed to prolong survival more than a few weeks, high doses tacrolimus could block acute rejection in 50% of the animals.[10] In that case, however, treatment withdrawal after 3 months maintenance resulted in an immediate (within 6 days) irreversible cell mediated rejection. In contrast, biotherapies combining FR104 with either low doses tacrolimus or with rapamycin allowed allograft maintenance, and treatment withdrawal did not result in the immediate rejection seen after tacrolimus administration. Rather, rejection episodes occurred in the weeks to months after treatment withdrawal.[34] This delayed rejection profile together with increased intragraft Treg cells and TGFb mRNA suggested that maintenance therapy with CD28 antagonists enhanced Treg functions. Another mechanism related to expression of indoleamine dioxygenase by perivascular smooth muscle cells was also identified.[10] Treg function has also been investigated in the baboon kidney graft model in a face to face study where FR104 was compared with belatacept: low doses tacrolimus plus either FR104 or belatacept was maintained for a month and then tacrolimus was weaned, leaving animals under FR104 or belatacept monotherapies. Intragraft FoxP3 TSDR analysis revealed a higher demethylation of the FoxP3 promoter in FR104-treated animals than in belatacept-treated animals. A better control of follicular helper T cells was also recorded.[35] These biological differences translated into strong clinical benefit since long

term survival was only observed in FR104-treated animals.[35] Therefore, in primates, such as in rodents, preservation of the CD80-CTLA-4 axis and the resulting amplification of Treg functions came along with a better control of acute rejection. To our knowledge, no other immunosuppressive agent than CD28 antagonists preserves the CD80-CTLA-4 axis and preserves/amplifies suppressive activity of Treg cells. Existing co-stimulation inhibitors (Belatacept) limit CD80-CTLA-4 interactions (Figure 2) and classical immunosuppressive agents do not discriminate between effector and regulatory T cells.

Selective CD28 inhibition has also been tested in a stringent model of heterotopic heart transplantation in the cynomolgus macaque. Here too, CD28 blockade delayed significantly acute rejection and synergized with low doses CNI, particularly preventing cardiac allograft vasculopathy, suggesting that it might be used as CNI-sparing regimen.[10] In this model, Treg cells were found increased in animals presenting better clinical outcomes.

Models of skin transplantation

Although skin graft rejection appears differentially impacted after selective CD28 blockade or after CD80/86 blockade (with CTLA4-Ig), the difference was not due to differential accumulation of CD8+ memory T cell into the skin in a model where OVA-expressing skin grafted animals received memory Thy1.1+CD8+ T cells. Rather, selective CD28 blockade differentially affected effector function with a better inhibition of the acquisition of IFN-γ, TNF, and IL-2 production by T cells. In these experiments, administration of CTLA-4 blocking antibodies reversed these effects, demonstrating that CTLA-4 coinhibitory function can modulate cytokine effector function of secondary CD8+ T cell responses. In contrast, anti–PD-L1 had no effect on the ability of selective CD28 blockade to attenuate donor-reactive memory CD8+ T cell cytokine function.[36]

In addition to effector CD8+ T cells, intragraft Treg activity directly impacts skin graft outcomes. To study the impact of CD28 vs. CD80/86 blockade directly on Treg cells, another study compared FR104 to abatacept after grafting human skin pieces onto NOD-scid mice reconstituted with allogeneic human PBMC and isolated Treg cells.[14] The study confirmed that Treg cells do mediate immunosuppression in vivo and that this suppressive activity is dampened (i.e. Treg become non-functional) by co-administration of abatacept whereas it is enhanced by co-administration of selective CD28 antagonists. Much of the enhanced effectiveness of FR104 over abatacept has been attributed to its ability to maintain immune regulation, as evidenced by the preservation of FOXP3+ cell infiltration into the skin both in this model and others.[36] These experiments confirmed previous in vitro experiments showing similar opposite effect on Treg suppression by abatacept versus selective CD28 antagonists.[10,11] The difference has been attributed to the impact of the CTLA4-CD80 interaction on the ability of Treg cells to form synapses with antigen-presenting cells and to mediated suppression.[11]

Model of bone-marrow transplantation

Infusion of human PBMC to NOD-scid mice lead to a severe xenogeneic graft-versus-host disease mainly targeting the lung, liver and the gut. An intriguing observation has been that abatacept or belatacept failed to inhibit this GVHD whereas the selective CD28 antagonist FR104 showed full efficacy in all animals. To understand this difference in potency, authors co-injected FR104 with a blocking anti-CTLA4 antibody, so as to mimic the blockade of both CD28 and CTLA-4 imposed by abatacept/belatacept, resulting in the loss of efficacy of FR104[25]. It was concluded that the mechanism of action of a selective CD28 antagonist is also CTLA4-dependent. The concept has been further assessed in a nonhuman primate acute GVHD model where FR104 was compared with belatacept. FR104 monoprophylaxis delayed the onset of acute GVHD with a survival advantage, but eventually

acute GVHD occurred. Gene set enrichment analysis (GSEA) revealed relative over- and underrepresentation of multiple gene sets in the FR104 monoprophylaxis.[37] These included gene sets indicating relative preservation of naive T cells and underrepresentation of gene sets associated with cell proliferation, T cell antigen–dependent activation, and effector differentiation. However, these genes were still not like healthy controls presumably linking this residual gene expression to immune escape occurring during FR104 monoprophylaxis. Importantly, this analysis uncovered evidence for presence of CTLA4 signaling in the FR104 cohort compared with the belatacept cohort. Combined prophylaxis with FR104/sirolimus led to enhanced control of effector T cell proliferation and activation and resulted in prolongation of GVHD-free survival compared with the use of belatacept or belatacept/sirolimus. However, overall survival was not improved, due to occurrence of sepsis possibly indicative of an overimmunosuppression. Flow cytometric and transcriptomic analyses indicated a synergistic control of T cell effector maturation with FR104/sirolimus. However, when FR104 was discontinued, and concomitant with desaturation of CD28 occupancy on donor T cells, loss of naive CD4+ T cells did occur. In addition, no increased expression of individual coinhibitory receptors (including PD-1, LAG3, 2B4, CTLA4 and TIM-3) in the FR104/sirolimus cohort was observed.

Here,[37] in contrast with kidney and heart transplant models, FR104/sirolimus did not lead to an amplification of Treg reconstitution or homeostasis. While combination prophylaxis with FR104/sirolimus initially preserved absolute Treg numbers as well as the Treg/Tconv ratio, this effect was not durable, with peripheral blood Treg counts dropping by approximately day 25 after transplant. Importantly, however, no negative impact of FR104 on the in vitro suppressive capacity of Tregs, was noted. This study suggests that FR104 could be useful to prevent GVHD in patients at risk of disease.

First in Human Experience

Two First in Human studies have been conducted so far with selective CD28 antagonists. FR104[38] and BMS-931699[39] have been administered in healthy volunteers to explore the safety and tolerability of single and multiple ascending iv doses to characterize the pharmacokinetics and pharmacodynamics aspects, immunological changes and to gain access to preliminary efficacy in controlling immune responses. First, there was a consensus to demonstrate that the monovalent format predicted to be antagonist of the CD28/CD80-86 liaison without residual agonist activity of T cells was indeed safe in human. In both studies, no significant cytokine or immune cell changes were observed, even in subject who might have developed anti-drug antibodies. There was no change considered significant in the total lymphocyte count and lymphocyte subsets, including naïve T cells, memory T cells, and natural Treg cells, including in volunteers exposed over a 3-months period. Second, both studies demonstrated that selective CD28 antagonists were potent enough to control IgG responses to a neoantigen in human. This evaluation was made possible in clinical Phase 1 through the availability of Immunothel (Biosyn, Carlsbad, CA), a KLH-based vaccine developed as a surrogate BCG-therapy for the treatment of bladder cancer, where patients are first sensitized to KLH and then receive an intravesical administration of KLH, leading to a local delayed-type hypersensitivity response of therapeutic impact. Volunteers who accepted the Immucothel vaccination presented a blunted IgG response evidenced at surprisingly low drug levels (<0.2 mg/Kg).[38,39] Despite this powerful control of anti-KLH responses, even volunteers dosed at the maximal dose (thus presenting a CD28 inhibition over more than 12 weeks) did not present increased EBV titers and a subject who developed a labial herpes resolved this herpes while still under exposure. Thus, within the limits of these Phase 1 studies, selective CD28 blockade might not compromise viral immunity. Since then, ex-vivo investigations explored the possible induction of T-cell tolerization to alloantigens through

exposure of selective CD28 antagonist antibodies. Authors concluded that despite effective and specific tolerization to the primary alloantigen, CD28 blockade did not impede C. albicans or third-party-specific reactions in re-stimulation cultures.[40] Phase 1 data and available ex-vivo data therefore indicate that exposure to viral and fungal pathogens while under CD28 antagonist treatment might not compromise immunity on the short term. In spite of these data, to our knowledge, no clinical experience has been gained so far in kidney transplant recipients with a CD28 antagonist.

Conclusions

The initial option of developing monovalent Fc-free molecules targeting an antagonist CD28 epitope, being unable to crosslink CD28, and showing no adverse effects in NHP and humans,[38,41] has rejuvenated the interest of blocking this major co-stimulation pathway of the immune response. The data reviewed above in the field of transplantation show a strong consensus for selective anti-CD28 blockade leading to a disturbed generation of effector T cells together with a preservation of regulatory mechanisms (Treg cells, stabilized TSDR, IDO). The available data demonstrate an advantage for selectively targeting CD28 over other molecules (cyclosporin A, tacrolimus, rapamycin, mycophelonate mofetil, abatacept, belatacept), as it uniformly blocks the activation arm and reinforces the CD80/86-mediated co-inhibition branches (interacting with CTLA-4 and PDL-1; see Figure 2). Because depriving the immune response of CTLA-4-mediated co-inhibition results in an inability to terminate T cell activation, selective CD28 blockade can terminate immune responses that are known to be mediated by memory phenotype T cells. In alloimmune pathology, the skew towards effector activity is associated with reduced immune regulatory activity. The ability to maintain Treg suppression in the presence of selective CD28 blockade reported by several groups[11,14,35,42,43] highlights the role that Treg-expressed CTLA-4 plays in promoting co-inhibition. In rodents, selective CD28 blockade induced kidney transplant tolerance through

an initial blockade of effector T cells followed by the induction of several types of regulatory cells (Treg, MDSC). In non-human primates, no such transplant tolerance induction could be recorded although it was clear that regulatory cells were part to the immunosuppressive mechanism of action. Antigen-specific tolerance could nevertheless be induced in non-human primates, for instance TH-1 type delayed-type hypersensitivity responses could be inhibited on the very long term.[43] However, tolerance could not be induced to other types of immune challenge of the skin,[44] indicating that immune tolerance induction by selective CD28 antagonist is antigen-dependent, adjuvant-dependent or protocol-dependent.

Important preclinical experience has been accumulated to better understand the mechanisms of action and the potency of selectively antagonizing CD28 in transplantation. In summary, the "Pros and Cons" could be as follows: agents blocking CD28 might be more potent than current co-stimulation inhibitors (belatacept) by blocking T cell functions while maintaining Treg activity. Like current co-stimulation inhibitors, as demonstrated in primates, agents blocking CD28 might also constitute the basis of a CNI-free maintenance therapy, while reducing the rejection risk that has been attributed to the use of belatacept.[45] A possible drawback of the stronger control of allogeneic T cell is the over immunosuppression and related infections. In addition, prior concerns in human clinical trials have been raised on the outcomes of CNI sparing strategies with belatacept[46] that might also be met if CD28 blocking agents are used. Only forthcoming clinical trials will allow fine-tuning to achieve the optimal clinical indications and to assess the risk-benefit ratio and the potential clinical advantage of this approach in organ and cell allotransplantation.

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Figure 1. Anti-CD28 antibody valency determines CD28 multimerization and T cell activation. A, Super-agonist anti-CD28 antibodies target the basolateral C''D domain, are not antagonist of binding of CD80/86 and induce a strong CD28 multimerization resulting in TCR-independent T cell activation (as described by Luhder et al 21). B, Divalent antibodies targeting the apical MYPPPY epitope of CD28 are antagonist of binding of CD80/86 but still induce CD28 capping/multimerization resulting in TCR-dependent T cell activation (as described by Mary et al 18). C, Monovalent antibodies targeting the apical MYPPPY epitope of CD28 are antagonist of binding of CD80/86 and do not induce CD28 multimerization resulting in prevention of T cell activation.

Figure 2. Costimulatory and coinhibitory molecules implicated in the targeting of CD80/86 vs CD28. Solid lines/arrows represent active signaling pathways. Dotted lines represent disrupted inhibitory pathways. APC, antigen-presenting cells.

Table 1 : Preclinical transplantation models deciphering efficacy and mechanisms of action of selective CD28 antagonists

Model	CD28 Antagonist	Outcome	Reference
Kidney transplantation in rat	JJ319 mAb	Tolerance induction in monotherapy	47
Heart transplantation in rat	JJ319	Synergizes with CD40Ig to reinforce tolerance	48
Graft versus host disease in humanized mice	FR104	Abrogates GVHD, in a CTLA-4-dependent manner	25 14
Skin transplantation in mice	BMS-1m74-14982	Inhibited cytokine production by $CD8^+$ memory T cells	36
Skin transplantation in humanized mice	FR104	Synergy with Treg	14
Heart transplantation in primates	FR104	Delays rejection and synergizes with CsA	10
Kidney transplantation in primates	FR104	Prevents rejection and synergizes with tacrolimus, rapamycin and mycophenolate mofetil	25, 35
Acute GVHD post HSCT in primates	FR104	Synergizes with sirolimus to prevent GVHD	37

Figure 1

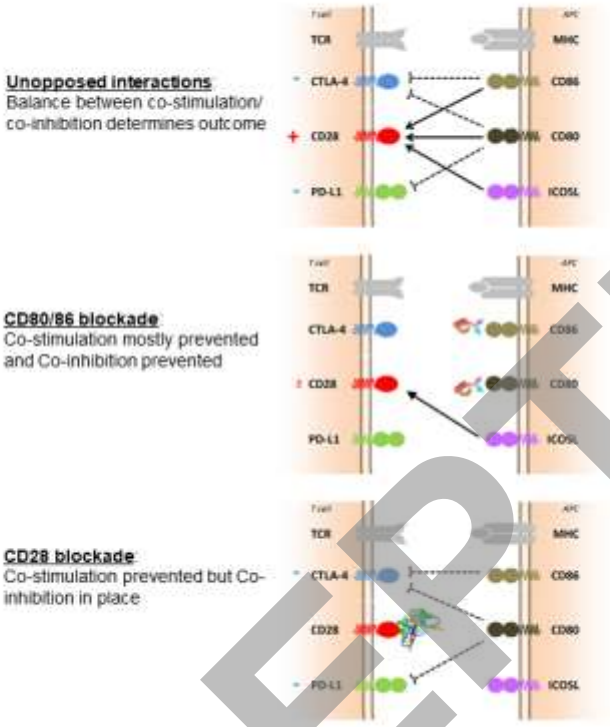

Figure 2