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Co-stimulatory blockade molecules and B cell-mediated immune response : Current knowledge and perspectives

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Abstract

There is an urgent need for therapeutic agents that target humoral alloimmunity in solid organ transplantation. This includes sensitized patients with preformed donor-specific human leukocyte antigen antibodies and patients who develop de-novo donor-specific antibodies, both of which are associated with acute and chronic antibody-mediated rejection and allograft loss. In the last decade both experimental and clinical studies highlighted the major impact of co-stimulation molecules in the control of immune responses both in the field of transplantation and autoimmune disease. Although these molecules have been initially developed to control the early steps of T cell activation, recent evidence also supports their influence at several steps of the humoral response. In this review we aim to provide an overview of the current knowledge of the effects of co-stimulatory blockade agents on humoral responses in both autoimmune and allogeneic contexts. We first present the effects of co-stimulatory molecules on the different steps of alloantibody production. We then summarize mechanisms and clinical results observed using CTLA4-Ig molecules both in transplantation and autoimmunity. Finally we present the potential interest and implications of other co-stimulatory family members as therapeutic targets, with emphasis on combinatorial approaches, for the optimal control of the alloantigen-specific humoral response.

Keywords: acute rejection, lymphocytes

Introduction

Antibody-mediated rejection (AMR) is now a well-recognized major contributor to graft loss in kidney transplant recipients. It accounts for up to 2/3 of death-censored graft failures beyond the first year of transplantation and is the leading cause of late transplant failure^{1,2}. AMR is likely to occur in sensitized kidney allograft recipients with anti-HLA donor-specific antibodies (DSA) before and after transplantation^{3,4}. Although our understanding of the mechanisms behind DSA development is far from complete, pathogenesis as well as clinical and histological features of DSA-mediated graft injury has been more extensively analyzed during the last decade⁵. Indeed experimental studies have shed light on the natural history of acute and chronic AMR⁶. The sequence starts with the generation of antibodies directed against the graft. Although highly polymorphic mismatched HLA molecules represent the most documented targets for DSA, it is clear that DSA can also be directed against other kinds of molecular targets including polymorphic minor histocompatibility antigens and, following a breakdown of B cell tolerance⁷, non-polymorphic autoantigens⁸. Binding of circulating DSA to directly accessible graft endothelial cells can trigger the activation of classical complement pathway, a central process in the pathophysiology of acute AMR⁹. By contrast, the pathogenesis of chronic AMR appears to be mainly related to antibody-dependent cell mediated cytotoxicity (ADCC)¹⁰. Binding of the Fc fragment of DSA complexes to innate immune cells causes their activation and inflammation, leading to smoldering endothelial cell damage¹¹. In turn, chronic vascular inflammation promotes the progressive development of typical vascular lesions, i.e. transplant glomerulopathy, allograft vasculopathy and lamination of the peritubular capillary basement membrane¹².

Improving long-term graft survival by targeting various aspects of the humoral immune response is a major goal in modern transplantation. To prevent both acute and chronic AMR, *de novo* DSA synthesis needs to be prevented or limited. Currently, conventional immunosuppressive protocols involve a short-term induction therapy with Basilixumab or Thymoglobulin, followed by a life-long maintenance immunosuppression combining anticalcineurine, antiproliferative drugs such as MMF or mTor inhibitors and steroids.

Although, these drugs target the different signaling pathways leading to alloantibody production by B cells, around 15–30% of renal transplant recipients still develop *de novo* DSA^{13–16}. Therefore they are not effective enough to inhibit *de novo* DSA production and new strategies need to be defined.

Additionally, currently up to 30-40% of transplant candidates are already sensitized, limiting their donor options and putting them at a higher risk for AMR. Therefore immunosuppressive protocols were tested to attempt to remove antibodies in sensitized allograft patients to expand their chance of receiving a compatible transplant and increasing graft survival¹⁷. These included numerous attempts to boost conventional immunosuppression, or to inhibit/remove B cells, plasma cells, antibodies, and/or complement¹⁸ through the use of B cell depleting agents (e.g., rituximab and anti-CD20), intravenous immunoglobulins, and plasmapheresis. Unfortunately, once DSAs are established, no current therapies can reliably reverse their pathogenic effects: DSA titers were incompletely reduced, and rebound of antibody production after treatment could be observed leading to an overall increased risk of AMR and graft loss in some patients. Moreover these controlled trials have used different treatment protocols, and have been underpowered and of low quality^{19,20}. These results strengthened the need to identify new molecules and optimal immunosuppressive strategies to target humoral immune responses.

Recent findings have highlighted the major impact of co-stimulation (co-stim) molecules for the control of immune responses. Targeting the second signal of T cell activation with co-stim blockade to inhibit activation, or checkpoint inhibitors to increase it, has been a revolutionary medical advance of the last decade. Unexpectedly, experimental data and clinical studies suggested co-stim molecules also play a large role in controlling humoral immune responses in both autoimmune and allogeneic settings. This review aims to describe immune mechanisms leading to DSA production and how co-stim blockade can modulate humoral immunity. We next consider clinical evidence for co-stim mediated control of humoral immune responses in transplantation and autoimmune diseases with a focus on the first clinically released drug, CTLA4-Ig. Finally, we discuss the potential involvement of the individual family members and suggest original combinatory strategies to optimally control allogenic humoral responses.

1. Immune mechanisms leading to DSA production: role of costimulatory molecules

General mechanisms

DSA are high affinity antibodies, typically IgG isotypes, mostly directed against donor HLA molecules. These characteristics imply that DSA generation results from a T cell-dependent B cell response. This type of humoral response, which primarily develops within secondary lymphoid organs (spleen or lymph nodes), involves two distinct events that are separated both spatially and temporally (Figure 1). Early events include, in parallel, the activation/priming of allogeneic T cells by dendritic cells, and allogeneic B cells by the antigen, in the secondary lymphoid organs (i.e. lymph nodes and spleen)²¹. Both activated B and T cells then migrate to the border of B cell follicles and T cell zones, respectively, where they undergo cognate interactions. These interactions lead to the development of the germinal center (GC) response. GC B cells will cyclically increase their affinity to the antigen and expand oligo-clonally^{22,23}. At one point, memory B cells and long lived plasma cells will be generated, but the exact signals that stop GC cycle and drive one or the other fate are unknown²⁴.

T follicular- B cell crosstalk

T follicular helper cells (Tfh) are now recognized to play a major role in the development of humoral responses²⁵⁻²⁷. Tfh are T helper cells specialized in helping GC B cells through cell-to-cell contact and the production of cytokines including IL-21. Their differentiation requires the transcription factor Bcl6 and they can be defined by ICOS expression with high CXCR5 and PD1 expression²⁸. Experimental models and human studies suggest that targeting Tfh, and particularly Tfh-B cell crosstalk, might be fundamental to control *de novo* humoral immune responses in transplantation. In several non-human primate (NHP) models, Kwun et al showed that GC size and frequency were positively correlated with DSA production and incidence of humoral rejection^{29,30}. Studies of Tfh-B cell crosstalk are limited in humans due to the difficulties to access human lymph nodes^{31,32}. Circulating blood subsets of "Tfh" were identified³³. They share some, but not all, phenotypic

characteristics with lymph node Tfh. These cells were found to express CXCR5, ICOS and PD-1 but to a lower level than lymph node Tfh, and don't express the transcription factor Bcl-6, known to be required for Tfh formation (in mice and humans³⁴). They can also interact with B cells, secrete IL-21 ex vivo, and induce plasmablasts differentiation and antibody secretion in vitro. The origins and role of this T cell population are still debated, and whether they can directly reflect ongoing follicular Tfh-B cell interactions remains to be fully determined. However, increased proportions of these circulating "Tfh-like" subsets were shown to precede seroconversion in human vaccination studies³⁵. S. Brouard's team recently showed that following kidney transplant, increased blood Tfh frequency preceded HLA sensitization in CNJ-treated patients³⁶. Conversely, operational tolerant recipients, a unique and rare clinical situation in which kidney graft function is preserved without immunosuppression, displayed lower DSA incidence, lower frequencies, and lower functional ability of circulating Tfh compared to control graft patients.

Interestingly, two of the main markers used to identify and characterize Tfh functions, ICOS and PD-1, belong to the co-stim molecule family. ICOS is known to be critical for Tfh formation in mice³⁷, and PD-1 was found to regulate the functional capacity of human blood Tfh³⁸. This highlights that a better understanding of the function of co-stim molecules on human Tfh and their role in Tfh-B cell crosstalk may be crucial for future therapies.

Role of co-stimulatory molecules in Tfh generation, Tfh-B cell crosstalk and B cell responses

- Family of co-stimulatory molecules

Co-stim molecules have historically been shown to be fundamental for T cell priming by DC. They are a complex set of stimulatory and inhibitory molecules, responsible for the fine-tuning of T cell activation³⁹⁻⁴¹. After antigen recognition and engagement of the TCR, the interaction of APC co-stim molecules with their ligands on T cells provides the additional signal required to fully achieve T cell activation. CD40 on APC binds to its T cell ligand CD40L resulting in the up-regulation of B7 molecules (CD80/CD86) and other co-stim molecules on APC⁴². CD28, which is constitutively expressed on T cells, then binds to

its ligands CD80 and CD86 on APC leading to T cell activation. Co-stim pathways also trigger the expression of inhibitory receptors that are responsible for turning off or shutting down the system to prevent uncontrolled activation. The inhibitory receptors share the same ligands as their stimulatory counterparts and thus the competition for ligands is a further mechanism of fine tuning T cell activation. For example, the inhibitory receptor CTLA4 is induced upon T cell activation, and binds to the B7 molecules CD80 and CD86 on APC with higher affinity than CD28. Thus its expression prevents further stimulatory CD28-ligation from the APC (T cell-extrinsically), and produces a T cell intrinsic inhibitory signal.

- Role of co-stim molecules in Tfh generation and function

It has recently been proven that co-stim molecules also play an important role in the different steps of Tfh differentiation and function (Figure 2A). Due to their complexity and involvement at different stages of this process (Figure 2B), the use of genetically deficient animal models was crucial to demonstrate their functions.

ICOS, a critical mediator of Tfh cell generation and function.

ICOS plays an important role at each stage of Tfh development and function. At an early stage, after DC priming, ICOS/COS-L interactions participates in the up-regulation of EBi2 leading to development of Tfh precursors⁴³. Further chemokine receptor changes allow them to migrate to the B cell follicle border^{28,37,44}. Interactions between ICOS on Tfh and ICOS-L on bystander B cells induce the activation of the PI3 kinase pathway, essential for Tfh localization in the GC⁴⁵. There, B cells offer repeated antigen presentation and co-stimulation through ICOS to promote full development of Tfh cells by inducing expression of the transcription factor Bcl6³⁷. ICOS may also play a role in Tfh differentiation or survival by regulating the induction of the transcription factor c-Maf, potentially through expression of IL-21^{46,47}. In the GC, ICOS-L is responsible for the entanglement between Tfh and GC B cells, a crucial feature to select B cell clones with the highest affinity for the antigen⁴⁸. Therefore, in mice ICOS plays an important role in multiple stages of humoral immunity.

Accordingly in humans, patients with ICOS deficiency have severely damaged GC and reduced blood CXCR5⁺ CD45RO⁺ memory cells. Therefore, ICOS signaling is also likely to play a fundamental role for Tfh differentiation in humans⁴⁹.

PD-1, a complex role in Tfh function

PD-1 is another key marker that defines Tfh phenotype. On “non-Tfh” T cells, PD-1 is a marker of exhaustion. PD-1 signaling down-regulates the activation of effector T cells by dampening CD28 signaling^{50,51}. However, the precise role of PD-1 in Tfh homeostasis and function is more complex. Good-Jacobson et al showed that PD-1 deficient mice displayed increased number of Tfh with decreased abilities to secrete cytokines (IL4 and IL21). On the B cell side, PD-1-deficient mice had increased GC B cell mortality, decreased number of plasma cells (PC) and overall decreased immunoglobulin production. However the remaining PC produced higher affinity antibodies than PD-1-sufficient mice⁵². Recently, Shi et al studied why PD-1 represses Tfh formation while it is also highly expressed on Tfh. They found that PD-1 signaling helps to restrain Tfh in the GC by repressing CXCR3 expression that would distract Tfh and potentially allow them to migrate out of the GC⁵³. Thus PD-1 functions in Tfh formation and GC localization.

CD28-CTLA4, additional players in regulating Tfh differentiation

The role of CD28 for T cell activation has been known for a long time. Recently it is also has been found that continuous CD28 expression is required for Tfh differentiation⁵⁴. Along parallel lines, CTLA4, the inhibitory competitor of CD28, restrains Tfh responses and inhibits their B cell stimulatory function. Specifically, deletion of CTLA4 on Tfh increased GC B cells and serum Ab titers^{55,56}.

- B cell intrinsic role of co-stimulatory molecules

Co-stim molecules also play a role in regulating B cell responses directly. CD40 signaling in B cells, triggered by its interaction with CD40L on Tfh, is a well-known example of a co-stim pathway directly influencing B cell fate by a cell-intrinsic mechanism. The combinatory

signals of CD40 and IL-21 in activated B cells induce the expression of Bcl6, a major transcription factor required in GC B cells⁵⁷. In GC B cells, Luo et al recently showed that CD40 ligation in conjunction with BCR signaling was required to induce c-Myc, a major transcription factor for GC B cell maintenance and survival⁵⁸. Moreover, it was demonstrated that CD40 was required on B cells intrinsically in the generation of antigen-specific GC B cells, high-affinity class-switched antibody production, and also for antigen-specific Tfh formation, suggesting that CD40-CD40L signaling is also bi-directional^{59–61}. Furthermore, CD40 signaling intrinsically regulates B cell homeostatic maintenance⁶². In contrast to GC B cells, CD40 ligation alone is sufficient to activate naïve B cells in vitro.

Although CD28-CTLA4/CD80-CD86 pathway has been thought to mainly control T cell activation, recent evidence suggest that they might also play a direct role on B cells. Both long-lived plasma cells (PC), that survive in the bone marrow and participate in sustaining long-term antibody levels, and short-lived plasma cells, re-express CD28 in mice and humans⁶³, due to the loss of the B cell master regulator Pax5 during terminal differentiation⁶⁴. Two teams have independently shown a role for CD28 in plasma cell function. However its exact function is still debated. Rozanski et al showed that specific CD28 deficiency in the B cell lineage was sufficient to decrease antigen-specific (NIP) IgG titers by 10-fold after immunization, even with adequate T cell help⁶⁵. They further showed a PC-intrinsic function of CD28 that supports the survival of long-lived PC in the bone marrow leading to increase Ab titers⁶⁵ through a Blimp-1 dependent mechanism⁶⁶. However, Njau et al found increased frequencies of PC and Ab production in CD28 deficient PC⁶⁷.

The current evidence supports that targeting co-stim molecules likely acts both on the T cells as well as on B cells (including plasma cells) to modulate the Tfh-B cell crosstalk. Thus, further development of strategies targeting these molecules will be important to further our ability to modulate humoral responses.

2. Co-stimulatory blockade immune mechanisms on B cell responses: the CTLA4-Ig experience.

Drugs targeting co-stim receptors have been developed in the past two decades to prevent T cell mediated allograft rejection, or to control the activation of autoreactive T cells in autoimmune settings (Figure 3). A thorough compilation of the relevant co-stim molecules, drugs which target them, and clinical trials can be found in Table 1. CD28/CTLA4/B7 axis was the first pathway to be therapeutically inhibited. The use of a recombinant CD28-Ig to bind CD80/CD86 and block natural CD28 signaling was ineffective due to its low affinity for its B7 ligands leading to the development of another recombinant protein: the CTLA4-Ig (Abatacept)⁴¹. This fusion protein comprises the extracellular domain of human CTLA-4, which has a higher affinity for CD80 and CD86 than CD28 does, fused with a fragment of the Fc portion of human IgG1, to increase its half-life. Because the majority of the first experimental and clinical data are related to the use of CTLA4-Ig (Abatacept) and its higher affinity version Belatacept (LEA29Y)⁶⁸ in auto-immunity and transplantation, we will focus first on what was learned from these studies.

Although Belatacept has been mainly developed to target DC-priming of T cells, pre-clinical models have interestingly shown that the use of Belatacept inhibited humoral responses in transplantation settings. Follicle size, GC proportion and IL-21 secretion were decreased in Belatacept treated primate recipients suggesting a specific role of Belatacept on Tfh-B cell crosstalk.

Anita Chong's studies further showed, in murine models, that delayed CTLA4-Ig could inhibit on-going humoral response, even if priming of allogenic T cells had already occurred^{69,70}. Indeed, introduction of CTLA4-Ig treatment 14 days after sensitization inhibited alloantibody production and collapsed GC responses. These results suggested that CTLA4-Ig action on B cell stimulatory capacities were important, independently of T cell priming by DC. Recently, in a murine skin transplant model, Baddell et al showed that Belatacept inhibited the function of adoptively transferred allogenic Tfh leading to decreased sensitization⁷¹.

We further studied mechanisms underlying Belatacept action on humoral response in humans⁷². We showed that Belatacept inhibited Tfh-B cell crosstalk *in vitro* by decreasing the proportion of activated PD1+ICOS+ Tfh cells, decreasing Tfh proliferation, and decreasing the differentiation of B cells into plasmablasts. We confirmed that Belatacept treated kidney-graft recipients displayed lower frequencies of circulating Tfh and terminally

differentiated B cells than control recipients, suggesting that Belatacept might also target and inhibit Tfh-B cell crosstalk *in vivo*.

Previously, Belatacept was mainly thought to alter B cell responses by blocking T cell priming. We unraveled an additional B cell intrinsic mechanism. We showed it could directly inhibit the immunoglobulin production of activated B cells *in vitro*, without any T cell intervention. The direct effect of Belatacept on B cells may imply a signaling through CD86, which was interestingly highly expressed in plasmablasts. It induced STAT3 activation in stimulated B cells but not the Akt/NFkB pathway, similar to CTLA4-Ig mediated reverse signaling described in DC^{73,74}.

The current evidence supports that targeting co-stim molecules likely acts both on the T cells as well as on B cells (including plasma cells) to modulate the Tfh-B cell crosstalk. Thus, further development of strategies targeting these molecules will be important to further our ability to modulate humoral responses.

Belatacept use in transplantation: one step toward a better control of humoral responses

Belatacept was initially developed as anticalcineurine sparing strategies to prevent their nephrotoxicity. Phase II and III clinical studies, BENEFIT and BENEFIT-EXT, which evaluated Belatacept efficiency in non-sensitized kidney graft recipients showed an increase in (i) overall survival, (ii) in graft survival leading to a 43% reduction in the risk of death or graft loss and (iii) an improved graft function when recipients were treated with Belatacept as compared to anticalcineurine regimens⁷⁵. Several hypotheses could be drawn to explain this clinical efficiency⁷⁶, including improved patient's compliance to monthly-infused treatment and anticalcineurine sparing toxicity.

Importantly, these studies also highlighted that Belatacept-treated recipients had a drastic reduction of DSA incidence. The cumulative rate for the development of DSA was 4.6% in the Belatacept group versus 17.8% in the control group, 84 months after transplantation⁷⁵. The lower incidence of HLA sensitization may account for the decreased risk of humoral rejection and clinical efficiency with Belatacept treatment.

Animal models also confirmed that Belatacept specifically inhibited humoral responses and T-dependent antibody production. Belatacept inhibited anti-sheep red blood cell antibodies secretion more efficiently than its predecessor Abatacept in a murine model⁶⁸.

Furthermore, in non-human primate kidney transplant model, Belatacept treatment was the only treatment able to prevent DSA formation⁷⁷, whereas combinatory treatment with anti-CD3, tacrolimus (anticalcineurine) and alefacept (anti-CD2) were ineffective at preventing DSA formation and AMR.

Overall, Belatacept effects on humoral responses led, for the first time, to improved clinical results as compared to the reference therapy (anticalcineurine+mycophenolate mofetil+corticosteroid) in non-sensitized kidney recipients that led to its US FDA approval in 2011. To more specifically inhibit CD28-mediated stimulatory pathway, while leaving CTLA-4 inhibitory signaling intact (especially on Tfh), a new generation of CD28 blockade agents was engineered: a pegylated Fab' antibody fragment antagonist of CD28, CD28-Fc (FR104)⁷⁸⁻⁸⁰. In a non-human primate transplantation model, CD28-Fc seems to have an even more potent effect in controlling Tfh-B cell crosstalk than Belatacept⁸¹: IL21 gene expression was decreased in FR104 treated animal biopsies at 1 month. FR104 also inhibited Tfh proliferation more potently than Belatacept in an *in vitro* model of Tfh-B cell co-culture. In a murine skin transplant model, anti-CD28 led to superior inhibition of donor targeted Tfh, GC B cells and DSA production than CTLA4-Ig⁷¹. Also promising, evaluation of its effects in human transplantation settings is warranted.

CTLA4-Ig in autoimmune disease

Though the previously described therapies were initially designed to target T cells, in light of their newly recognized effects on humoral responses, it is important to reconsider their mechanism of action in autoimmune diseases. Whereas generation of DSA is the driving process of AMR pathogenesis, the precise roles of autoantibody in the physiopathology of autoimmune diseases is still poorly understood. One reason for this is that in transplantation settings, the source of the initial exposure to the antigens, and the pathophysiological mechanisms of T and B cell mediated damage are known. However, at the time of diagnosis of an autoimmune disease, the progression and evolution of the immunological processes underlying it are unknown. Moreover, "autoimmune diseases" refers to a variety of conditions with very different pathological contexts and pathogenesis.

Based on the finding that CD28 deficient mice were resistant to arthritis in a model of collagen-induced arthritis (CIA)⁸², targeting autoreactive T cells with CTLA4-Ig demonstrated striking reduction of disease manifestations in lupus disease⁸³, and CIA⁸² mouse models. According to the timing of administration, CTLA4-Ig administration either prevented the development of CIA, or was able to ameliorate CIA^{82,84}.

The promising pre-clinical results in murine models of lupus, especially for the treatment of arthritis, spurred the initial human clinical trials of CTLA4-Ig. They mainly focused on the treatment of patients with psoriasis⁸⁵ and rheumatoid arthritis (RA). Successful clinical trials with abatacept (human CTLA4-Ig) led to abatacept approval by the US Food and Drug Administration for the treatment of RA in 2005⁸⁶. These trials showed a reduction in autoantibody production, but it's unknown whether it is a direct effect of CTLA4-Ig treatment on the humoral response, or an indirect effect of reduced disease. CTLA4-Ig has been reported to inhibit antibody responses in humans in the context of autoimmunity. Antibody titers induced after vaccination with the monovalent pandemic 2009 influenza A H1N1 virus vaccine was severely reduced in abatacept-treated rheumatoid patients as compared to control patients treated with Methotrexate (9% versus 58%)⁸⁷.

Using the NZB/NZW.F1 experimental murine model of Systemic Lupus Erythematosus (SLE), it was observed that administration of murine CTLA4-Ig prolonged survival and prevented autoantibody production⁸³. However, clinical trials of abatacept in SLE patients have not shown any clear efficacy⁸⁸. In lupus nephritis, abatacept did not clearly improve the outcome, despite some evidence of biological activity, including decreased anti-double-stranded DNA antibody, C3, and C4 levels, but the results were inconsistent⁸⁹⁻⁹¹. Collectively, a more thorough understanding of both the pathophysiology of individual autoimmune diseases, and the precise mechanisms of function of abatacept is needed for a more targeted approach to treatment.

3. Other co-stimulatory blockade agents

Although CD28 was the first co-stim pathway to be therapeutically targeted, current knowledge involving other co-stim pathways in T cell activation led to the development of novel co-stim blockade agents. They have mainly been tested in animal models. We will review their potential impact on humoral responses in light of recent advances of functional mechanisms.

CD40/CD154

CD40/CD154 (CD40L) pathway seemed one of the most promising therapeutic targets in pre-clinical murine and non-human primate models of transplantation. It belongs to the TNF superfamily (Figure 2). As discussed in chapter 2, in contrast to other co-stim pathways where the ligands on the APC mainly amplify T cell activation signaling pathways, activation of CD40 on B cells by CD40L is crucial for T cell dependent B cell activation, and at several steps of their differentiation⁵⁸. Consequently, the blockade of this pathway has drastic consequences on humoral responses. In humans, the genetic deficiency of CD40L leads to a severe immune disorder: hyper IgM syndrome. Patients genetically deficient in CD40L are severely immune-compromised; they lack GC reactions and produce decreased levels of class switched antibodies⁹²⁻⁹⁴, demonstrating the importance of CD40/CD40L in immune responses.

In numerous experimental models of transplantation, inhibition of the CD40/CD40L pathway led to remarkably increased graft survival^{95,96}. Interestingly, using anti-CD154 (anti-CD40L) therapy has drastic consequence in reducing DSA production in mouse models: treatment with anti-CD154 was able to terminate ongoing germinal center reactions⁶⁹ and effectively reduced alloantibody production following renal transplantation in rhesus macaques⁷⁷. Finally, blocking this pathway is a potent means to induce tolerance, i.e. stable graft function without any continuous immunosuppressive therapies, in experimental models⁹⁷.

However, human clinical trials evaluating the safety and efficiency of anti-CD154 reagents had to be prematurely stopped due to severe thromboembolic complications, related to

CD154 expression on human platelets⁹⁸. Anti-human CD40, ASKP1240, has now been developed as a safe and efficient alternative^{99,100}.

Importantly, anti-CD40 is also very potent in controlling humoral responses. In non-human primates, it inhibited the production of antibodies against tetanus toxoid (TT) in a dose-dependent manner, with a complete inhibition of anti-TT IgG generation at the highest dose¹⁰⁰. These results suggest it also effectively blocks GC and the production of high affinity antibodies in this model. Moreover, production of DSA was abolished in ASKP1240 treated monkeys in a model of pancreatic islet transplantation⁹⁹. ASKP1240 appears to be a promising candidate for immunosuppression in clinical transplantation. Several clinical trials in humans are on-going^{101,102}.

PD-1/PD-L1

PD-1/PD-L1 is a major inhibitory pathway of T-cell activation. The use of PD-1 inhibitors to boost T cell immunity has revolutionized the treatment and prognosis of several metastatic cancers¹⁰³. As discussed in chapter1, Tfh cells express high levels of PD-1 but its function on this specific T cell subset is complex. It dampens TCR and CD28 signaling and the accumulation of Tfh. However it also influences Tfh migration and the stringency of B cells affinity exiting the GC^{52,53}. Therefore, in contrast to CTLA4-Ig, which mainly blocks T cells from receiving co-stim signals, administering PDL1-Ig is hypothesized to induce an inhibitory signal through PD-1 on T cells. Since PDL1-Ig is designed to mimic a stronger PD-1 signal, it may also inadvertently increase the affinity of B cells that effectively seed a GC. PDL1-Ig efficacy to prevent organ rejection was studied in some experimental models^{104,105}. PDL1-Ig, used in combination with anti-CD154, greatly increased organ survival to 140 days versus 40 days and 13 days with the anti-CD154 or PDL1-Ig alone respectively. Its impact on humoral response has not been assessed yet and will be critical information.

ICOS/ICOSL

ICOS-ICOS-L pathway is essential for Tfh differentiation, GC development, and

459 maintenance^{37,48}. However, robust antigen delivery by cognate B cells may overcome
460 ICOS-L requirement on B cells using compensatory signals¹⁰⁶. Thus targeting ICOS-ICOS-
461 L pathway in autoimmunity or organ transplantation should be closely monitored.

462 To target this pathway, two therapeutic strategies have been developed and are in pre-
463 clinical studies; the use of recombinant ICOS-Ig protein, and the anti-ICOS blocking
464 antibody. ICOS-Ig was designed to out-compete binding of surface-bound ICOS on T cells
465 to its cognate receptor ICOS-L on APC, thus preventing the delivery of co-stim signal from
466 APC to T cells (Figure 2). ICOS-Ig was subsequently found to prevent the binding of
467 ICOS-L to CD28. Pre-clinical studies evaluating ICOS-Ig efficacy on preventing allograft
468 rejection produced disappointing results. In kidney transplanted non-human primates, the
469 use of ICOS-Ig alone as a short-term treatment did not modify overall survival as
470 compared to untreated animals¹⁰⁷. Furthermore, co-administration of ICOS-Ig with
471 Belatacept did not improve graft survival as compared to Belatacept alone. More recently,
472 co-administration of ICOS-Ig with anti-CD40 also did not improve survival as compared to
473 anti-CD40 alone in a model of cardiac transplantation in non-human primates¹⁰⁸.

474 The use of anti-ICOS, however, seemed to more effectively block humoral responses in
475 pre-clinical models. It led to a decreased production of DSA in a model of islet xeno-
476 transplantation¹⁰⁹. Moreover, in combination with anti-CD40 in a model of chronic cardiac
477 humoral rejection, anti-ICOS led to decreased DSAs titer and ameliorated histology as
478 compared to anti-CD40 alone¹¹⁰. Hence anti-ICOS treatment may be a promising
479 candidate for clinical trials. It may be particularly effective when used in combinatory
480 therapies, as compensatory pathways may overrule its requirement. Optimal combinatory
481 therapies still need to be defined and tested in humans.

483 **B7-H4**

484 B7-H4 is a new receptor of the B7 family, mainly expressed on B cells¹¹¹, however its
485 ligand is unknown¹¹². B7-H4 triggering on B cells in the context of EBV infection led to
486 increased expression of Fas-L inducing their apoptosis¹¹³ and reduced the proliferation of
487 EBV transformed lymphoma B cells¹¹⁴. B7-H4 also appeared to play a role in modulating
488 autoimmune disease. B7-H4 deficient lupus-prone mice have more severe disease and

increased production of autoantibodies¹¹⁵. Inhibiting B7-H4 in a model of cardiac transplantation increased DSA production without any influence on graft survival¹¹⁶. Conversely injection of B7-H4 recombinant protein led to ameliorated disease in a lupus-prone model¹¹⁵. Moreover in a murine pancreatic islet transplantation model, overexpression of B7-H4 on donor cells led to increased graft survival and prolonged euglycemia^{116–118}. In both models, B7-H4 seems to interact and synergize with CD28-CTLA4/B7 pathway: overexpression of B7-H4 increased CTLA4-Ig mediated graft survival¹¹⁸ and conversely blocking B7-H4 in CD28-deficient mice decreased graft survival¹¹⁹. B7-H4 targeting may give interesting results when used in conjunction with CTLA4-Ig/CD28-Fc regimen.

CD27/CD70

Compared to other TNF α family members, few studies have evaluated the impact of targeting this pathway in transplantation. However it has been shown that CD27/CD70 plays a major role in B cell hematopoiesis and differentiation¹²⁰. Indeed, CD27 is expressed on early B cell progenitors¹²¹. In humans, CD27 is mainly known as a memory B cell marker¹²². CD70 is transiently expressed on activated B cells but more permanently on plasma cells¹²³. Blocking CD70 signaling *in vitro* leads to decreased B cell activation and immunoglobulin production¹²⁴. CD70 pathway may also synergize with the CD40 pathway^{124,125}.

In experimental models, blocking CD70 led to increased-cardiac graft survival¹²⁶. However clinical efficacy relied on the use of a depleting antibody¹²⁷. Another study suggested that anti-CD27 therapy might efficiently control memory humoral responses in pre-sensitized cardiac transplantation leading to increased graft survival¹²⁸. Hence, targeting CD27/CD70 may lead to interesting results toward controlling humoral responses¹²⁹.

4. Future strategies

Single agent-based therapy may not be sufficient to reach the ultimate goal of controlling humoral responses that most likely rely on redundant mechanisms. Moreover, multiple

barriers exist toward effectively targeting humoral responses. These include; (i) cellular barriers and niches that prevent drug penetration, such as GC in secondary lymphoid organs or plasmablast niches in bone marrow, (ii) the complementarity and redundancy of certain pathways and (iii) potentially variable effects of the drugs at different stages of the immune response, the basic mechanisms of which are still incompletely understood. Due to these complexities, a combinatorial approach to co-stim blocking may be more effective, and defining the best combinations is the ultimate goal.

4.a. Combination of co-stimulatory blockades

Co-stimulatory pathways are closely related³⁹. For example, different ligands share the same receptor (Figure 2) and signaling of a given pathway influences the others. Hui et al recently showed that PD-L1-induced-signaling of PD-1 inhibited T cell activation through CD28 signaling⁵⁰. We also showed that using Belatacept modifies the expression of other co-stim molecules, particularly PD-L1, in vitro⁷².

This implies that targeting one co-stim pathway may lead to the induction of a new equilibrium of all co-stim molecule expression and function. These changes have to be studied and taken into account when designing future therapeutic strategies.

With the use and development of new molecules directly targeting B and plasma cells in the autoimmune, transplantation, or hematological fields, the main challenge will be to design the best complementary strategies (Figure 3).

4.b. Costimulatory blockade agents and other B cell targeting therapies

Bortezomib, a broad-spectrum proteasome inhibitor (PI), inhibits alloantibody production in patients with kidney transplantation through induction of apoptosis in plasma cells¹³⁰. In the context of AMR, PI seems to reduce the titers of immunodominant DSA by 50%, especially for early AMR¹³¹. A similar, although transient, reduction rate has also been observed for pre-formed anti-HLA antibodies in a prospective trial of PI-based desensitization¹³². Interestingly, the authors also reported a low rate of *de novo* DSA production (12.5%) in the 43% of desensitized patients. In another study of 18 patients

undergoing clonal deletion with donor-specific transfusion followed by treatment with bortezomib, rATG, rituximab, and steroids, 4 patients developed *de novo* DSA, whereas 4 could be weaned off immunosuppression¹³³. Similar results were reported in a small series of pediatric heart transplant cases, where Bortezomib has been associated with a marked reduction in *de novo* DSA and resolution of AMR¹³⁴. More recently a randomized trial of Bortezomib in late AMR failed to demonstrate that bortezomib could prevent glomerular filtration rate loss, improve histologic or molecular disease features, or reduce DSA¹³⁵. The limited benefit of Bortezomib could be related to the nonselective nature of proteasome inhibition. In a pre-clinical model, Kwun et al evaluated the combination of a proteasome inhibitor (bortezomib) to deplete bone marrow PC, in conjunction with two co-stim blockade agents (Belatacept and anti-CD40 (2C10)) to target GC, in a model of pre-sensitized non-human primate kidney transplantation. This strategy increased allograft survival and led to sustainable decreased DSA titers³⁰. Hence targeting B cells at different maturation stages might be useful.

Daratumumab binds with high affinity to human CD38 engaging diverse mechanisms of action, including complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, programmed cell death, modulation of enzymatic activity, and immunomodulatory activity¹³⁶. Daratumumab monotherapy induced at least a partial response in around 30% of heavily pretreated and refractory patients with multiple myeloma, with a favorable safety profile¹³⁷. While CD38-targeting antibodies were initially developed to kill malignant plasma cells, these monoclonal antibodies may also abrogate the production of autoantibodies in autoimmune disorders. Indeed, daratumumab was effective in the treatment of refractory autoimmune hemolytic anemia. Similarly, daratumumab reduced total and allergen-specific IgE levels by depleting IgE-producing plasma cells, and this suggests potential value of CD38 antibodies in the management of severe allergy¹³⁸.

Both drugs targeting terminally differentiated B cells and particularly plasma cells might be particularly interesting to use in combination therapies when these cells play a detrimental role: in the context of antibody-mediated rejection or in pre-sensitized patients.

4c. co-B and cytokine inhibitors

Another therapeutic possibility would be to combine co-stim blockade with cytokine inhibitors. In this context we will consider the 3 major cytokines implied in B cell functions: IL-21, BAFF and IL-6.

IL-21 is an important cytokine secreted by Tfh. It sustains terminally differentiated B cell generation. Blocking the IL-21 pathway in conjunction with co-stim blockade might be an efficient means to control humoral responses. In an *in vitro* model, in which lymphocytes of the graft recipient are stimulated with donor antigens, blocking IL-21R led to a decrease in plasmablasts differentiation¹³⁹. In pre-clinical models of islet transplantation, infusion of soluble IL-21R to neutralize IL-21, or the IL-21 blocking antibody, doubled CTLA4-Ig mediated graft survival¹⁴⁰.

The B cell-stimulating molecules, BAFF (B cell activating factor) and APRIL (a proliferation-inducing ligand), are critical factors in the maintenance of the B cell pool and humoral immunity. BAFF and APRIL bind to transmembrane activator and CAML interactor (TACI) and B cell maturation antigen (BCMA), while BAFF additionally binds to a third receptor, BAFF-R; all three receptors are expressed by B lineage cells¹⁴¹. BAFF maintains B cell homeostasis by acting as a survival and fitness factor for B cells from the transitional stage of development onwards, while APRIL appears to act at a later stage, modulating the function and survival of antigen-experienced B cells^{142–145}. BAFF is elevated in various autoimmune diseases including SLE, rheumatoid arthritis (RA) and Sjögren's syndrome, and BAFF elevation is associated with autoantibody production¹⁴⁶. Several therapeutics targeting BAFF as a treatment for autoimmune diseases have been developed¹⁴⁷. FDA approved belimumab, a monoclonal anti-BAFF antibody, for the treatment of SLE in 2011, and Atacicept, blisibimod, and tabalumab, a recombinant soluble TACI receptor, and two selective antagonists of BAFF respectively are currently being tested in phase III trials for the therapy of SLE.

Recently Kwun et al tested the ability of Atacicept to prevent alloantibody production and prolong allograft survival²⁹ after kidney transplantation in non-human primates. They showed that Atacicept prevented early DSA formation and AMR development in an induced AMR model, although more T cell rejection findings were observed. Whether

targeting BAFF/APRIL could be useful for prevention and/or treatment of AMR in humans remain to be established.

IL-6 also plays a major role in B cell differentiation into plasma cells or memory B cells. Moreover IL-6 is needed for the long-term survival of plasma cells^{148–150}. Blocking IL-6 with Tocilizumab (anti IL-6R) seems to be an efficient method to block unwanted immune responses in autoimmunity^{151,152}, thus several studies have evaluated its role in the prevention of graft rejection. Jordan et al recently reviewed the role of anti IL-6 therapy on AMR in transplantation models¹⁴⁹. Briefly, tocilizumab significantly reduced the risk of AMR in pre-sensitized kidney transplant recipients¹⁵³. However it was ineffective at treating AMR. A new generation of IL-6 blocking agents, an anti-IL-6 blocking antibody (NCT03380962), is being developed and tested. Several pre-clinical observations suggest that the combined use of co-stim blockade with IL-6 blockade might be highly beneficial for graft survival. In combination with co-stim blockade (anti-CD154 and CTLA4-Ig), both genetic deficiency in IL-6 and treatment with anti-IL-6 blocking antibodies led to increased graft survival in multiple transplantation models^{154,155}. This association must be better understood and may improve transplant outcomes in humans but need to be evaluated.

Conclusion

Co-stimulatory molecules are now well-recognized therapeutic targets for controlling immune activation. They are central to allo-and auto-reactive T cell responses and thus are viable targets for inhibiting graft rejection and auto-reactive T cells. There are now strong arguments that co-stimulation pathways may also play active roles in controlling the pathways leading to DSA and autoantibody production. Both experimental models and clinical data in solid organ transplantation indicate that CTLA4-Ig may uniquely control B cell responses. It modulates both their stimulatory capacities, leading to the impairment of Tfh-B cell crosstalk, and the production of antibodies by effector B cells. Other costimulatory molecules including CD40/CD40-L, ICOS/ICOS-L, PD-1/PD-L1 are also involved in Tfh function, Tfh-B cell crosstalk, and plasma cell homeostasis, and may be promising therapeutic options.

Effective control of the humoral responses during transplantation may require targeting of

both B cell APC function and antibody production. Now with the expansion of tools that can target both functions, and the demonstration of increased effectiveness of combination strategies, real improvement in graft survival may be within reach.

Disclosure

All the authors declared no competing interests

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Figure legends

Figure1 : Cellular mechanisms involved in antibody mediated rejection.

Antigen recognition initiates priming of T cells and B cells. T cells begin to differentiate into Tfh as they move to the T cell zone B cell follicle border, where cognate T-B interactions lead to the development of the germinal center. Two zones can be anatomically observed, the dark zone where B cells proliferate very rapidly and the light zone where Tfh B cell interactions are thought to primarily take place. Affinity maturation occurs in the GC in a cyclical manner alternating between mutating the BCR and acquiring Tfh help. Throughout this process, memory B cells and long-lived plasma cells are formed. DSA: Donor-specific antibody; Ag: antigen; DC: dendritic cells; DZ: Dark Zone; GC: germinal center; LZ: light zone; Tfh: T follicular helper cells

Figure 2: Co-stimulatory molecules

A: Co-stimulatory molecules expression and interaction patterns between Tfh and B cell are shown to illustrate the complexity of their interactions. Red molecules indicate TNF α family members, and blue molecules are B7 family members. Green arrows indicate stimulatory interactions and red indicate inhibitory interactions. Some drugs, which target these interactions, are also shown. B: Key molecules of the GC Tfh B cell interactions are depicted. An emphasis is made on the timing of expression of these molecules through the different stages of B cell differentiation leading to DSA production. MHC: Major histocompatibility complex-II; CTLA-4: cytotoxic T lymphocyte antigen 4; BCR: B cell receptor; ICOS: inducible T cell costimulator ; ICOS-L: ICOS ligand; PD-1: program death 1; PD-L: PD-1 ligand

Figure 3: Immunosuppressant agents and classes in kidney Transplantation

This diagram is a summary of our discussion of the potential therapeutic targets and their effects on different cells key to humoral responses. New data show that co-stim blockade targets multiple stages of cell-cell interactions. In contrast to Tfh-B cells interactions, relative few drugs target plasma cells. Furthermore, bortezomib and anti-CD38, which do affect plasma cells actually have much broader effects. Therapeutic compounds are depicted in red font. **APC: antigen presenting cell; BCR: B cell receptor; CoB : Co-stimulatory blockade; IL: interleukin; MHC: Major histocompatibility complex; TCR: T cell receptor;**

pathway		Name		Agent	Target
		INNs	Commercial		
CYTOKINE	IL-6	Tocilizumab Clazakizumab (BMS-945429)	ROACTEMRA®	Humanized mAbs Humanized mAbs	IL-6-R IL-6
	Baff	Belimumab Atacicept blisibimod tabalumab VAY736	BENLYSTA®	Human mAbs TACI-IG Selective antagonist Human mAbs Human mAbs	BAFF BAFF BAFF BAFF BAFF-R
CO-STIMULATORY MOLECULES	CD28/CD80-CD86	Abatacept Belatacept ASP2409 Iulizumab FR104	ORENCIA® NULOJIX®	CTLA4-IG CTLA4-IG CTLA4-IG Humanized mAbs nanobodies	CD80/CD86 CD86/CD80 CD86 CD28 CD28
	CD40/CD154	Ruplizumab (BG9588) Dapirolizumab (CDP7657) MEDI4920 Bleselumab (ASKP1240) CFZ533		Humanized mAbs Humanized mAbs nanobodies Human mAbs Human mAbs	CD154 CD154 CD154 CD40 CD40
	ICOS/ICOSL	Prezalumab (AMG 557) MEDI-570		Human mAbs Humanized mAbs	ICOSL ICOS
Other target	B cells	Rituximab	MabThera®	chimeric mAbs	CD20
	CD38	Daratumumab	DARZALEX®	Human mAbs	CD38
	CD25	Basilixumab	SIMULEC®	Humanized mAbs	CD25
		Bortezomib	VELCADE®	inhibitor	Proteasome

Table 1: **B cell targeting therapies** : Recombinant protein (**RP**) Monoclonal antibodies (**mAbs**)