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Novel Immunosuppressive Strategies for the Control of T_{FH} Cell-Driven *de novo* DSA Responses

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Immunology and Molecular Pathogenesis

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B.S. Biology and Chemistry, University of Vermont, 2012

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An abstract of

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in

Graduate Division of Biological and Biomedical Sciences

Immunology and Molecular Pathogenesis

2019

Abstract

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By: G. Michael La Muraglia II

Organ transplantation remains as the only curative treatment for end stage organ disease. Despite recent advances in medicine and technology within the field of transplantation, chronic immunological graft rejection remains to be a significant barrier to the success of organ transplantation. Recent clinical evidence has shown that donor-specific antibodies (DSAs) contribute significantly to the progression of chronic allograft rejection. However, robust therapeutic options to control DSA formation in the post-transplant phase have yet to reach widespread clinical application.

CD28 blockade with CTLA-4-Ig has the ability to reduce the incidence of these donor-specific antibodies (DSA), but its mechanism is suboptimal for the inhibition of alloimmunity in that CTLA-4-Ig blocks both CD28 costimulation and CTLA-4 coinhibition. Thus, selective CD28 blockade that spares CTLA-4 has potential to result in improved inhibition of humoral alloimmunity. To test this possibility, we utilized both antigen-specific TCR transgenic T cells for donor-specific studies, and a full allogeneic mismatch murine transplant model in combination with a T follicular helper (Tfh):B cell co-culture system.

We observed that selective blockade with an anti-CD28 domain antibody (dAb) compared to CTLA-4-Ig led to superior inhibition of Tfh cell, germinal center and DSA responses *in vivo*, and better control of B cell responses *in vitro*. CTLA-4 blockade enhanced the humoral alloresponse, and in combination with anti-CD28 dAb abrogated the effects of selective blockade. This CTLA-4-dependent inhibition was Tfh cell-specific in that CTLA-4 expression by Tfh cells was necessary and sufficient for the improved humoral inhibition observed with selective blockade. As CD28 blockade attracts interest for the control of alloantibodies in the clinic, these data support selective blockade as a superior strategy to address DSA via the sparing of CTLA-4 and more potent targeting of Tfh cells.

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1.1 The Current Burden of End Stage Organ Failure: *A Preface*

Organ transplantation remains as the only curative treatment for end stage organ disease. Data from the Organ Procurement and Transplantation Network (OPTN) and the United Network of Organ Sharing (UNOS) reports that an average of 30,000 transplantations have been performed each year in the United States over the past ten years(1). However, due to the critical shortage of available donor organs, approximately 110,000 patients remain on the waiting list at the end of each year. Despite recent advances in medicine and technology within the field of clinical transplantation, chronic immunological graft rejection remains to be a significant barrier to the success of organ transplantation. While great strides have been made to expand the available donor pool through the advancements in the potential of xenogeneic graft transplantation and the wide acceptance of living donor protocols, the goal of one transplant for life remains to be of the utmost importance.

Over the last several decades the discovery of potent systemic immunosuppressive drugs such as calcineurin inhibitors (CNI), have significantly improved graft survival through their superior control of T cell activation and resistance towards acute rejection. However, these potent immunosuppressants have proven to effectuate substantial off target effects; including, but not limited to, nephrotoxicity, hypertension, dyslipidemia and opportunistic infection. These undesirable side effects compose a significant source of morbidity and mortality within the transplant recipient population, with the only course of treatment centering on the reduction of immunosuppression, rendering the graft more susceptible to immunologic rejection. Current research endeavors within the field of transplantation have

sought to explore new pathways to directly target the activity of alloreactive immune responses to mitigate the severe complications that can arise during treatment with calcineurin inhibitors.

Through our improved understanding of the role that the adaptive immune response plays in the rejection of donor organs, new strategies have been developed to potently inhibit the *de novo* activation of T cells through the specific blockade of costimulatory signaling. These costimulation blockade therapies provide the potential for potent control of chronic rejection through their increased efficacy in inhibiting antibody-mediated rejection as compared to CNI treatment. While these preliminary treatment protocols have yielded improved graft function and resistance to antibody-mediated rejection over classic CNI immunosuppression, they have been accompanied by increased rates of acute rejection. Ultimately, combating the potential for both acute and chronic graft rejection while maintaining optimal graft function remain the keystones in the discovery of a next generation immunosuppressive therapy.

1.2 A Brief History of Clinical Organ Transplantation

The first major technical breakthroughs that laid the framework for clinical organ transplantation as it exists today were not discovered until the mid-19th century, when anesthesia in the form of ether was first utilized by William T.G. Morton at Massachusetts General Hospital to anesthetize patients undergoing surgical procedures(2). This was followed by British surgeon Joseph Lister's publication of *Antiseptic Principle in the Practice of Surgery* which extolled the virtues of cleanliness in the surgical arena leading to the adoption of aseptic surgical technique(3). However, despite various medical advancements throughout the 19th

century, it was not until the early 20th century that solid organ transplantation was first experimentally described.

The origins of vascularized organ transplantation were started by French surgeon Alexis Carrel in his pioneering of a technique in which the lumen of two blood vessels could be sutured together, a process termed vascular anastomoses(4). It was through this technique that he was able to perform the what was called the first homoplastic kidney transplant between a canine donor and recipient pair, later earning him the Nobel Prize in Physiology and Medicine in 1912(5). Interestingly, while Carrel had performed these experiments with the focus on arterial and venous stenosis surrounding his anastomoses, he noted that these organs failed to function for more than a few days following reperfusion, free of any stenotic complications. Never further pursuing the mechanism underlying this phenomenon of spontaneous graft failure, the fundamental immunologic basis of organ rejection would remain clandestine for decades to come.

While the milestone of the vascular anastomoses allowed for the surgical feasibility of solid organ transplantation, further experimentation in animal models of transplantation began the uncover the robust immunological barrier present in models of allotransplantation. The first implications of an immunologic component to transplantation were described by Sir Peter Medawar through his observations of skin transplantation performed on burn patients during the height of World War II(6). He described that patients receiving autografts or self-tissue encountered complication free skin engraftment. However, patients receiving homografts experienced rapid tissue degeneration leading to dermal necrosis and graft bed inflammation. Through further investigation in animal studies of skin transplantation, Medawar established a

rabbit skin transplant model that was able to recapitulate his observations of autograft acceptance and homograft rejection in human patients(7). He was able to expand upon his previous findings, describing that recipients of two successive skin allografts displayed accelerated graft rejection kinetics on the second graft(8). This observation of accelerated graft loss led to the hypothesis that an "actively acquired immune reaction" was the mechanism underlying the accelerated rejection of the grafts based on the antigenic disparity of the donor-recipient pair.

Independent studies performed by veterinarian Ray Owen during the 1940's led to his seminal publication in *Science* where he described his observations of stable hematologic chimerism in dizygotic cattle twins(9). These findings demonstrated that parabiotic anastomoses leading to the free transfer of red blood cells between dizygotic littermates allowed for reciprocal transplantation of hematopoietic stem cells, thus establishing the first evidence of immunological tolerance. Medawar expanded upon the findings of Owen through extrapolation of his dizygotic cattle model to define specificity of this acquired "immunological tolerance", noting that skin grafts transplanted between dizygotic cattle twins went on to be accepted whereas 3rd party skin grafts were rejected(10). Further studies by Medawar in murine models of acquired tolerance described that early exposure to alloantigens in the form of splenocytes during development *in utero* was capable of inducing a state of antigen-specific immune tolerance(11) leading to the spontaneous acceptance of skin allografts from the same splenocyte donor strain(12). These groundbreaking studies performed by Owen and Medawar galvanized the principal of "acquired immune tolerance", which established transplant rejection as an immunologic phenomenon with the capacity of being suppressed.

While the early animal studies clearly delineated an immunological component of graft rejection on the basis of self vs. non-self-tissue recognition, it was only a matter of brief time before these lessons were explored through clinical transplantation. In December of 1954, Joseph Murray performed the first successful renal transplant at Brigham and Women's Hospital between two identical twin brothers, one of whom experienced chronic nephritis leading to end-stage renal failure(13). The success of this operation was predicated on the fact that the recipient was genetically identical to the donor, providing no immunologic barrier to prevent the graft from being accepted as self-tissue. Nonetheless, this remarkable surgical achievement afforded the first demonstration that a human renal graft could be physically transplanted, and provide life-supporting function within a new recipient. Subsequent to this milestone in transplant history, several other institutions were also able to exchange organs between pairs of twins, recapitulating the work of Murray, but transplantation remained an experimental procedure with a multitude of compromising risks involved during this era.

1.3 The Advent of Clinical Immunosuppression

The first efforts towards achieving immunosuppression came in the form of total body irradiation (TBI) and the administration of the corticosteroid prednisone. However, TBI was found to be associated with a high incidence of morbidity and mortality leading to its abandonment as a potential therapy for post-transplant immunosuppression. Investigations led by Gertrude Elion and George Hitchings working in the field of leukemia research, led to the eventual discovery of an anti-proliferative drug, 6-mercaptopurine(14). The application of a derivative of this anti-proliferative agent, azathioprine (AZA) to experimental transplantation, displayed the capability of inhibiting immune responses in animal models(15, 16). A decade

later these early attempts of pharmacological immunosuppression gave way to Dr. Thomas Starzl's adoption of AZA and prednisone as a dual therapy immunosuppressive treatment for clinical renal transplantation, resulting in unparalleled success compared to all previous protocols(17). It was during this era of experimental therapeutics that animal models of transplantation also began to identify lymphocytes as the cells responsible for graft rejection(18-20). In response to these findings, Starzl later introduced a ground-breaking therapeutic for induction therapy in the form of anti-thymocyte globulin (ATG) isolated from equine tissues, which further improved outcomes through its action of depleting lymphocytes within the transplant recipient(21, 22). While these advances in transplant therapy improved short-term outcomes compared to earlier attempts, the life expectancy of grafts remained poor.

The discovery and subsequent adoption of a new class of immunosuppressive therapy termed calcineurin inhibitors (CNI) in the form of Cyclosporine A (CyA) was revolutionary for the field of transplantation. CyA was first described by Borel in 1976 as having immunosuppressive activity, which was later defined through its inhibition of the lymphocyte signaling molecule calcineurin(23-25). Through the interruption of calcineurin signaling, CyA prevents the activation of alloreactive T cells through rendering them unable to competently mediate graft rejection(26, 27). The application of CyA to clinical transplant protocols drastically reduced the incidence of acute rejection(28, 29), however, it comes at the cost of significant off target effects ranging from nephrotoxicity to pulmonary hypertension(30). In addition to these harmful side effects, CNIs act systemically and inhibit all T cell responses regardless of their specificity, thus patients become more vulnerable to opportunistic infection while also having

impaired tumor surveillance due to the global suppression of the adaptive immune system. While further improvement has been made in CNI therapy with the introduction of Tacrolimus (FK-506) in the early 90's(31), the overall half-life of grafted organs has remained relatively unchanged since the adoption of CNI therapy in the 80's(32, 33). Therefore, it remains imperative within the field of transplantation to push for the discovery and implementation of next generation immunosuppressive therapies aimed at specifically targeting alloreactive immune responses, while limiting off target toxicity and impairment of protective immunity.

1.4 Innate and Acquired Immune Mechanisms of Graft Rejection

The physical process of organ transplantation, consisting of; tissue procurement, processing, implantation and reperfusion initiates significant injury to the donor organ(34, 35). Depending on the relative health of the donor at the time of tissue harvest these effects can be exacerbated through either acute or chronic circumstances(36). These effects manifest in the form of inflammation and the release of markers of tissue injury referred to as damage associated molecular patterns (DAMPs). These DAMPs can be sensed by cells expressing innate pattern recognition receptors (PRRs) which can be localized intracellularly or on the cell surface(37). The binding of these DAMPs by PRRs on innate immune cells can trigger the activation of the inflammasome(38, 39), resulting in the upregulation of genes associated with inflammatory function leading activation of bystander cells. The outcome of this response is the production of proinflammatory cytokines including IL-1, IL-6, IL-12, IFNg and TNFα(39) along with various chemokines promoting the recruitment of other immune cell subsets(40). This

inflammatory response marks the donor tissue as a site of injury, promoting the diffusion of donor lymphocytes and recruitment of recipient proinflammatory leukocytes(41).

The activation of the innate immune response post-transplantation is mainly a non-specific reaction to tissue damage, occurring regardless of potential genetic disparities between the donor and recipient pair(40). While the tissue damage sustained by grafts during the innate immune response can be significant, it is seldom observed that this process can lead to rejection in the absence of the adaptive immune system. Studies have shown that animals devoid of T cells are incapable of effectuating graft rejection of genetically disparate transplants, however, through the adoptive transfer of wild type T cells to these recipients, rejection is able to proceed(42). Similarly, in clinical transplantation the depletion of peripheral blood mononuclear cells (PBMC) has proven to be effective in both the prevention and reversal of acute rejection(31, 43). The initial steps of the of the adaptive immune response to a transplanted organ therefore consist of recipient T-cell recognition of a donor derived alloantigen, termed allorecognition.

Transplantation induces a dynamic immune response, highlighted by polyclonality generated through a relatively high precursor frequency of T Cell Receptors (TCRs) capable of responding to the mismatched Major Histocompatibility Complex (MHC) present on donor derived cells. MHC which is also referred to as Human Leukocyte Antigen (HLA) functions to present processed peptides derived from intra- and extracellular sources on the surface of cells for specific recognition by cognate T cells, providing part of the signal essential to their activation(39). MHC was first described in the seminal publication by Zinkernagel and Doherty where they demonstrated that for a cytotoxic CD8+ T cell to kill an infected target cell, the T cell

and target cell must share a class I MHC allele, leading to the definition of MHC restriction(44). The elevated level of reactivity observed in the adaptive response is afforded by a combination of specific TCR allorecognition for donor peptide-MHC complex (pMHC) and cross reactive TCRs specific for other pMHC capable of activation in response to alloantigen. While MHC molecules unequivocally represent the greatest immunologic barrier to success in transplantation, instances of haplo-identical transplantation in which MHC molecules are identical have still precipitated rejection, leading to the discovery and characterization of minor histocompatibility antigens(45).

The process of T cell priming, as first described by Charles Janeway involves; antigen sequestration and processing, expression of antigen derived peptide within the MHC (pMHC), followed by interaction between TCR and pMHC resulting in proliferation and differentiation of T cells(46). The process of T cell priming has since been further defined, characterized by the interaction of TCR-pMHC composing the first signal followed by a required second signal in the form of costimulatory receptor ligation for proper activation(39). The provision of this second signal, for which the ligands are inducibly expressed on antigen presenting cells (APCs) under inflammatory conditions prevents the improper activation of T cells that may be responding to self-antigen(47). The absence of this costimulatory signal under the circumstance of TCR-pMHC engagement results in a state of T cell anergy, rendering affected T cells incapable of responding to antigen. One of the best described costimulatory receptors in T cell biology is the surface molecule CD28, known to provide the required costimulatory signaling for the activation of T cells through engagement with the B7 family of ligands(CD80/CD86)(48). The ligation of CD28 on T cells by CD80/86 leads to the upregulation of transcription and

stabilization of IL-2 mRNA, the canonical growth factor for T cells undergoing clonal expansion (49-51). In addition, CD28 costimulation results in the provision of a reduced activation threshold for T cells, permitting an increase in responsiveness to lower quantities of antigen(52). Through these discoveries in the field of T cell biology, a better understanding could be garnered of the process of T cell allorecognition by recipient T cells following transplantation.

The process of transplantation provides a unique environment under which recipient T cells can be primed and activated by alloantigens through multiple distinct pathways(53). The method of T cell priming exclusive to transplantation is the direct allorecognition pathway, where a recipient T cells TCR interacts with intact allogeneic pMHC complexes presented on donor derived APCs resulting in clonal expansion of donor specific T cells(54). This direct pathway plays a dominant role during the acute phase of transplantation; however, it is dependent on the short half-life of donor derived passenger leukocytes. Indirect allorecognition is the process by which peptides derived from donor MHC are processed by recipient APCs that in turn present these donor antigens to recipient T cells, remaining the dominant active pathway of cellular rejection throughout the life of the graft(53). A third, rather enigmatic pathway termed semi-direct allorecognition occurs through the capture and presentation of intact donor pMHC complexes by recipient APCs. Potential methods for this phenomenon include cellular trogocytosis(55) or the fusion of donor derived exosomes with recipient APCs(56), however, the significance of this semi-direct pathway of allorecognition in the context of graft rejection remains to be determined. Together, these pathways of

allorecognition result in the activation and clonal expansion of donor-specific T cells capable of effectuating graft rejection.

Humoral immunity also plays a pivotal role in the pathology of graft rejection, mediated through the actions of antibodies generated by way of donor-reactive B cells responses. Contrary to the methods of T cell priming, B cells are activated through the crosslinking of native conformation antigens by membrane bound immunoglobulins, referred to as the B cell receptor (BCR)(39). Activation of B cells, much like T cells results in clonal expansion, however, B cells can refine the specificity of their BCR through the process of affinity maturation requiring multiple interactions with the antigen in combination with cognate T cell help(57), to be discussed further in the proceeding section. Following the initial events of activation, B cells begin to differentiate into specialized antibody secreting cells called plasma cells that are responsible for effectuating the processes of antibody-mediated graft rejection.

Through the combination of these processes in innate and acquired immunity following transplantation, three distinct pathways of graft rejection can be defined. The first, and most destructive pathway is hyper-acute rejection, characterized by a massive inflammatory reaction driven by the presence of pre-existing donor reactive antibody, referred to as donor specific alloantibodies(58). This reaction normally occurs immediately following the reperfusion of the donor graft with the recipient's arterial blood supply, initiating with severe arteritis and interstitial edema resulting in significant thromboembolic complications precipitated by the activation of platelets and the complement system(59). Through improvements of pre-transplant screening for HLA sensitization and anti-donor antibody titers(60), hyper-acute rejection only seldom occurs in clinical settings(61). The second pathway, acute rejection is

mediated mainly through the actions alloreactive T cells, however recent studies have implicated a significant role for antibodies(62, 63). Acute rejection is characterized by graft infiltrating lymphocytes that elicit direct damage to the graft through cytotoxic processes against parenchymal cells and indirect damage through the promotion of an inflammatory environment leading to graft dysfunction(64). While acute rejection episodes still occur in the clear majority of clinical organ transplantation, they are easily reversed through the increase in intensity of immunosuppressive therapy(65).

The final pathway, and perhaps the least understood, is that of chronic graft rejection. Chronic rejection is defined as the gradual deterioration of graft function, characterized by the development of obliterative fibrosis, arteriosclerosis, and inflammation(66, 67). Initially, it was proposed that chronic rejection was simply the progression of multiple acute rejection episodes, culminating in eventual loss of graft function through repeated immunological insults(68, 69). However, the reasons behind suboptimal long-term transplant outcomes are multifactorial, and it has been increasingly recognized that antibody-mediated rejection (AMR) is a leading cause of chronic allograft injury and loss(70, 71). The antibodies in question are anti-HLA donor-specific antibodies (DSA), which can be generated following alloantigen exposure during or prior transplant, pregnancy or blood transfusion, and are known to cause early and late immunologic injury of allografts(72, 73). These anti-HLA antibodies are pre-formed in approximately 40% of transplant candidates and develop de novo in 20% of transplant recipients. Despite the highly recognized deleterious role antibodies play in kidney transplant outcomes, large knowledge deficits exist regarding the mechanisms underlying their development and maintenance following transplantation, and

robust therapeutic strategies to control DSA-mediated allograft dysfunction have yet to be developed(70, 74, 75).

1.5 Costimulation Blockade Therapy: Next Generation Immunosuppression

To address the significant off target toxicities of CNI immunosuppression commonly precipitating non-immunologic graft dysfunction, novel immunosuppressive strategies are required. Given the new insights into T cell activation and the requirement of T cell costimulation by the activating APC, new targets for therapeutic intervention were proposed. Through the manipulation of the CD28 costimulatory pathway during T cell priming, it was known that T cell anergy could be induced, rendering cells incapable of responding to their cognate pMHC upon secondary interaction in the presence of costimulation(76, 77). Therefore, it was postulated that blockade of CD28 costimulation following organ transplantation would result in anergy of alloreactive T cell clones resulting in long-term graft acceptance through taper of immunosuppression following the induction phase.

One of the first costimulatory blockade reagents developed aimed to exploit the high affinity advantage of Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) over CD28 for their B7 family ligands (CD80/86)(78). This was achieved through the generation of a fusion protein composed of two CTLA-4 molecules fused to an Ig tail, appropriately named CTLA-4Ig(78, 79). CTLA-4Ig showed dramatic reduction in the rate of both acute and chronic rejection in models of rodent transplantation(80-83). However, translation to large animal models showed significantly reduced efficacy in transplantation models(84, 85). Adaption of the molecule through

mutagenic approaches lead to the development of Belatacept (LEA29Y) which was able to recapitulate the advantages of CTLA-4Ig in models of non-human primate renal transplantation(86). Clinical trials of Belatacept began in the early 2000's demonstrating significant advantages in renal graft function over CNI immunosuppression, leading to its FDA approval for use in transplantation(87). Interestingly, the use of belatacept provided increased protection from the generation of DSA post transplantation as compared to CNI immunosuppression(88). Unfortunately, while CTLA-4Ig has significantly improved renal graft function post-transplant, increased incidence of acute rejection has also been noted leading to concerns over its safety *versus* traditional CNI therapy(89-91).

The mechanisms underlying this increased incidence of acute rejection have yet to be determined. One hypothesis proposed is the presence of heterologous immunity and the potential for cross-reactivity of alloantigens with preexisting memory T cells(92, 93). The potential for CTLA-4Ig also having negative effects in the fact that is blocks the action of T cell intrinsic CD28 costimulatory and CTLA-4 coinhibitory signals, rendering CTLA-4 coinhibitory action ineffective(94). The blockade of CTLA-4 function also has significantly detrimental effects on T_{REG} cell populations, which are responsible for the control of alloreactive responses(95). These ideas have spurred the development of anti-CD28 domain antibodies (dAb) that can spare CTLA-4 coinhibitory signaling while more potently inducing anergy in alloreactive T cells through CD28 antagonism(96-98).

The introduction of belatacept and other costimulatory blockade agents represent a significant step forward in transplantation immunosuppression since the adoption of CNI. However, the use of costimulation blockade therapy requires further investigation to optimize

targeting in the effort of providing potent immunosuppression of alloreactive cells in the absence of acute rejection episodes.

1.6 A Brief History of T Cell Help: The Generation of High Affinity Alloantibodies

The generation of high-affinity antibodies and long-lived memory B cells constitute the hallmarks of an effective humoral immune response. The provision of T cell help to B cells was one of the first functions discovered in T cell biology(99), which lead to the adoption of the term helper T cells regarding the CD4 T cell subset. Through cell transfer experiments, Miller and Mitchell described that in the absence of either bone marrow derived B cells or thymus derived T cells results in the incapacity of the immune system to develop an antibody response following immunization(100, 101). These findings were met with a number of confirmatory studies applying the hypothesis to a myriad of experimental systems further exemplifying the requirement of T cells in the generation of robust antibody responses(102). However, conflicting studies were also published, such as the report on B cell responses to salmonella derived flagellin, noting a lack for the requirement of T cell help in antibody formation(103). The discoveries described in these seminal publications underlie the basis for our eventual definition of T-dependent and T-independent antibody responses.

For the vast majority of protein antigens, the production of antibodies by B cells in a primary immune response is dependent upon the procurement of stimulation from cognate helper T cells. However, antigens do exist, primarily from bacterial byproducts that have B cell mitogenic properties through their capability of ligating PRRs on B cells such as Toll Like

Receptors (TLRs) providing a B cell intrinsic stimulatory signal, allowing for the generation of an extra-follicular antibody response(104). Other antigens lacking PRR ligation ability, have also been described through their triggering of innate immune responses to promote production of B lymphocyte stimulator (BlyS) by macrophages and monocytes(105). BlyS binds to the B cell growth factor receptors TACI and BCMA which are constitutively expressed on mature B cells, resulting in their proliferation and increased production of serum antibody(106, 107). Humoral responses generated through the T-independent pathway typically produce short-lived, low affinity antibodies with little to no memory component(108). While this pathway provides unique insights into the generation of antibodies, its role in transplantation and the generation of DSA has yet to be elucidated.

The first signs of a plausible molecule executing T cell help to B lymphocytes came through the discovery of IL-4 in combination with BCR signaling leading to the proliferation of B cells *in vitro*(109). However, later *in vivo* studies revealed IL-4 to not be essential in T-dependent antibody responses describing that genetic deletion of the IL-4 gene does not result in the loss of germinal center formation following immunization(110). During the late 1980's, the description of CD40 and CD40L represented the first discovery of a ligand-receptor pair involved in T cell help, essential to the progression of T-dependent antibody responses(111). This was demonstrated through the generation of an agonistic CD40 specific antibody that in combination with tonic BCR signaling resulted in B cell proliferation, further studies targeting germinal center B cells with this method displayed reduced apoptosis(112). CD40L was eventually cloned and found the be highly expressed on CD4 T helper cells, indicating it as a dominant mechanism of T cell help to B cells(113). This concept was further reinforced through

the discovery that X linked Hyper-IgM syndrome correlated with a high incidence of mutations within the gene for CD40-L(114, 115), directly implicating CD4 T cells as the main component of T cell help to B cells. In an effort to confirm these novel mechanistic findings, a murine antagonistic CD40-L antibody was generated, showing that blockade of CD40-L on T cells prevented the formation of germinal centers(116).

Germinal centers (GCs) are transient microanatomical structures composed of actively dividing B lymphocytes localized within the B cell follicles of secondary and tertiary lymphoid organs(117). GCs form in the response to T-dependent antigens, where follicular dendritic cells (fDCs) initiate formation through their secretion of the chemokine CXCL13, which promotes chemotaxis of all cells expressing the receptor CXCR5(118). The morphology of GCs is composed of two zones: a light zone where fDCs, helper T cells and non-actively dividing GC B cells termed centrocytes are localized, and a dark zone densely packed with actively proliferating GC B cells called centroblasts(118). Within the dark zone of the germinal center somatic hypermutation (SHM) occurs, which is the process of mutation within the immunoglobulin's variable region genes, generating diversity and increasing affinity of the Ig for the immunizing agent(119, 120). Light zone interactions focus mainly on the engagement of centrocytes with helper T cells, where B cells receive essential survival signals in the form of CD40 stimulation, along with polarizing cytokines that promote class switch recombination (CSR) of antibody isotypes(121). The light zone also promotes the ontogeny of centrocytes signaling differentiation of GC B cells into plasmablasts, plasma cells and memory B cells(122). Light-Dark zone cycling of GC B cells is achieved through the transient expression of either CXCR4 which attracts GC B cells to the dark zone though fibroblastic reticular cells secreting CXCL12, or CXCR5 resulting in the chemotaxis

to the light zone through secretion of CXCL13 by fDCs(123). Expression of CXCR5 is tightly governed by the transcription factor BCL-6 which is upregulated in response to the reception of T cell help, allowing for recirculation into the dark zone(124). The transient activity of the germinal center reaction generates antibodies of high affinity and avidity in response to T-dependent antigens.

Further research into the provision of B cell help by T cells led to the discovery and subsequent cloning of the cytokine IL-21 which was shown to potentiate B cell proliferation(125, 126). Through immunohistochemical tissue staining, IL-21 was found to be localized within B cell follicles of active germinal center reactions(127). Interestingly, IL-21 staining was found to be positive on some CD4+ T cells within the light zones(128). Furthermore, mice deficient in the IL-21 gene display significantly lower germinal center activity in combination with lower levels of affinity maturation and an inadequacy of IgG production (129, 130). Investigation into the signaling of IL-21 in germinal center B cells revealed a direct relationship between the control of BCL-6, and IL-21R ligation leading to maximum expression of BCL-6(129). Together, these data implicate IL-21 as an essential molecule in the communication of help from T cells to B cells, driving forward the germinal center response promoting robust humoral immunity.

Further mechanisms of T cell help to B cells have been described relating to contact dependent interactions. The first of which, inducible T cell co-stimulator (ICOS) found on CD4 T cells can interact with ICOS-L (CD275) on GC B cells, providing proximity for sustained CD40-CD154 signaling and directed cytokine stimulation(131). Mutations within the gene for ICOS-L result in the onset of immunodeficiency(132), with mouse models revealing defects in germinal

center generation and the formation of B cell memory(133). A second receptor found on T cells, SLAM-associated protein (SAP) is also important in T to B cell communication(134). Studies have described that mutations in the SAP protein result in the impairment of germinal center formation(135), manifesting in the inability to clear certain infections in children(136). Additional understanding of these dynamic interactions between T and B cells in the germinal center response have been garnered through the utilization of intra-vital microscopy, lending greater insight to the specialized T cell subset responsible for B cell help(137). These studies have reinforced the fact that extensive interactions between B cells and their cognate T cells occur within the light zones of the germinal center driving the formation of long lived humoral immunity(124).

In conclusion, the provision of T cell help to B cells in the germinal center response is a complex interaction composed of many diverse factors and processes. Through the exchange of stimulatory and inhibitory signals between T cells and B cells occurs massive proliferation, mutation and selection of antigen-specific B cells. T cell derived signals are then capable of signaling the differentiation of specialized antibody secreting cells, capable of effectuating high affinity antibody responses.

1.7 Emergence of the T Follicular Helper Cell Subset

Initial descriptions of CD4 T cell subsets by Terry Mossman in 1986 laid the foundations of what is referred to today as the T_H1 / T_H2 Paradigm(138). Further ground was broken in the establishment of the T regulatory (T_{REG}) cells as a unique CD4 T cell subset, which was catalyzed

by the discovery of the transcription factor forkhead box P3 (FoxP3)(139). These advances in our understanding of CD4 T cell subsets lead to the early descriptions of a specialized set of CD4 T cells responsible for the provision of B cell help. These cells, named T follicular helper cells (T_{FH}) were initially described in human tonsil tissues through their high expression of the chemokine receptor CXCR5(140-142). Tonsils are considered secondary lymphoid organs with significant exposure of antigens from the upper respiratory tract, lending to high frequencies of germinal center responses. Given the previous discussion of germinal center responses, the tonsil provides a viable and common tissue for the study of T cell help to B cells(141).

While these initial descriptions of $CXCR5^+$ T_{FH} cells were important, the early-published data did not garner wide acceptance of a distinct program of CD4 T cell differentiation. The chief complaint of skeptics revolved around the lack of a defined master transcription factor regulator like had been described for T_H1, T_H2, T_{REG} and T_H17 cells with T-bet, GATA3, FoxP3 and RORyt respectively(143). Combining this shortfall with the fact that the phenotype established was simply an elevation in the expression of CXCR5 led many scientists to question the validity and plasticity of initial T_{FH} cell data. However, the recent identification of BCL-6 as the master transcription factor regulator of T_{FH} cell commitment and establishment of the requirement of T_{FH} cells for B cell help *in vivo* has laid the foundation for T_{FH} cells as a unique subset of CD4 T cells(144-146). Further significance of T_{FH} cells as a unique subset was garnered through the identification of the importance of IL-21 in the function of these cells(127, 129, 147).

The transcriptional repressor BCL-6 was originally identified as the master regulator of GC B cell differentiation(148), with subsequent discovery in T_{FH} cells(149). While BCL-6 is required for the progression of differentiation in both GC B cells and T_{FH} cells, its serves distinct

roles in each cell type. BCL-6 is involved in the control of GC B cells through the suppression of a myriad of signaling pathways, the regulation of the cell cycle, and the programming of DNA damage response genes(149, 150). In T_{FH} cells our understanding of the role of BCL-6 is limited, however in the absence of BCL-6, T_{FH} cell differentiation does not proceed(144, 145). Additionally, it has been well described that BCL-6 can modulate microRNA expression(146) leading to the inhibition of differentiation to other CD4 T cell subsets(145). BCL-6 has been shown to antagonize T-bet involved in T_H1 commitment(145, 146), GATA-3 for T_H2 commitment(145, 151), and RORyt in T_H17 commitment(145). One of the common targets of BCL-6 shared by these programs is Blimp-1(144) which has been implicated in the polarization of T cells to an effector phenotype(149). BCL-6 exerts control of T_H cell lineage commitment during naïve CD4 T cell ontogeny.

While BCL-6 can antagonize the differentiation pathways of other CD4 T cell subsets, the inhibition is normally incomplete, giving rise to T_{FH} cells with T_H1, T_H2 or T_H17 characteristics. Evidence of the expression of IFNy(144) and IL-4(141) by T cells in the GC have previously been described(152), lending to theory that T_H1 and T_H2 cytokine profiles can be induced in T_{FH} cells during TCR priming. These adapted cytokine profiles have been implicated in the promotion of CSR(153) in GC B cells with IFNy stimulating activation of the IgG switch region, while IL-4 stimulates the IgE switch region(121). While evidence exists that the event of CSR may occur prior to the formation of GCs in the humoral response, GCs remain to be the major site of CSR in B cells(153, 154). It can be argued that cytokines from non-T_{FH} cells within the T cell zones may diffuse to the GC to instruct B cell CSR. However, studies have shown evidence of the

importance of focal cytokine expression, favoring contact dependent interactions between T_{FH} and GC B cells(153, 155, 156).

The differentiation program of T_{FH} cells has also been a point of contention amongst scientists in the field. This was in part, due to the mounting evidence of CD4 T cell plasticity(157, 158) at the time that T_{FH} cells were becoming widely accepted. Our initial understanding of T_{FH} cell differentiation is derived from *in vitro* studies where purified CD4 T cells exposed to IL-6 or IL-21 incudes the production of IL-21 by naïve CD4 T cells(147). Additionally, this group has published data showing that splenic derived APCs cultured with CD4 T cells in the presence of IL-21 promotes the expression of CXCR5 and BCL-6 mRNA in T cells, lending to enhanced B cell help capacity when adoptively transferred as compared to other T cell subsets(147). Given the fact that both IL-6 and IL-21 signal intracellularly through the recruitment and activation of STAT3, it was found that STAT3 deficient mice were incapable of T_{FH} cell ontogeny(147). Together these data support a model of T_{FH} cell differentiation similar to other CD4+ T cell subsets, where exposure to a single cytokine (IL-6 or IL-21) drives the differentiation of the distinct T_{FH} cell subset. However, other experimental models have shown that in instances of IL-21 or IL-21R deficiencies that mice are still capable of producing normal frequencies of T_{FH} cells(127, 129). While other models of T_{FH} differentiation have argued that it is a B cell contact dependent phenomenon orchestrated through the interaction of ICOS and ICOS-L(137, 149, 159), noting a lack of T_{FH} cells in the absence of B cells following immunization(160) or infection(144).

Additional studies in the field of T_{FH} biology have garnered a greater understanding of the unique phenotype these cells display. Some of the earlier experiments with T_{FH} cells

described their dependence on CD28, noting that in CD28 deficient mice, GCs do not develop due to a lack of T_{FH} cells(161, 162). Further investigation of T_{FH} phenotype suggested a significant role for the expression of ICOS on T_{FH} cells in contact-dependent interactions with GC B cells expressing ICOS-L(147). Mice deficient in either ICOS or ICOS-L display a lack of T_{FH} cell populations(147, 153, 163), with further confirmation in instances of human ICOS deficiencies(132, 164). It was then found that ICOS expression on T_{FH} cells was controlled through a CD28 dependent and independent mechanisms(165) involving the intracellular signaling cascades of NFAT(166) and PI3K(167). Taken together, these data indicate that CD28 and ICOS expression are critical for the differentiation and function of T_{FH} cells.

Various levels of T_{FH} cell polarization have also been well described in the literature, leading to the establishment of two distinct populations of BCL-6 expressing T_{FH} cells. Current nomenclature defines these two subsets as T_{FH} and GC T_{FH} cells, the latter of the two indicating greater polarization of a T_{FH} phenotype. T_{FH} cells are defined as $CXCR5^+$ $BCL\text{-}6^+$ CD4 T cells expressing elevated levels of ICOS and PD-1(160, 168). GC T_{FH} can be identified through their further differentiation, expressing elevated levels of CXCR5 and BCL-6 over that of the Non-GC T_{FH} cell population(160, 168). Additional markers such as GL-7(168) have been proposed to provide unique identity of GC T_{FH} cells, but have not garnered wide acceptance. These phenotypes lend insight to the localization of GC T_{FH} to be within the GC, while T_{FH} cells will localize closer to T cell zones on the edge of follicles(160). Non-GC T_{FH} cells express elevated CXCR5, BCL-6, PD-1, ICOS, BTLA (B and T lymphocyte attenuator), CD200, and SAP as compared to naïve ($CD44^-CD62L^+$) CD4 T cells or antigen experienced non-T_{FH} cells, while GC T_{FH} express all these markers at higher levels than T_{FH} cells(160, 168). *In vitro* studies have demonstrated the

ability of both T_{FH} subsets to provide adequate B cell help(168, 169). However, in vivo T_{FH} cells have only been shown to effectuate early B cell help(168), requiring GC T_{FH} cells for the generation of the GC response and generation of robust humoral immunity(134-136).

While nomenclature and methods for the identification of unique phenotypic markers on T_{FH} cells have been a significant source of contention within the field(170), the functionality of these cells have been well established. T_{FH} cells are essential for the formation and maintenance of GCs(171), while also providing cues for B cell ontogeny, stimulating the differentiation of plasma cells and memory B cells. T_{FH} cells initiate the GC formation through the stimulation of BCL-6 expression in cognate B cells(148, 150). Maintenance of the GC is carried out through the provision of cell survival and proliferation signals by T_{FH} cells in the form of CD40L, and more recently described PD-1 interactions with PD-L1/2(160, 172). These pro-survival stimuli are responsible for the activity of activation induced cytidine deaminase (AID) upon which the activity of SHM and CSR in GC B cells is dependent(121, 173). In instances of limited T_{FH} help to B cells, GC T_{FH} expressing Fas-L will interact with GC B cell expressed Fas to initiate the extrinsic apoptotic pathway, resulting in the collapse of the GC(174). Finally, T_{FH} cells also shape the products of the GC response, stimulating the generation of plasma cells through the promotion of Blimp-1(175, 176) and the generation of memory B cells through a mechanism that remains elusive(177, 178). While T_{FH} cells can potently promote the generation of high affinity antibodies in response pathogens, it also harnesses the potential for the development of autoimmunity(179), requiring the presence of tight multilayer regulation.

The hallmark of an effective adaptive immune response is the generation of long-lasting immunity capable of protection from subsequent antigen exposure(180). This is achieved

through the generation of antigen specific memory cells capable of surviving in the absence of antigenic stimulation through homeostatic proliferation(181). Early studies have established that memory can be formed following the contracture of an immune response in T_{FH} cells, utilizing murine models of acute viral infection(182). Investigation of acute viral infection in the LCMV model allowed for the tracking of LCMV-specific TCR transgenic memory T_{FH} cells adoptively transferred into B cell deficient mice. Upon LCMV challenge in these recipients, memory T_{FH} cells recalled an effector T_{FH} phenotype, while adoptively transferred naïve CD4 T cells did not generate any T_{FH} characteristic markers(182). Further studies modeling protein immunization have provided evidence for accelerated T_{FH} function of B cell help in recall responses that lead to increased IgG titers following immunization as compared to naïve CD4 T cell adoptive transfers(183). The identification of memory T_{FH} cells has been described through expression of CXCR5 in the absence of BCL-6, PD-1 and ICOS(182, 184). Interestingly, one unique difference observed in memory T_{FH} is the loss of CD69 expression(185), a sign indicative of the absence of sustained antigen exposure which is typical in the resting memory pool. These data indicate that effector T_{FH} cells have the capacity to form memory with cell-intrinsic programming that promotes the recall of an effector-like T_{FH} cell phenotype and function following antigen-specific reactivation.

Recent identification of a regulatory subset of follicular T helper cells (T_{FR}) capable of inhibiting T-dependent B cell activation has furthered our knowledge regarding the control of antibody production in GC responses(186). T_{FR} cells comprise a small frequency of approximately 6% of all T_{REG} cells in secondary lymphoid organs following antigenic exposure(187). However, within localization of the B cell follicle at a basal state the frequency

of T_{FR} cells can encompass upwards of 50% of all follicular T cells(187). The T_{FH}:T_{FR} ratio within the GC has been shown to correlate with antibody responses, with polarization towards T_{FH} dominance in the GC is observed during active GC responses(186). T_{FR} cells are identified phenotypically though their expression of CXCR5, PD-1, ICOS and the co-expression of FoxP3 and BCL-6 in the effector phase(188). Much like T_{FH} cells, T_{FR} cells also display varying levels of GC polarization through their loss of CD25 expression, a phenotype consistent with the absence of IL-2 within GC responses(189). Early studies have postulated that T_{FR} differentiation occurs in a manner similar to T_{FH} cells through contact dependent interactions with cognate B cells, solidifying the T_{FR} program(190, 191). Interestingly, T_{FR} cells can only differentiate from natural T_{REG} precursors, rather than naïve CD4 T cells that give rise to the T_{FH} cell lineage(192). Recent studies have confirmed this observation in T_{FR} cell ontogeny, showing an inability of peripherally induced T_{REG} cells to generate a T_{FR} cell phenotype under T_{FR} cell polarizing conditions(186).

The chief function of T_{FR} cells is the regulation of the GC response, through which they mediate inhibition of both GC B cells and T_{FH} cells by multiple mechanisms. The first descriptions of T_{FR} function were described in murine models devoid of T_{FR} cells utilizing CXCR5 or BCL-6 deficient mice, resulting in increased antigen-specific serum Ig, GC B cells and plasma cells(187, 188, 193). Further studies corroborated these findings of B cell inhibition in both *in vitro* suppression and *in vivo* T_{FR} adoptive transfer models(190, 191, 194, 195). *In vitro* data has also shown inhibitory action by T_{FR} cells on T_{FH} cells, noting down regulation of the proliferation marker Ki-67 in T_{FH} cells along with attenuation of cytokine production following co-culture with T_{FR} cells(190, 194). The coinhibitory molecule CTLA-4 is significantly upregulated on T_{FR} cells as

compared to T_{FH} and has been implicated in the control of follicular cell differentiation though its modulation of CD28 engagement(196). Adoptive transfer studies have shown that T_{FR} cells devoid of the co-inhibitor CTLA-4 lead to greater expansion of T_{FH} cells and GC responses(191, 197). Finally, like T_{FH} cells, T_{FR} cells also develop a circulating memory component(191). However, experiments addressing the recall capacity of circulating memory T_{FR} cells have described them as being less suppressive than effector T_{FR} cells localized to B cell follicles(190). This was found to be in stark contrast to circulating memory T_{FH} cells that proved to be more potent than primary effector T_{FH} cells following antigen exposure.

Much remains to be understood regarding how T_{FH} and T_{FR} cells stimulate and regulate humoral immune responses through their control of the germinal center reaction. Nonetheless, the discovery of these cell subsets has revolutionized our understanding of T cell help to B cells. Further insights into the differentiation and function of the follicular T cell subsets are critical to the development of new therapeutics aimed at controlling or augmenting antibody responses.

1.8 T Follicular Cells in Transplantation

Through the previously outlined studies, T_{FH} cells have been established as a distinct lineage of $CD4^+$ T cells that provide T cell help to B cells for GC formation, affinity maturation and the generation of memory B cells and long-lived plasma cells to effectuate an adequate humoral immune response. The critical role T_{FH} cells play in the generation of antibody responses to vaccines and in autoimmune disease has been established (174, 198), but little is known regarding their role in the generation of DSAs in the setting of transplantation (199,

200). The costimulatory and coinhibitory molecules CD28, ICOS and CTLA-4 are critical mediators of the T cell-mediated alloimmune response (84). Coincidentally, T_{FH} cells heavily depend on the costimulatory molecules CD28 and ICOS for differentiation and GC formation, and loss of either CD28 or ICOS results in inadequate T cell dependent antibody responses (174). Less is known about the role of the coinhibitor CTLA-4 on T_{FH} cells, but its importance as a key regulator of T_{FH} cell function has recently been elucidated (196, 201). While these costimulatory and coinhibitory molecules have been extensively studied, and are known to play important roles in transplantation, there is little knowledge regarding the role these molecules play in $CD4^+$ T_{FH} cell-mediated alloimmunity following transplantation.

Costimulatory interactions, namely CD28 and CD40-L are essential for the for the development of T_{FH} cells and the effectuation of T-dependent antibody responses. Studies investigating the requirement of CD40 in murine heart transplant recipients have described the generation of DSA in a CD40-independent manner, however DSA titers did not remain robust long term, indicative of potential extrafollicular antibody formation(202). Later studies investigating the use of anti-CD40-L therapy established IFNy and B cell activating factor (BAFF) production by memory T cells as the mechanism of CD40 independent DSA generation(203, 204). While many of these studies were not predicated on the action of T_{FH} or T_{FR} cells, the conclusions remain relevant as they examined memory populations comprised of approximately 20% CXCR5+ CD4 T cells, indicating the presence of a follicular T cell phenotype(205). Recent studies in NHP transplant models have further established the potency of anti-CD40-L therapy, describing mitigation of the generation of DSA following renal transplantation(206).

Costimulation blockade of the CD28 pathway has been achieved using CTLA-4Ig, as previously described. The use of CTLA-4Ig has shown marked reduction in the formation of DSA following transplantation in both animal(207) and human models of transplantation(88). However, the published data on CTLA-4 expression on T_{FH} cells acting as a critical mediator of T_{FH} regulation and inhibition suggests that this blockade therapy could be further improved(194, 196, 208). Our group's recently published data show that selective CD28 blockade exhibits a significant reduction of donor-specific T_{FH} cells, a marked increase in skin allograft survival and a superior suppression of DSA following transplantation as compared to CTLA-4Ig(209). Other groups have postulated that the advantage of selective CD28 blockade is mediated through the enhanced function of T_{REG} cells through a T_{FH} extrinsic mechanism(210). These data suggest that selective CD28 blockade in the presence of intact CTLA-4 coinhibitory signaling facilitates improved suppression of T_{FH} cell accumulation and T-dependent antibody responses following transplantation.

The idea of monitoring for T_{FH} cells in clinical transplantation is just beginning to be explored(211). Early reports of these efforts have isolated circulating T_{FH} cells (cT_{FH}) from the periphery of renal transplant patients at both pre-and post-transplant timepoints (212, 213). They described these T_{FH} cells as having suppressed *ex vivo* function in IL-21 production following the induction of immunosuppression, however T_{FH} cells maintained their ability to provide B cell help under these conditions(214). Interestingly, kidney biopsies in rejecting renal allografts in this study also developed Ig positive follicular like structures rich in T_{FH} and B cells. This establishes the idea that functional T_{FH} cells circulate in renal transplant patients on immunosuppression with the potential to effectuate T-dependent humoral responses. Based on

these observations it is hypothesized by Baan et al. that TFH cell derived IL-21 production mediates the generation of DSA following transplantation leading to increased incidence of AMR, and that the targeting of the IL-21 axis may hold great therapeutic potential in the control of these responses(215). In conclusion, these data support continued investigations aimed to develop cT_{FH} cells as a biomarker for the clinical assessment of humoral alloimmunity to guide DSA management for improvement of long-term kidney transplant outcomes.

Conclusions

Despite our significant strides in understanding the cellular and molecular mechanisms that govern T_{FH} cells and their role in human disease, a vast knowledge gap remains regarding the balance of costimulatory and coinhibitory signaling on activated $CD4^+$ T cells committed to T_{FH} and T_{FR} lineages. Additionally, further investigation is required into their regulation by cellular mechanisms that determine their ultimate function and potential subset plasticity, particularly in the context of solid organ transplantation. While it is now well established that circulating T_{FH} and T_{FR} cells and their subsets have been linked to the state of the humoral immune response, further examination of these cells in the context of transplantation is imminent and heavily warranted for the improvement of long-term graft survival.

References

1. Israni AK, Zaun D, Bolch C, Rosendale JD, Schaffhausen C, Snyder JJ, Kasiske BL. OPTN/SRTR 2015 Annual Data Report: Deceased Organ Donation. Am J Transplant. 2017;17 Suppl 1:503-42. Epub 2017/01/05. doi: 10.1111/ajt.14131. PubMed PMID: 28052608.

2. William T.G. Morton (1819-1868), demonstrator of ether anesthesia. JAMA. 1965;194(2):190-1. Epub 1965/10/11. PubMed PMID: 5319582.

3. Lister J. On the Antiseptic Principle in the Practice of Surgery. Br Med J. 1867;2(351):246-8. Epub 1867/09/21. PubMed PMID: 20744875; PMCID: PMC2310614.

4. Carrel D. [Operative Technic of Vascular Anastomoses and Visceral Transplantation]. Lyon Med. 1964;212:1561-8. Epub 1964/12/20. PubMed PMID: 14253645.

5. Carrel A, Guthrie CC. Functions of a Transplanted Kidney. Science. 1905;22(563):473. Epub 1905/10/13. doi: 10.1126/science.22.563.473. PubMed PMID: 17753234.

6. Gibson T, Medawar PB. The fate of skin homografts in man. J Anat. 1943;77(Pt 4):299-310 4. Epub 1943/07/01. PubMed PMID: 17104936; PMCID: PMC1252734.

7. Medawar PB. The behaviour and fate of skin autografts and skin homografts in rabbits: A report to the War Wounds Committee of the Medical Research Council. J Anat. 1944;78(Pt 5):176-99. Epub 1944/10/01. PubMed PMID: 17104960; PMCID: PMC1272490.

8. Medawar PB. A second study of the behaviour and fate of skin homografts in rabbits: A Report to the War Wounds Committee of the Medical Research Council. J Anat. 1945;79(Pt 4):157-76 4. Epub 1945/10/01. PubMed PMID: 17104981; PMCID: PMC1272582.

9. Owen RD. Immunogenetic Consequences of Vascular Anastomoses between Bovine Twins. Science. 1945;102(2651):400-1. Epub 1945/10/19. doi: 10.1126/science.102.2651.400. PubMed PMID: 17755278.

10. Medawar PB. Relationship between the antigens of blood and skin. Nature. 1946;157:161. Epub 1946/02/09. PubMed PMID: 21015116.

11. Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. Nature. 1953;172(4379):603-6. Epub 1953/10/03. PubMed PMID: 13099277.

12. Billingham RE, Brent L, Medawar PB. Acquired tolerance of skin homografts. Ann N Y Acad Sci. 1955;59(3):409-16. Epub 1955/01/24. PubMed PMID: 14362286.

13. Merrill JP, Murray JE, Harrison JH, Guild WR. Successful homotransplantation of the human kidney between identical twins. J Am Med Assoc. 1956;160(4):277-82. Epub 1956/01/28. PubMed PMID: 13278189.

14. Elion GB. The George Hitchings and Gertrude Elion Lecture. The pharmacology of azathioprine. Ann N Y Acad Sci. 1993;685:400-7. Epub 1993/06/23. PubMed PMID: 8363248.

15. Elion GB, Bieber S, Hitchings GH. The fate of 6-mercaptopurine in mice. Ann N Y Acad Sci. 1954;60(2):297-303. Epub 1954/12/06. PubMed PMID: 14350535.

16. Calne RY, Alexandre GP, Murray JE. A study of the effects of drugs in prolonging survival of homologous renal transplants in dogs. Ann N Y Acad Sci. 1962;99:743-61. Epub 1962/10/24. PubMed PMID: 14017936.

17. Starzl TE, Marchioro TL, Waddell WR. The Reversal of Rejection in Human Renal Homografts with Subsequent Development of Homograft Tolerance. Surg Gynecol Obstet. 1963;117:385-95. Epub 1963/10/01. PubMed PMID: 14065716; PMCID: PMC2581919.

18. McGregor DD, Gowans JL. The Antibody Response of Rats Depleted of Lymphocytes by Chronic Drainage from the Thoracic Duct. J Exp Med. 1963;117(2):303-20. Epub 1963/01/31. PubMed PMID: 19867225; PMCID: PMC2137611.

19. Gowans JL. The Role of Lymphocytes in the Destruction of Homografts. Br Med Bull. 1965;21:106-10. Epub 1965/05/01. PubMed PMID: 14313580.

20. McGregor DD, Gowans JL. Survival of Homografts of Skin in Rats Depleted of Lymphocytes by Chronic Drainage from the Thoracic Duct. Lancet. 1964;1(7334):629-32. Epub 1964/03/21. PubMed PMID: 14107928.

21. Iwasaki Y, Porter KA, Amend JR, Marchioro TL, Zuhlke V, Starzl TE. The preparation and testing of horse antidog and antihuman antilymphoid plasma or serum and its protein fractions. Surg Gynecol Obstet. 1967;124(1):1-24. Epub 1967/01/01. PubMed PMID: 6016025; PMCID: PMC2682216.

22. Starzl TE, Marchioro TL, Porter KA, Iwasaki Y, Cerilli GJ. The use of heterologous antilymphoid agents in canine renal and liver homotransplantation and in human renal homotransplantation. Surg Gynecol Obstet. 1967;124(2):301-8. Epub 1967/02/01. PubMed PMID: 4163340; PMCID: PMC2675827.

23. Borel JF. Comparative study of in vitro and in vivo drug effects on cell-mediated cytotoxicity. Immunology. 1976;31(4):631-41. Epub 1976/10/01. PubMed PMID: 824198; PMCID: PMC1445361.

24. Borel JF, Feurer C, Gubler HU, Stahelin H. Biological effects of cyclosporin A: a new antilymphocytic agent. Agents Actions. 1976;6(4):468-75. Epub 1976/07/01. PubMed PMID: 8969.

25. Borel JF. Immunosuppressive properties of cyclosporin A (CY-A). Transplant Proc. 1980;12(2):233. Epub 1980/06/01. PubMed PMID: 6771901.

26. Walsh CT, Zydowsky LD, McKeon FD. Cyclosporin A, the cyclophilin class of peptidylprolyl isomerases, and blockade of T cell signal transduction. J Biol Chem. 1992;267(19):13115-8. Epub 1992/07/05. PubMed PMID: 1618811.

27. Wiesinger D, Borel JF. Studies on the mechanism of action of cyclosporin A. Immunobiology. 1980;156(4-5):454-63. Epub 1980/01/01. doi: 10.1016/S0171-2985(80)80078-7. PubMed PMID: 6154645.

28. Calne RY, White DJ, Thiru S, Evans DB, McMaster P, Dunn DC, Craddock GN, Pentlow BD, Rolles K. Cyclosporin A in patients receiving renal allografts from cadaver donors. Lancet. 1978;2(8104-5):1323-7. Epub 1978/12/23. PubMed PMID: 82836.

29. Calne RY, Rolles K, White DJ, Thiru S, Evans DB, McMaster P, Dunn DC, Craddock GN, Henderson RG, Aziz S, Lewis P. Cyclosporin A initially as the only immunosuppressant in 34 recipients of cadaveric organs: 32 kidneys, 2 pancreases, and 2 livers. Lancet. 1979;2(8151):1033-6. Epub 1979/11/17. PubMed PMID: 91781.

30. Issa N, Kukla A, Ibrahim HN. Calcineurin inhibitor nephrotoxicity: a review and perspective of the evidence. Am J Nephrol. 2013;37(6):602-12. Epub 2013/06/26. doi: 10.1159/000351648. PubMed PMID: 23796509.

31. Fung JJ. Tacrolimus and transplantation: a decade in review. Transplantation. 2004;77(9 Suppl):S41-3. Epub 2004/06/18. PubMed PMID: 15201685.

32. Lamb KE, Lodhi S, Meier-Kriesche HU. Long-term renal allograft survival in the United States: a critical reappraisal. Am J Transplant. 2011;11(3):450-62. Epub 2010/10/27. doi: 10.1111/j.1600-6143.2010.03283.x. PubMed PMID: 20973913.

33. Meier-Kriesche HU, Schold JD, Kaplan B. Long-term renal allograft survival: have we made significant progress or is it time to rethink our analytic and therapeutic strategies? Am J Transplant. 2004;4(8):1289-95. Epub 2004/07/23. doi: 10.1111/j.1600-6143.2004.00515.x. PubMed PMID: 15268730.

34. Pallet N, Fougeray S, Beaune P, Legendre C, Thervet E, Anglicheau D. Endoplasmic reticulum stress: an unrecognized actor in solid organ transplantation. Transplantation. 2009;88(5):605-13. Epub 2009/09/11. doi: 10.1097/TP.0b013e3181b22cec. PubMed PMID: 19741454.

35. Muhlberger I, Perco P, Fechete R, Mayer B, Oberbauer R. Biomarkers in renal transplantation ischemia reperfusion injury. Transplantation. 2009;88(3 Suppl):S14-9. Epub 2009/12/02. doi: 10.1097/TP.0b013e3181af65b5. PubMed PMID: 19667956.

36. Kim IK, Bedi DS, Denecke C, Ge X, Tullius SG. Impact of innate and adaptive immunity on rejection and tolerance. Transplantation. 2008;86(7):889-94. Epub 2008/10/15. doi: 10.1097/TP.0b013e318186ac4a. PubMed PMID: 18852649.

37. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140(6):805-20. Epub 2010/03/23. doi: 10.1016/j.cell.2010.01.022. PubMed PMID: 20303872.

38. Ogura Y, Sutterwala FS, Flavell RA. The inflammasome: first line of the immune response to cell stress. Cell. 2006;126(4):659-62. Epub 2006/08/23. doi: 10.1016/j.cell.2006.08.002. PubMed PMID: 16923387.

39. Murphy K, Weaver C. Janeway's immunobiology2017.

40. Carvalho-Gaspar M, Billing JS, Spriewald BM, Wood KJ. Chemokine gene expression during allograft rejection: comparison of two quantitative PCR techniques. J Immunol Methods. 2005;301(1-2):41-52. Epub 2005/07/16. doi: 10.1016/j.jim.2005.03.003. PubMed PMID: 16018884.

41. Larsen CP, Morris PJ, Austyn JM. Migration of dendritic leukocytes from cardiac allografts into host spleens. A novel pathway for initiation of rejection. J Exp Med. 1990;171(1):307-14. Epub 1990/01/01. PubMed PMID: 2404081; PMCID: PMC2187651.

42. Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, Morris PJ, Powrie F, Wood KJ. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. J Immunol. 2001;166(6):3789-96. Epub 2001/03/10. PubMed PMID: 11238621.

43. Cai J, Terasaki PI. Induction immunosuppression improves long-term graft and patient outcome in organ transplantation: an analysis of United Network for Organ Sharing registry data. Transplantation. 2010;90(12):1511-5. Epub 2010/11/09. doi: 10.1097/TP.0b013e3181fecfcb. PubMed PMID: 21057388.

44. Zinkernagel RM, Doherty PC. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature. 1974;248(5450):701-2. Epub 1974/04/19. PubMed PMID: 4133807.

45. Dierselhuis M, Goulmy E. The relevance of minor histocompatibility antigens in solid organ transplantation. Curr Opin Organ Transplant. 2009;14(4):419-25. Epub 2009/05/16. doi: 10.1097/MOT.0b013e32832d399c. PubMed PMID: 19444105.

46. Janeway CA, Jr. The priming of helper T cells. Semin Immunol. 1989;1(1):13-20. Epub 1989/09/01. PubMed PMID: 15630955.

47. Johnson JG, Jenkins MK. Accessory cell-derived signals required for T cell activation. Immunol Res. 1993;12(1):48-64. Epub 1993/01/01. PubMed PMID: 8099942.

48. Jenkins MK, Taylor PS, Norton SD, Urdahl KB. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. J Immunol. 1991;147(8):2461-6. Epub 1991/10/15. PubMed PMID: 1717561.

49. Lindstein T, June CH, Ledbetter JA, Stella G, Thompson CB. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. Science. 1989;244(4902):339-43. Epub 1989/04/21. PubMed PMID: 2540528.

50. Fraser JD, Irving BA, Crabtree GR, Weiss A. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. Science. 1991;251(4991):313-6. Epub 1991/01/18. PubMed PMID: 1846244.

51. Norton SD, Zuckerman L, Urdahl KB, Shefner R, Miller J, Jenkins MK. The CD28 ligand, B7, enhances IL-2 production by providing a costimulatory signal to T cells. J Immunol. 1992;149(5):1556-61. Epub 1992/09/01. PubMed PMID: 1380533.

52. Viola A, Schroeder S, Sakakibara Y, Lanzavecchia A. T lymphocyte costimulation mediated by reorganization of membrane microdomains. Science. 1999;283(5402):680-2. Epub 1999/01/29. PubMed PMID: 9924026.

53. Afzali B, Lombardi G, Lechler RI. Pathways of major histocompatibility complex allorecognition. Curr Opin Organ Transplant. 2008;13(4):438-44. Epub 2008/08/08. doi: 10.1097/MOT.0b013e328309ee31. PubMed PMID: 18685342; PMCID: PMC3815495.

54. Boardman DA, Jacob J, Smyth LA, Lombardi G, Lechler RI. What Is Direct Allorecognition? Curr Transplant Rep. 2016;3(4):275-83. Epub 2016/12/03. doi: 10.1007/s40472-016-0115-8. PubMed PMID: 27909647; PMCID: PMC5107184.

55. Joly E, Hudrisier D. What is trogocytosis and what is its purpose? Nat Immunol. 2003;4(9):815. Epub 2003/08/28. doi: 10.1038/ni0903-815. PubMed PMID: 12942076.

56. Morelli AE, Bracamonte-Baran W, Burlingham WJ. Donor-derived exosomes: the trick behind the semidirect pathway of allorecognition. Curr Opin Organ Transplant. 2017;22(1):46-54. Epub 2016/11/30. doi: 10.1097/MOT.0000000000000372. PubMed PMID: 27898464; PMCID: PMC5407007.

57. Neu KE, Wilson PC. Taking the Broad View on B Cell Affinity Maturation. Immunity. 2016;44(3):518-20. Epub 2016/03/18. doi: 10.1016/j.immuni.2016.03.001. PubMed PMID: 26982359.

58. Higgins RM, Bevan DJ, Carey BS, Lea CK, Fallon M, Buhler R, Vaughan RW, O'Donnell PJ, Snowden SA, Bewick M, Hendry BM. Prevention of hyperacute rejection by removal of antibodies to HLA immediately before renal transplantation. Lancet. 1996;348(9036):1208-11. Epub 1996/11/02. PubMed PMID: 8898038.

59. Puttarajappa C, Shapiro R, Tan HP. Antibody-mediated rejection in kidney transplantation: a review. J Transplant. 2012;2012:193724. Epub 2012/05/12. doi: 10.1155/2012/193724. PubMed PMID: 22577514; PMCID: PMC3337620.

60. Gebel HM, Bray RA, Nickerson P. Pre-transplant assessment of donor-reactive, HLA-specific antibodies in renal transplantation: contraindication vs. risk. Am J Transplant. 2003;3(12):1488-500. Epub 2003/11/25. PubMed PMID: 14629279.

61. Pereira M, Guerra J, Goncalves J, Santana A, Nascimento C, da Costa AG. Hyperacute Rejection in a Kidney Transplant With Negative Crossmatch: A Case Report. Transplant Proc. 2016;48(7):2384-6. Epub 2016/10/16. doi: 10.1016/j.transproceed.2016.06.021. PubMed PMID: 27742303.

62. Pape L, Becker JU, Immenschuh S, Ahlenstiel T. Acute and chronic antibody-mediated rejection in pediatric kidney transplantation. Pediatr Nephrol. 2015;30(3):417-24. Epub 2014/05/29. doi: 10.1007/s00467-014-2851-2. PubMed PMID: 24865478.

63. Uber WE, Self SE, Van Bakel AB, Pereira NL. Acute antibody-mediated rejection following heart transplantation. Am J Transplant. 2007;7(9):2064-74. Epub 2007/07/07. doi: 10.1111/j.1600-6143.2007.01900.x. PubMed PMID: 17614978.

64. Wu O, Levy AR, Briggs A, Lewis G, Jardine A. Acute rejection and chronic nephropathy: a systematic review of the literature. Transplantation. 2009;87(9):1330-9. Epub 2009/05/09. doi: 10.1097/TP.0b013e3181a236e0. PubMed PMID: 19424033.

65. Zheng J, Xue W, Qing X, Jing X, Hou J, Tian X, Guo Q, He X, Cai J. Treatment of Biopsy-Proven Acute Antibody-Mediated Rejection Using Thymoglobulin (ATG) Monotherapy and a Combination of Rituximab, Intravenous Immunoglobulin, and Plasmapheresis: Lesson Learned from Primary Experience. Clin Transpl. 2014:223-30. PubMed PMID: 26281149.

66. Nankivell BJ, Kuypers DR. Diagnosis and prevention of chronic kidney allograft loss. Lancet. 2011;378(9800):1428-37. Epub 2011/10/18. doi: 10.1016/S0140-6736(11)60699-5. PubMed PMID: 22000139.

67. Matas AJ. Chronic rejection--definition and correlates. Clin Transplant. 1994;8(2 Pt 2):162-7. Epub 1994/04/01. PubMed PMID: 8019032.

68. Humar A, Kerr S, Hassoun A, Granger D, Suhr B, Matas A. The association between acute rejection and chronic rejection in kidney transplantation. Transplant Proc. 1999;31(1-2):1302-3. Epub 1999/03/20. PubMed PMID: 10083581.

69. Matas AJ, Humar A, Payne WD, Gillingham KJ, Dunn DL, Sutherland DE, Najarian JS. Decreased acute rejection in kidney transplant recipients is associated with decreased chronic rejection. Ann Surg. 1999;230(4):493-8; discussion 8-500. Epub 1999/10/16. PubMed PMID: 10522719; PMCID: PMC1420898.

70. Salvadori M, Bertoni E. Impact of donor-specific antibodies on the outcomes of kidney graft: Pathophysiology, clinical, therapy. World J Transplant. 2014;4(1):1-17. doi: 10.5500/wjt.v4.i1.1. PubMed PMID: 24669363; PMCID: PMC3964192.

71. Loupy A, Hill GS, Jordan SC. The impact of donor-specific anti-HLA antibodies on late kidney allograft failure. Nat Rev Nephrol. 2012;8(6):348-57. doi: 10.1038/nrneph.2012.81. PubMed PMID: 22508180.

72. Baldwin WM, 3rd, Valujskikh A, Fairchild RL. Mechanisms of antibody-mediated acute and chronic rejection of kidney allografts. Curr Opin Organ Transplant. 2016;21(1):7-14. doi: 10.1097/MOT.0000000000000262. PubMed PMID: 26575854; PMCID: PMC4701597.

73. Thomas KA, Valenzuela NM, Reed EF. The perfect storm: HLA antibodies, complement, FcgammaRs, and endothelium in transplant rejection. Trends Mol Med. 2015;21(5):319-29. doi: 10.1016/j.molmed.2015.02.004. PubMed PMID: 25801125; PMCID: PMC4424065.

74. Djamali A, Kaufman DB, Ellis TM, Zhong W, Matas A, Samaniego M. Diagnosis and management of antibody-mediated rejection: current status and novel approaches. Am J Transplant. 2014;14(2):255-71. doi: 10.1111/ajt.12589. PubMed PMID: 24401076; PMCID: PMC4285166.

75. Knechtle SJ, Kwun J, Iwakoshi N. Prevention trumps treatment of antibody-mediated transplant rejection. J Clin Invest. 2010;120(4):1036-9. Epub 2010/03/26. doi: 10.1172/JCI42532. PubMed PMID: 20335653; PMCID: PMC2846071.

76. Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. J Exp Med. 1987;165(2):302-19. Epub 1987/02/01. PubMed PMID: 3029267; PMCID: PMC2188516.

77. Harding FA, McArthur JG, Gross JA, Raulet DH, Allison JP. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. Nature. 1992;356(6370):607-9. Epub 1992/04/16. doi: 10.1038/356607a0. PubMed PMID: 1313950.

78. Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA. CTLA-4 is a second receptor for the B cell activation antigen B7. J Exp Med. 1991;174(3):561-9. Epub 1991/09/01. PubMed PMID: 1714933; PMCID: PMC2118936.

79. Linsley PS, Wallace PM, Johnson J, Gibson MG, Greene JL, Ledbetter JA, Singh C, Tepper MA. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. Science. 1992;257(5071):792-5. Epub 1992/08/07. PubMed PMID: 1496399.

80. Turka LA, Linsley PS, Lin H, Brady W, Leiden JM, Wei RQ, Gibson ML, Zheng XG, Myrdal S, Gordon D, et al. T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection in vivo. Proc Natl Acad Sci U S A. 1992;89(22):11102-5. Epub 1992/11/15. PubMed PMID: 1332070; PMCID: PMC50492.

81. Sayegh MH, Turka LA. The role of T-cell costimulatory activation pathways in transplant rejection. N Engl J Med. 1998;338(25):1813-21. Epub 1998/06/19. doi: 10.1056/NEJM199806183382506. PubMed PMID: 9632449.

82. Azuma H, Chandraker A, Nadeau K, Hancock WW, Carpenter CB, Tilney NL, Sayegh MH. Blockade of T-cell costimulation prevents development of experimental chronic renal allograft rejection. Proc Natl Acad Sci U S A. 1996;93(22):12439-44. Epub 1996/10/29. PubMed PMID: 8901600; PMCID: PMC38010.

83. Larsen CP, Elwood ET, Alexander DZ, Ritchie SC, Hendrix R, Tucker-Burden C, Cho HR, Aruffo A, Hollenbaugh D, Linsley PS, Winn KJ, Pearson TC. Long-term acceptance of skin and cardiac allografts

after blocking CD40 and CD28 pathways. Nature. 1996;381(6581):434-8. doi: 10.1038/381434a0. PubMed PMID: 8632801.

84. Kirk AD, Harlan DM, Armstrong NN, Davis TA, Dong Y, Gray GS, Hong X, Thomas D, Fechner JH, Jr., Knechtle SJ. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. Proc Natl Acad Sci U S A. 1997;94(16):8789-94. Epub 1997/08/05. PubMed PMID: 9238056; PMCID: PMC23132.

85. Levisetti MG, Padrid PA, Szot GL, Mittal N, Meehan SM, Wardrip CL, Gray GS, Bruce DS, Thistlethwaite JR, Jr., Bluestone JA. Immunosuppressive effects of human CTLA4Ig in a non-human primate model of allogeneic pancreatic islet transplantation. J Immunol. 1997;159(11):5187-91. Epub 1998/04/21. PubMed PMID: 9548454.

86. Larsen CP, Pearson TC, Adams AB, Tso P, Shirasugi N, Strobert E, Anderson D, Cowan S, Price K, Naemura J, Emswiler J, Greene J, Turk LA, Bajorath J, Townsend R, Hagerty D, Linsley PS, Peach RJ. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. Am J Transplant. 2005;5(3):443-53. doi: 10.1111/j.1600-6143.2005.00749.x. PubMed PMID: 15707398.

87. Vincenti F, Larsen C, Durrbach A, Wekerle T, Nashan B, Blancho G, Lang P, Grinyo J, Halloran PF, Solez K, Hagerty D, Levy E, Zhou W, Natarajan K, Charpentier B, Belatacept Study G. Costimulation blockade with belatacept in renal transplantation. N Engl J Med. 2005;353(8):770-81. Epub 2005/08/27. doi: 10.1056/NEJMoa050085. PubMed PMID: 16120857.

88. Vincenti F, Rostaing L, Grinyo J, Rice K, Steinberg S, Gaite L, Moal MC, Mondragon-Ramirez GA, Kothari J, Polinsky MS, Meier-Kriesche HU, Munier S, Larsen CP. Belatacept and Long-Term Outcomes in Kidney Transplantation. N Engl J Med. 2016;374(4):333-43. doi: 10.1056/NEJMoa1506027. PubMed PMID: 26816011.

89. Vincenti F, Charpentier B, Vanrenterghem Y, Rostaing L, Bresnahan B, Darji P, Massari P, Mondragon-Ramirez GA, Agarwal M, Di Russo G, Lin CS, Garg P, Larsen CP. A phase III study of

belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). Am J Transplant. 2010;10(3):535-46. doi: 10.1111/j.1600-6143.2009.03005.x. PubMed PMID: 20415897.

90. Grinyo J, Charpentier B, Pestana JM, Vanrenterghem Y, Vincenti F, Reyes-Acevedo R, Apanovitch AM, Gujrathi S, Agarwal M, Thomas D, Larsen CP. An integrated safety profile analysis of belatacept in kidney transplant recipients. Transplantation. 2010;90(12):1521-7. Epub 2010/11/20. doi: 10.1097/TP.0b013e3182007b95. PubMed PMID: 21088650.

91. Durrbach A, Pestana JM, Pearson T, Vincenti F, Garcia VD, Campistol J, Rial Mdel C, Florman S, Block A, Di Russo G, Xing J, Garg P, Grinyo J. A phase III study of belatacept versus cyclosporine in kidney transplants from extended criteria donors (BENEFIT-EXT study). Am J Transplant. 2010;10(3):547-57. doi: 10.1111/j.1600-6143.2010.03016.x. PubMed PMID: 20415898.

92. Adams AB, Williams MA, Jones TR, Shirasugi N, Durham MM, Kaech SM, Wherry EJ, Onami T, Lanier JG, Kokko KE, Pearson TC, Ahmed R, Larsen CP. Heterologous immunity provides a potent barrier to transplantation tolerance. J Clin Invest. 2003;111(12):1887-95. doi: 10.1172/JCI17477. PubMed PMID: 12813024; PMCID: PMC161424.

93. Adams AB, Pearson TC, Larsen CP. Heterologous immunity: an overlooked barrier to tolerance. Immunol Rev. 2003;196:147-60. Epub 2003/11/18. PubMed PMID: 14617203.

94. Poirier N, Azimzadeh AM, Zhang T, Dilek N, Mary C, Nguyen B, Tillou X, Wu G, Reneaudin K, Hervouet J, Martinet B, Coulon F, Allain-Launay E, Karam G, Soulillou JP, Pierson RN, 3rd, Blancho G, Vanhove B. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. Sci Transl Med. 2010;2(17):17ra0. doi: 10.1126/scitranslmed.3000116. PubMed PMID: 20371478; PMCID: PMC2860737.

95. Riella LV, Liu T, Yang J, Chock S, Shimizu T, Mfarrej B, Batal I, Xiao X, Sayegh MH, Chandraker A. Deleterious effect of CTLA4-Ig on a Treg-dependent transplant model. Am J Transplant. 2012;12(4):846-55. Epub 2012/02/04. doi: 10.1111/j.1600-6143.2011.03929.x. PubMed PMID: 22300534.

96. Poirier N, Dilek N, Mary C, Ville S, Coulon F, Branchereau J, Tillou X, Charpy V, Pengam S, Nerriere-Daguin V, Hervouet J, Minault D, Le Bas-Bernardet S, Renaudin K, Vanhove B, Blancho G. FR104, an antagonist anti-CD28 monovalent fab' antibody, prevents alloimmunization and allows calcineurin inhibitor minimization in nonhuman primate renal allograft. Am J Transplant. 2015;15(1):88-100. doi: 10.1111/ajt.12964. PubMed PMID: 25488654.

97. Ville S, Poirier N, Branchereau J, Charpy V, Pengam S, Nerriere-Daguin V, Le Bas-Bernardet S, Coulon F, Mary C, Chenouard A, Hervouet J, Minault D, Nedellec S, Renaudin K, Vanhove B, Blancho G. Anti-CD28 Antibody and Belatacept Exert Differential Effects on Mechanisms of Renal Allograft Rejection. J Am Soc Nephrol. 2016;27(12):3577-88. doi: 10.1681/ASN.2015070774. PubMed PMID: 27160407; PMCID: PMC5118475.

98. Liu D, Krummey SM, Badell IR, Wagener M, Schneeweis LA, Stetsko DK, Suchard SJ, Nadler SG, Ford ML. 2B4 (CD244) induced by selective CD28 blockade functionally regulates allograft-specific CD8+ T cell responses. J Exp Med. 2014;211(2):297-311. doi: 10.1084/jem.20130902. PubMed PMID: 24493803; PMCID: PMC3920565.

99. Claman HN, Chaperon EA, Triplett RF. Thymus-marrow cell combinations. Synergism in antibody production. Proc Soc Exp Biol Med. 1966;122(4):1167-71. Epub 1966/08/01. PubMed PMID: 5918139.

100. Miller JF, Mitchell GF. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. J Exp Med. 1968;128(4):801-20. Epub 1968/10/01. PubMed PMID: 5691985; PMCID: PMC2138540.

101. Mitchell GF, Miller JF. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. J Exp Med. 1968;128(4):821-37. Epub 1968/10/01. PubMed PMID: 5691986; PMCID: PMC2138546.

102. Katz DH, Benacerraf B. The regulatory influence of activated T cells on B cell responses to antigen. Adv Immunol. 1972;15:1-94. Epub 1972/01/01. PubMed PMID: 4116319.

103. Nossal GJ, Lederberg J. Antibody production by single cells. Nature. 1958;181(4620):1419-20. Epub 1958/05/17. PubMed PMID: 13552693.

104. Schreiber JR. Role of Toll like receptors in the antibody response to encapsulated bacteria. Front Biosci (Elite Ed). 2012;4:2638-46. Epub 2012/06/02. PubMed PMID: 22652666.

105. Moore PA, Belvedere O, Orr A, Pieri K, LaFleur DW, Feng P, Soppet D, Charters M, Gentz R, Parmelee D, Li Y, Galperina O, Giri J, Roschke V, Nardelli B, Carrell J, Sosnovtseva S, Greenfield W, Ruben SM, Olsen HS, Fikes J, Hilbert DM. BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. Science. 1999;285(5425):260-3. Epub 1999/07/10. PubMed PMID: 10398604.

106. Gross JA, Johnston J, Mudri S, Enselman R, Dillon SR, Madden K, Xu W, Parrish-Novak J, Foster D, Lofton-Day C, Moore M, Littau A, Grossman A, Haugen H, Foley K, Blumberg H, Harrison K, Kindsvogel W, Clegg CH. TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. Nature. 2000;404(6781):995-9. Epub 2000/05/09. doi: 10.1038/35010115. PubMed PMID: 10801128.

107. Fagarasan S, Honjo T. T-Independent immune response: new aspects of B cell biology. Science. 2000;290(5489):89-92. Epub 2000/10/06. PubMed PMID: 11021805.

108. Adderson EE. Antibody repertoires in infants and adults: effects of T-independent and T-dependent immunizations. Springer Semin Immunopathol. 2001;23(4):387-403. Epub 2002/02/06. PubMed PMID: 11826616.

109. Howard M, Farrar J, Hilfiker M, Johnson B, Takatsu K, Hamaoka T, Paul WE. Identification of a T cell-derived b cell growth factor distinct from interleukin 2. J Exp Med. 1982;155(3):914-23. Epub 1982/03/01. PubMed PMID: 6977612; PMCID: PMC2186613.

110. Kopf M, Le Gros G, Coyle AJ, Kosco-Vilbois M, Brombacher F. Immune responses of IL-4, IL-5, IL-6 deficient mice. Immunol Rev. 1995;148:45-69. Epub 1995/12/01. PubMed PMID: 8825282.

111. Clark EA, Ledbetter JA. Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. Proc Natl Acad Sci U S A. 1986;83(12):4494-8. Epub 1986/06/01. PubMed PMID: 3487090; PMCID: PMC323760.

112. Liu YJ, Joshua DE, Williams GT, Smith CA, Gordon J, MacLennan IC. Mechanism of antigen-driven selection in germinal centres. Nature. 1989;342(6252):929-31. Epub 1989/12/21. doi: 10.1038/342929a0. PubMed PMID: 2594086.

113. Armitage RJ, Fanslow WC, Strockbine L, Sato TA, Clifford KN, Macduff BM, Anderson DM, Gimpel SD, Davis-Smith T, Maliszewski CR, et al. Molecular and biological characterization of a murine ligand for CD40. Nature. 1992;357(6373):80-2. Epub 1992/05/07. doi: 10.1038/357080a0. PubMed PMID: 1374165.

114. Allen RC, Armitage RJ, Conley ME, Rosenblatt H, Jenkins NA, Copeland NG, Bedell MA, Edelhoff S, Disteche CM, Simoneaux DK, et al. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. Science. 1993;259(5097):990-3. Epub 1993/02/12. PubMed PMID: 7679801.

115. Fuleihan R, Ramesh N, Loh R, Jabara H, Rosen RS, Chatila T, Fu SM, Stamenkovic I, Geha RS. Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM. Proc Natl Acad Sci U S A. 1993;90(6):2170-3. Epub 1993/03/15. PubMed PMID: 7681587; PMCID: PMC46047.

116. Foy TM, Laman JD, Ledbetter JA, Aruffo A, Claassen E, Noelle RJ. gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. J Exp Med. 1994;180(1):157-63. Epub 1994/07/01. PubMed PMID: 7516405; PMCID: PMC2191546.

117. MacLennan IC. Germinal centers. Annu Rev Immunol. 1994;12:117-39. Epub 1994/01/01. doi: 10.1146/annurev.iy.12.040194.001001. PubMed PMID: 8011279.

118. Shlomchik MJ, Weisel F. Germinal centers. Immunol Rev. 2012;247(1):5-10. Epub 2012/04/17. doi: 10.1111/j.1600-065X.2012.01125.x. PubMed PMID: 22500827.

119. Jacob J, Kelsoe G, Rajewsky K, Weiss U. Intraclonal generation of antibody mutants in germinal centres. Nature. 1991;354(6352):389-92. Epub 1991/12/05. doi: 10.1038/354389a0. PubMed PMID: 1956400.

120. Berek C, Berger A, Apel M. Maturation of the immune response in germinal centers. Cell. 1991;67(6):1121-9. Epub 1991/12/20. PubMed PMID: 1760840.

121. Stavnezer J, Schrader CE. IgH chain class switch recombination: mechanism and regulation. J Immunol. 2014;193(11):5370-8. Epub 2014/11/21. doi: 10.4049/jimmunol.1401849. PubMed PMID: 25411432; PMCID: PMC4447316.

122. Blink EJ, Light A, Kallies A, Nutt SL, Hodgkin PD, Tarlinton DM. Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. J Exp Med. 2005;201(4):545-54. Epub 2005/02/16. doi: 10.1084/jem.20042060. PubMed PMID: 15710653; PMCID: PMC2213050.

123. Allen CD, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, Cyster JG. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. Nat Immunol. 2004;5(9):943-52. Epub 2004/08/10. doi: 10.1038/ni1100. PubMed PMID: 15300245.

124. Victora GD, Nussenzweig MC. Germinal centers. Annu Rev Immunol. 2012;30:429-57. Epub 2012/01/10. doi: 10.1146/annurev-immunol-020711-075032. PubMed PMID: 22224772.

125. Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, Johnston J, Madden K, Xu W, West J, Schrader S, Burkhead S, Heipel M, Brandt C, Kuijper JL, Kramer J, Conklin D, Presnell SR, Berry J, Shiota F, Bort S, Hambly K, Mudri S, Clegg C, Moore M, Grant FJ, Lofton-Day C, Gilbert T, Rayond F, Ching A, Yao L, Smith D, Webster P, Whitmore T, Maurer M, Kaushansky K, Holly RD, Foster D. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature. 2000;408(6808):57-63. Epub 2000/11/18. doi: 10.1038/35040504. PubMed PMID: 11081504.

126. Ozaki K, Kikly K, Michalovich D, Young PR, Leonard WJ. Cloning of a type I cytokine receptor most related to the IL-2 receptor beta chain. Proc Natl Acad Sci U S A. 2000;97(21):11439-44. Epub 2000/10/04. doi: 10.1073/pnas.200360997. PubMed PMID: 11016959; PMCID: PMC17218.

127. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, Corcoran LM, Godfrey DI, Toellner KM, Smyth MJ, Nutt SL, Tarlinton DM. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. J Exp Med. 2010;207(2):365-78. Epub 2010/02/10. doi: 10.1084/jem.20091777. PubMed PMID: 20142430; PMCID: PMC2822601.

128. Weinstein JS, Herman EI, Lainez B, Licona-Limon P, Esplugues E, Flavell R, Craft J. TFH cells progressively differentiate to regulate the germinal center response. Nat Immunol. 2016;17(10):1197-205. Epub 2016/08/31. doi: 10.1038/ni.3554. PubMed PMID: 27573866; PMCID: PMC5030190.

129. Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, Verma NK, Smyth MJ, Rigby RJ, Vinuesa CG. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. J Exp Med. 2010;207(2):353-63. Epub 2010/02/10. doi: 10.1084/jem.20091738. PubMed PMID: 20142429; PMCID: PMC2822609.

130. Ozaki K, Spolski R, Feng CG, Qi CF, Cheng J, Sher A, Morse HC, 3rd, Liu C, Schwartzberg PL, Leonard WJ. A critical role for IL-21 in regulating immunoglobulin production. Science. 2002;298(5598):1630-4. Epub 2002/11/26. doi: 10.1126/science.1077002. PubMed PMID: 12446913.

131. Liu D, Xu H, Shih C, Wan Z, Ma X, Ma W, Luo D, Qi H. T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. Nature. 2015;517(7533):214-8. doi: 10.1038/nature13803. PubMed PMID: 25317561.

132. Grimbacher B, Hutloff A, Schlesier M, Glocker E, Warnatz K, Drager R, Eibel H, Fischer B, Schaffer AA, Mages HW, Kroczek RA, Peter HH. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. Nat Immunol. 2003;4(3):261-8. Epub 2003/02/11. doi: 10.1038/ni902. PubMed PMID: 12577056.

133. Dong C, Temann UA, Flavell RA. Cutting edge: critical role of inducible costimulator in germinal center reactions. J Immunol. 2001;166(6):3659-62. Epub 2001/03/10. PubMed PMID: 11238604.

134. Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. SAP is required for generating long-term humoral immunity. Nature. 2003;421(6920):282-7. Epub 2003/01/17. doi: 10.1038/nature01318. PubMed PMID: 12529646.

135. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. Nature. 2008;455(7214):764-9. Epub 2008/10/10. doi: 10.1038/nature07345. PubMed PMID: 18843362; PMCID: PMC2652134.

136. Cannons JL, Tangye SG, Schwartzberg PL. SLAM family receptors and SAP adaptors in immunity. Annu Rev Immunol. 2011;29:665-705. Epub 2011/01/12. doi: 10.1146/annurev-immunol-030409-101302. PubMed PMID: 21219180.

137. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, Lao C, Crotty S. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. Immunity. 2011;34(6):932-46. doi: 10.1016/j.immuni.2011.03.023. PubMed PMID: 21636296; PMCID: PMC3124577.

138. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol. 1986;136(7):2348-57. Epub 1986/04/01. PubMed PMID: 2419430.

139. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003;299(5609):1057-61. Epub 2003/01/11. doi: 10.1126/science.1079490. PubMed PMID: 12522256.

140. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J Exp Med. 2000;192(11):1553-62. Epub 2000/12/06. PubMed PMID: 11104798; PMCID: PMC2193097.

141. Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. J Exp Med. 2001;193(12):1373-81. Epub 2001/06/20. PubMed PMID: 11413192; PMCID: PMC2193300.

142. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, Forster R. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J Exp Med. 2000;192(11):1545-52. Epub 2000/12/06. PubMed PMID: 11104797; PMCID: PMC2193094.

143. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). Annu Rev Immunol. 2010;28:445-89. Epub 2010/03/03. doi: 10.1146/annurev-immunol-030409-101212. PubMed PMID: 20192806; PMCID: PMC3502616.

144. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, Dent AL, Craft J, Crotty S. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science. 2009;325(5943):1006-10. Epub 2009/07/18. doi: 10.1126/science.1175870. PubMed PMID: 19608860; PMCID: PMC2766560.

145. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, Wang YH, Dong C. Bcl6 mediates the development of T follicular helper cells. Science. 2009;325(5943):1001-5. Epub 2009/07/25. doi: 10.1126/science.1176676. PubMed PMID: 19628815; PMCID: PMC2857334.

146. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, Srivastava M, Linterman M, Zheng L, Simpson N, Ellyard JI, Parish IA, Ma CS, Li QJ, Parish CR, Mackay CR, Vinuesa CG. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. Immunity. 2009;31(3):457-68. Epub 2009/07/28. doi: 10.1016/j.immuni.2009.07.002. PubMed PMID: 19631565.

147. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, Wang YH, Watowich SS, Jetten AM, Tian Q, Dong C. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity. 2008;29(1):138-49. Epub 2008/07/05. doi: 10.1016/j.immuni.2008.05.009. PubMed PMID: 18599325; PMCID: PMC2556461.

148. Klein U, Dalla-Favera R. Germinal centres: role in B-cell physiology and malignancy. Nat Rev Immunol. 2008;8(1):22-33. Epub 2007/12/22. doi: 10.1038/nri2217. PubMed PMID: 18097447.

149. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. Nat Immunol. 2010;11(2):114-20. Epub 2010/01/20. doi: 10.1038/ni.1837. PubMed PMID: 20084069; PMCID: PMC2864556.

150. Basso K, Saito M, Sumazin P, Margolin AA, Wang K, Lim WK, Kitagawa Y, Schneider C, Alvarez MJ, Califano A, Dalla-Favera R. Integrated biochemical and computational approach identifies BCL6 direct target genes controlling multiple pathways in normal germinal center B cells. Blood. 2010;115(5):975-84. Epub 2009/12/08. doi: 10.1182/blood-2009-06-227017. PubMed PMID: 19965633; PMCID: PMC2817639.

151. Kusam S, Toney LM, Sato H, Dent AL. Inhibition of Th2 differentiation and GATA-3 expression by BCL-6. J Immunol. 2003;170(5):2435-41. Epub 2003/02/21. PubMed PMID: 12594267.

152. Butch AW, Chung GH, Hoffmann JW, Nahm MH. Cytokine expression by germinal center cells. J Immunol. 1993;150(1):39-47. Epub 1993/01/01. PubMed PMID: 7678034.

153. Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. Nat Immunol. 2009;10(4):385-93. Epub 2009/03/03. doi: 10.1038/ni.1715. PubMed PMID: 19252490; PMCID: PMC2714053.

154. Coffman RL, Seymour BW, Lebman DA, Hiraki DD, Christiansen JA, Shrader B, Cherwinski HM, Savelkoul HF, Finkelman FD, Bond MW, et al. The role of helper T cell products in mouse B cell differentiation and isotype regulation. Immunol Rev. 1988;102:5-28. Epub 1988/02/01. PubMed PMID: 2966762.

155. Huse M, Quann EJ, Davis MM. Shouts, whispers and the kiss of death: directional secretion in T cells. Nat Immunol. 2008;9(10):1105-11. Epub 2008/09/19. doi: 10.1038/ni.f.215. PubMed PMID: 18800163; PMCID: PMC2905669.

156. Kupfer A, Mosmann TR, Kupfer H. Polarized expression of cytokines in cell conjugates of helper T cells and splenic B cells. Proc Natl Acad Sci U S A. 1991;88(3):775-9. Epub 1991/02/01. PubMed PMID: 1825141; PMCID: PMC50896.

157. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. Science. 2010;327(5969):1098-102. Epub 2010/02/27. doi: 10.1126/science.1178334. PubMed PMID: 20185720; PMCID: PMC2997673.

158. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. Immunity. 2009;30(5):646-55. Epub 2009/05/26. doi: 10.1016/j.immuni.2009.05.001. PubMed PMID: 19464987.

159. Xu H, Li X, Liu D, Li J, Zhang X, Chen X, Hou S, Peng L, Xu C, Liu W, Zhang L, Qi H. Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. Nature. 2013;496(7446):523-7. doi: 10.1038/nature12058. PubMed PMID: 23619696.

160. Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. J Immunol. 2007;179(8):5099-108. Epub 2007/10/04. PubMed PMID: 17911595.

161. Shahinian A, Pfeffer K, Lee KP, Kundig TM, Kishihara K, Wakeham A, Kawai K, Ohashi PS, Thompson CB, Mak TW. Differential T cell costimulatory requirements in CD28-deficient mice. Science. 1993;261(5121):609-12. Epub 1993/07/30. PubMed PMID: 7688139.

162. Ferguson SE, Han S, Kelsoe G, Thompson CB. CD28 is required for germinal center formation. J Immunol. 1996;156(12):4576-81. Epub 1996/06/15. PubMed PMID: 8648099.

163. Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, Ma J, Tezuka K, Yagita H, Okumura K. The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo. J Immunol. 2005;175(4):2340-8. Epub 2005/08/06. PubMed PMID: 16081804.

164. Warnatz K, Bossaller L, Salzer U, Skrabl-Baumgartner A, Schwinger W, van der Burg M, van Dongen JJ, Orlowska-Volk M, Knoth R, Durandy A, Draeger R, Schlesier M, Peter HH, Grimbacher B. Human ICOS deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. Blood. 2006;107(8):3045-52. Epub 2005/12/31. doi: 10.1182/blood-2005-07-2955. PubMed PMID: 16384931.

165. Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, Yu D, Domaschenz H, Whittle B, Lambe T, Roberts IS, Copley RR, Bell JI, Cornall RJ, Goodnow CC. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. Nature. 2005;435(7041):452-8. Epub 2005/05/27. doi: 10.1038/nature03555. PubMed PMID: 15917799.

166. Tan AH, Wong SC, Lam KP. Regulation of mouse inducible costimulator (ICOS) expression by Fyn-NFATc2 and ERK signaling in T cells. J Biol Chem. 2006;281(39):28666-78. Epub 2006/08/02. doi: 10.1074/jbc.M604081200. PubMed PMID: 16880206.

167. Gigoux M, Shang J, Pak Y, Xu M, Choe J, Mak TW, Suh WK. Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase. Proc Natl Acad Sci U S A. 2009;106(48):20371-6. Epub 2009/11/17. doi: 10.1073/pnas.0911573106. PubMed PMID: 19915142; PMCID: PMC2787139.

168. Yusuf I, Kageyama R, Monticelli L, Johnston RJ, Ditoro D, Hansen K, Barnett B, Crotty S. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). J Immunol. 2010;185(1):190-202. Epub 2010/06/08. doi: 10.4049/jimmunol.0903505. PubMed PMID: 20525889; PMCID: PMC2913439.

169. Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KM, Lanzavecchia A, Sallusto F. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. Immunity. 2013;38(3):596-605. doi: 10.1016/j.immuni.2012.11.020. PubMed PMID: 23499493.

170. Iyer SS, Latner DR, Zilliox MJ, McCausland M, Akondy RS, Penaloza-Macmaster P, Hale JS, Ye L, Mohammed AU, Yamaguchi T, Sakaguchi S, Amara RR, Ahmed R. Identification of novel markers for mouse CD4(+) T follicular helper cells. Eur J Immunol. 2013;43(12):3219-32. Epub 2013/09/14. doi: 10.1002/eji.201343469. PubMed PMID: 24030473; PMCID: PMC3947211.

171. Rolf J, Bell SE, Kovesdi D, Janas ML, Soond DR, Webb LM, Santinelli S, Saunders T, Hebeis B, Killeen N, Okkenhaug K, Turner M. Phosphoinositide 3-kinase activity in T cells regulates the magnitude of the germinal center reaction. J Immunol. 2010;185(7):4042-52. Epub 2010/09/10. doi: 10.4049/jimmunol.1001730. PubMed PMID: 20826752.

172. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. Immunity. 2007;27(1):111-22. doi: 10.1016/j.immuni.2007.05.016. PubMed PMID: 17629517; PMCID: PMC2707944.

173. Di Noia JM, Neuberger MS. Molecular mechanisms of antibody somatic hypermutation. Annu Rev Biochem. 2007;76:1-22. Epub 2007/03/03. doi: 10.1146/annurev.biochem.76.061705.090740. PubMed PMID: 17328676.

174. Crotty S. Follicular helper CD4 T cells (TFH). Annu Rev Immunol. 2011;29:621-63. doi: 10.1146/annurev-immunol-031210-101400. PubMed PMID: 21314428.

175. Tarlinton D, Radbruch A, Hiepe F, Dorner T. Plasma cell differentiation and survival. Curr Opin Immunol. 2008;20(2):162-9. Epub 2008/05/06. doi: 10.1016/j.coi.2008.03.016. PubMed PMID: 18456483.

176. Roth K, Oehme L, Zehentmeier S, Zhang Y, Niesner R, Hauser AE. Tracking plasma cell differentiation and survival. Cytometry A. 2014;85(1):15-24. Epub 2014/04/05. doi: 10.1002/cyto.a.22355. PubMed PMID: 24700574.

177. Tangye SG, Tarlinton DM. Memory B cells: effectors of long-lived immune responses. Eur J Immunol. 2009;39(8):2065-75. Epub 2009/07/29. doi: 10.1002/eji.200939531. PubMed PMID: 19637202.

178. Good-Jacobson KL, Shlomchik MJ. Plasticity and heterogeneity in the generation of memory B cells and long-lived plasma cells: the influence of germinal center interactions and dynamics. J Immunol. 2010;185(6):3117-25. Epub 2010/09/04. doi: 10.4049/jimmunol.1001155. PubMed PMID: 20814029.

179. King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. Annu Rev Immunol. 2008;26:741-66. Epub 2008/01/05. doi: 10.1146/annurev.immunol.26.021607.090344. PubMed PMID: 18173374.

180. Sallusto F, Lanzavecchia A, Araki K, Ahmed R. From vaccines to memory and back. Immunity. 2010;33(4):451-63. Epub 2010/10/30. doi: 10.1016/j.immuni.2010.10.008. PubMed PMID: 21029957; PMCID: PMC3760154.

181. Williams MA, Bevan MJ. Effector and memory CTL differentiation. Annu Rev Immunol. 2007;25:171-92. Epub 2006/11/30. doi: 10.1146/annurev.immunol.25.022106.141548. PubMed PMID: 17129182.

182. Hale JS, Youngblood B, Latner DR, Mohammed AU, Ye L, Akondy RS, Wu T, Iyer SS, Ahmed R. Distinct memory CD4+ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection. Immunity. 2013;38(4):805-17. Epub 2013/04/16. doi: 10.1016/j.immuni.2013.02.020. PubMed PMID: 23583644; PMCID: PMC3741679.

183. MacLeod MK, David A, McKee AS, Crawford F, Kappler JW, Marrack P. Memory CD4 T cells that express CXCR5 provide accelerated help to B cells. J Immunol. 2011;186(5):2889-96. Epub 2011/01/29. doi: 10.4049/jimmunol.1002955. PubMed PMID: 21270407; PMCID: PMC3069687.

184. Choi YS, Yang JA, Yusuf I, Johnston RJ, Greenbaum J, Peters B, Crotty S. Bcl6 expressing follicular helper CD4 T cells are fate committed early and have the capacity to form memory. J Immunol. 2013;190(8):4014-26. doi: 10.4049/jimmunol.1202963. PubMed PMID: 23487426; PMCID: PMC3626566.

185. Hale JS, Ahmed R. Memory T follicular helper CD4 T cells. Front Immunol. 2015;6:16. Epub 2015/02/24. doi: 10.3389/fimmu.2015.00016. PubMed PMID: 25699040; PMCID: PMC4313784.

186. Sage PT, Sharpe AH. T follicular regulatory cells. Immunol Rev. 2016;271(1):246-59. Epub 2016/04/19. doi: 10.1111/imr.12411. PubMed PMID: 27088919.

187. Sage PT, Sharpe AH. T follicular regulatory cells in the regulation of B cell responses. Trends Immunol. 2015;36(7):410-8. Epub 2015/06/21. doi: 10.1016/j.it.2015.05.005. PubMed PMID: 26091728; PMCID: PMC4508020.

188. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, Wang YH, Lim H, Reynolds JM, Zhou XH, Fan HM, Liu ZM, Neelapu SS, Dong C. Follicular regulatory T cells expressing Foxp3 and Bcl-6

suppress germinal center reactions. Nat Med. 2011;17(8):983-8. Epub 2011/07/26. doi: 10.1038/nm.2426. PubMed PMID: 21785430; PMCID: PMC3151340.

189. Wing JB, Kitagawa Y, Locci M, Hume H, Tay C, Morita T, Kidani Y, Matsuda K, Inoue T, Kurosaki T, Crotty S, Coban C, Ohkura N, Sakaguchi S. A distinct subpopulation of CD25(-) T-follicular regulatory cells localizes in the germinal centers. Proc Natl Acad Sci U S A. 2017;114(31):E6400-E9. Epub 2017/07/13. doi: 10.1073/pnas.1705551114. PubMed PMID: 28698369; PMCID: PMC5547636.

190. Sage PT, Alvarez D, Godec J, von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. J Clin Invest. 2014;124(12):5191-204. Epub 2014/10/28. doi: 10.1172/JCI76861. PubMed PMID: 25347469; PMCID: PMC4348955.

191. Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. Nat Immunol. 2013;14(2):152-61. Epub 2012/12/18. doi: 10.1038/ni.2496. PubMed PMID: 23242415; PMCID: PMC3788614.

192. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, Srivastava M, Divekar DP, Beaton L, Hogan JJ, Fagarasan S, Liston A, Smith KG, Vinuesa CG. Foxp3+ follicular regulatory T cells control the germinal center response. Nat Med. 2011;17(8):975-82. Epub 2011/07/26. doi: 10.1038/nm.2425. PubMed PMID: 21785433; PMCID: PMC3182542.

193. Wollenberg I, Agua-Doce A, Hernandez A, Almeida C, Oliveira VG, Faro J, Graca L. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. J Immunol. 2011;187(9):4553-60. Epub 2011/10/11. doi: 10.4049/jimmunol.1101328. PubMed PMID: 21984700.

194. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. Immunity. 2014;41(6):1026-39. doi: 10.1016/j.immuni.2014.12.005. PubMed PMID: 25526313; PMCID: PMC4309019.

195. Sage PT, Ron-Harel N, Juneja VR, Sen DR, Maleri S, Sungnak W, Kuchroo VK, Haining WN, Chevrier N, Haigis M, Sharpe AH. Suppression by TFR cells leads to durable and selective inhibition of B cell effector function. Nat Immunol. 2016;17(12):1436-46. Epub 2016/11/01. doi: 10.1038/ni.3578. PubMed PMID: 27695002; PMCID: PMC5502675.

196. Wang CJ, Heuts F, Ovcinnikovs V, Wardzinski L, Bowers C, Schmidt EM, Kogimtzis A, Kenefeck R, Sansom DM, Walker LS. CTLA-4 controls follicular helper T-cell differentiation by regulating the strength of CD28 engagement. Proc Natl Acad Sci U S A. 2015;112(2):524-9. doi: 10.1073/pnas.1414576112. PubMed PMID: 25548162; PMCID: PMC4299196.

197. Tivol EA, Boyd SD, McKeon S, Borriello F, Nickerson P, Strom TB, Sharpe AH. CTLA4Ig prevents lymphoproliferation and fatal multiorgan tissue destruction in CTLA-4-deficient mice. J Immunol. 1997;158(11):5091-4. PubMed PMID: 9164923.

198. Goodnow CC, Sprent J, Fazekas de St Groth B, Vinuesa CG. Cellular and genetic mechanisms of self tolerance and autoimmunity. Nature. 2005;435(7042):590-7. doi: 10.1038/nature03724. PubMed PMID: 15931211.

199. Badell IR, Ford ML. T follicular helper cells in the generation of alloantibody and graft rejection. Curr Opin Organ Transplant. 2016;21(1):1-6. doi: 10.1097/MOT.0000000000000260. PubMed PMID: 26727455; PMCID: PMC4702514.

200. Walters GD, Vinuesa CG. T Follicular Helper Cells in Transplantation. Transplantation. 2016;100(8):1650-5. doi: 10.1097/TP.0000000000001217. PubMed PMID: 27362303.

201. Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity. 2014;41(4):529-42. Epub 2014/11/05. doi: 10.1016/j.immuni.2014.10.004. PubMed PMID: 25367570; PMCID: PMC4223692.

202. Rabant M, Gorbacheva V, Fan R, Yu H, Valujskikh A. CD40-independent help by memory CD4 T cells induces pathogenic alloantibody but does not lead to long-lasting humoral immunity. Am J

Transplant. 2013;13(11):2831-41. Epub 2013/10/10. doi: 10.1111/ajt.12432. PubMed PMID: 24102790; PMCID: PMC4019209.

203. Gorbacheva V, Fan R, Wang X, Baldwin WM, 3rd, Fairchild RL, Valujskikh A. IFN-gamma production by memory helper T cells is required for CD40-independent alloantibody responses. J Immunol. 2015;194(3):1347-56. Epub 2014/12/31. doi: 10.4049/jimmunol.1401573. PubMed PMID: 25548230; PMCID: PMC4297685.

204. Gorbacheva V, Ayasoufi K, Fan R, Baldwin WM, 3rd, Valujskikh A. B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) mediate CD40-independent help by memory CD4 T cells. Am J Transplant. 2015;15(2):346-57. Epub 2014/12/17. doi: 10.1111/ajt.12984. PubMed PMID: 25496308; PMCID: PMC4304935.

205. Su CA, Fairchild RL. Memory T Cells in Transplantation. Curr Transplant Rep. 2014;1(3):137-46. Epub 2014/12/02. doi: 10.1007/s40472-014-0018-5. PubMed PMID: 25435071; PMCID: PMC4235994.

206. Kim EJ, Kwun J, Gibby AC, Hong JJ, Farris AB, 3rd, Iwakoshi NN, Villinger F, Kirk AD, Knechtle SJ. Costimulation blockade alters germinal center responses and prevents antibody-mediated rejection. Am J Transplant. 2014;14(1):59-69. Epub 2013/12/21. doi: 10.1111/ajt.12526. PubMed PMID: 24354871; PMCID: PMC3985346.

207. Badell IR, Russell MC, Cardona K, Shaffer VO, Turner AP, Avila JG, Cano JA, Leopardi FV, Song M, Strobert EA, Ford ML, Pearson TC, Kirk AD, Larsen CP. CTLA4Ig prevents alloantibody formation following nonhuman primate islet transplantation using the CD40-specific antibody 3A8. Am J Transplant. 2012;12(7):1918-23. Epub 2012/03/31. doi: 10.1111/j.1600-6143.2012.04029.x. PubMed PMID: 22458552; PMCID: PMC3387300.

208. de Graav GN, Hesselink DA, Dieterich M, Kraaijeveld R, Verschoor W, Roelen DL, Litjens NHR, Chong AS, Weimar W, Baan CC. Belatacept Does Not Inhibit Follicular T Cell-Dependent B-Cell

Differentiation in Kidney Transplantation. Front Immunol. 2017;8:641. Epub 2017/06/18. doi: 10.3389/fimmu.2017.00641. PubMed PMID: 28620390; PMCID: PMC5450507.

209. Badell IR, La Muraglia GM, 2nd, Liu D, Wagener ME, Ding G, Ford ML. Selective CD28 Blockade Results in Superior Inhibition of Donor-Specific T Follicular Helper Cell and Antibody Responses Relative to CTLA4-Ig. Am J Transplant. 2017. Epub 2017/06/22. doi: 10.1111/ajt.14400. PubMed PMID: 28637095.

210. Ville S, Poirier N, Branchereau J, Charpy V, Pengam S, Nerriere-Daguin V, Le Bas-Bernardet S, Coulon F, Mary C, Chenouard A, Hervouet J, Minault D, Nedellec S, Renaudin K, Vanhove B, Blancho G. Anti-CD28 Antibody and Belatacept Exert Differential Effects on Mechanisms of Renal Allograft Rejection. J Am Soc Nephrol. 2016. doi: 10.1681/ASN.2015070774. PubMed PMID: 27160407.

211. Yan L, de Leur K, Hendriks RW, van der Laan LJW, Shi Y, Wang L, Baan CC. T Follicular Helper Cells As a New Target for Immunosuppressive Therapies. Front Immunol. 2017;8:1510. Epub 2017/11/23. doi: 10.3389/fimmu.2017.01510. PubMed PMID: 29163552; PMCID: PMC5681999.

212. Shi J, Luo F, Shi Q, Xu X, He X, Xia Y. Increased circulating follicular helper T cells with decreased programmed death-1 in chronic renal allograft rejection. BMC Nephrol. 2015;16:182. Epub 2015/11/04. doi: 10.1186/s12882-015-0172-8. PubMed PMID: 26525294; PMCID: PMC4630917.

213. Chen W, Bai J, Huang H, Bi L, Kong X, Gao Y, Han Y, Xiao L, Shi B. Low proportion of follicular regulatory T cell in renal transplant patients with chronic antibody-mediated rejection. Sci Rep. 2017;7(1):1322. Epub 2017/05/04. doi: 10.1038/s41598-017-01625-3. PubMed PMID: 28465534; PMCID: PMC5431051.

214. de Graav GN, Dieterich M, Hesselink DA, Boer K, Clahsen-van Groningen MC, Kraaijeveld R, Litjens NH, Bouamar R, Vanderlocht J, Tilanus M, Houba I, Boonstra A, Roelen DL, Claas FH, Betjes MG, Weimar W, Baan CC. Follicular T helper cells and humoral reactivity in kidney transplant patients. Clin

Exp Immunol. 2015;180(2):329-40. Epub 2015/01/06. doi: 10.1111/cei.12576. PubMed PMID: 25557528; PMCID: PMC4408167.

215. Baan CC, de Graav GN, Boer K. T Follicular Helper Cells in Transplantation: The Target to Attenuate Antibody-Mediated Allogeneic Responses? Curr Transplant Rep. 2014;1(3):166-72. Epub 2014/01/01. doi: 10.1007/s40472-014-0019-4. PubMed PMID: 25927019; PMCID: PMC4405228.

Chapter 2:

Selective CD28 blockade results in superior inhibition of donor-specific T follicular helper cell and antibody responses relative to CTLA-4-Ig

Abstract

Donor-specific antibodies (DSA) are a barrier to improved long-term outcomes following kidney transplantation. Costimulation blockade with CTLA-4-Ig has shown promise as a potential therapeutic strategy to control DSA. T follicular helper (Tfh) cells, a subset of $CD4^+$ T cells required for optimum antibody production, are reliant on the CD28 costimulatory pathway. We have previously shown that selective CD28 blockade leads to superior allograft survival through improved control of $CD8^+$ T cells relative to CTLA-4-Ig, but the impact of CD28-specific blockade on $CD4^+$ Tfh cells is unknown. Thus, we identified and characterized donor-reactive Tfh cells in a murine skin transplant model, and then utilized this model to evaluate the impact of selective CD28 blockade with an anti-CD28 domain antibody (dAb) on the donor-specific Tfh-mediated immune response. We observed that the anti-CD28 dAb led to superior inhibition of donor-reactive $CXCR5^+PD\text{-}1^{hi}$ Tfh and $CD95^+GL7^+$ germinal center B cells, and DSA formation as compared to CTLA-4-Ig. Interestingly, donor-reactive Tfh cells differentially upregulated CTLA-4 expression, suggesting a key role for CTLA-4 in mediating the superior inhibition observed with the anti-CD28 dAb. Therefore, selective CD28 blockade as a novel approach to control Tfh cell responses and prevent DSA after kidney transplantation warrants further study.

Introduction

Anti-human leukocyte antigen (HLA) donor-specific antibodies (DSA) are an increasingly recognized barrier to improved long-term outcomes following kidney transplantation (1, 2). Both pre-existing and *de novo* DSA portend inferior kidney transplant outcomes (3-6). Currently 14% of the renal transplant waiting list is comprised of highly sensitized (cPRA > 80%) patients (7), and *de novo* DSA rates under calcineurin inhibitor (CNI)-based immunosuppression are estimated to be 20% (6, 8). Despite the large burden of DSA, existing therapeutic options to combat either pre-formed or *de novo* alloantibodies are suboptimal and mostly unproven with limited efficacy (9). Thus, a better understanding of the mechanistic underpinnings of DSA formation and persistence is needed to guide the development of novel strategies to control DSA and improve outcomes.

CTLA-4-Ig in the form of belatacept, a first in class costimulation blocker FDA approved in 2011 for maintenance immunosuppression following kidney transplantation, offers a new therapeutic option to improve long-term outcomes (10). Seven year results from a phase III study showed that patient and graft survivals were significantly higher with belatacept than with a CNI-based regimen. The reasons underlying improved outcomes with belatacept are likely multifactorial and include less nephrotoxicity and metabolic toxicity (11, 12), but improved prevention of *de novo* DSA may be a contributing factor (2, 5). Lower rates of DSA were observed with belatacept as compared to CNI (10, 13), but the ultimate effect of belatacept on DSA is not known, nor is the effect on highly sensitized recipients with or without pre-existing DSA (14). Moreover, preliminary data from an ongoing clinical trial designed to

evaluate the ability of belatacept monotherapy to prevent DSA formation in kidney transplant recipients with failed allografts indicate that belatacept alone may not be sufficient to completely prevent DSA in this setting (unpublished data, IR Badell). Therefore, optimization of current costimulation blockade (i.e. CTLA-4-Ig) as a tool to treat anti-HLA antibodies remains an important goal.

Long-standing experimental evidence suggests that costimulation blockade of the CD28 pathway with CTLA-4-Ig in mice and primates is an effective means of preventing alloantibody formation (15, 16), but the underlying mechanisms responsible for this observation are not known. Tfh cells are a newly defined $CD4^+$ T cell subset required for mature, high affinity antibody responses through the formation of germinal centers and provision of optimum B cell help (17). Tfh cells are distinguishable by their unique expression of CXCR5, high levels of PD-1, and the transcription factor Bcl6. This lineage of $CD4^+$ T cells has been largely defined in the setting of vaccine responses, pathogen infections and autoimmunity (18), but its role in allograft rejection and alloantibody responses following transplantation has been largely unexplored (19, 20).

We and others have previously reported that selective blockade of the CD28 pathway leads to improved allograft survival compared to CTLA-4-Ig in preclinical murine and nonhuman primate (NHP) transplant models (21-23). Several mechanisms to explain this survival benefit have been put forward. Poirier et al. have postulated that the survival benefit of selective CD28 blockade is a result of improved CTLA-4-dependent, Treg-mediated immune regulation (21, 24), while we

have reported that anti-CD28 treatment with a selective CD28 domain antibody (dAb) mediates CTLA-4 dependent upregulation of the 2B4 coinhibitor that leads to improved control of graft-reactive $CD8^+$ T cells (22). However, the impact of selective CD28 blockade on the Tfh-mediated humoral response and DSA production has not been carefully examined in an *in vivo* preclinical transplant model, and whether selective blockade can differentially impact this arm of the alloimmune response compared to CTLA-4-Ig is not known.

In this study, we identified and characterized both TCR transgenic and endogenous, polyclonal donor-reactive Tfh cells in our established mOVA murine skin transplant model. We observed that selective CD28 blockade resulted in superior inhibition of the Tfh cell response as compared to CTLA-4-Ig, and found that donor-specific Tfh cells differentially upregulated the coinhibitor CTLA-4 relative to activated, non-Tfh $CD4^+$ T cells, as well as in response to a mismatched donor graft. Selective CD28 blockade also resulted in better control of GC B cell responses and prevention of *de novo* DSA formation relative to CTLA-4-Ig. These findings support the development of next generation costimulation blockade in the form of selective CD28 blockers that spare the inhibitory and regulatory functions of CTLA-4 as a more potent immunosuppressive strategy to combat DSA in clinical transplantation to improve long-term kidney transplant outcomes.

Materials and Methods

Mice

C57BL/6 mice were obtained from the National Cancer Institute and ovalbumin (OVA)-specific TCR transgenic OT-I (25) and OT-II (26) mice purchased from Taconic Farms were bred to Thy1.1$^+$ background at Emory University. C57BL/6 mice that constitutively express membrane-bound OVA under the beta-actin promoter (mOVA mice) (27) were a gift from M. Jenkins (University of Minnesota). All animals were housed in pathogen-free facilities and maintained in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

T cell adoptive transfers, skin transplantation and immunosuppression

For adoptive transfers of OVA-specific T cells, spleen and mesenteric lymph nodes isolated from Thy1.1$^+$ OT-I and Thy1.1$^+$ OT-II mice were stained with mAbs for CD4, CD8, Thy1.1, and Vα2 for quantification by TruCount bead analysis (BD Biosciences). In order to mimic the higher precursor frequencies of alloreactive cells that are observed in fully MHC mismatched models (28), 1.0 x 10^6 of each Thy1.1$^+$ OT-I and Thy1.1$^+$ OT-II T cells were resuspended in PBS and injected intravenously into naïve C57BL/6 mice. 1-2 days after the adoptive transfers, full thickness tail and ear skin grafts were transplanted onto the dorsal thorax of recipient mice (29). Skin graft recipients then received no treatment, human CTLA-4-Ig (200 μg, Bristol-Myers Squibb), or anti-CD28 dAb (100 μg, Bristol-Myers Squibb). CTLA-4-Ig and anti-CD28 dAb were administered i.p. on post-transplant days 0, 2, 4, 6 and then weekly thereafter until sacrifice. Reagent dosing was based on molecular weight, serum half-life and murine mixed lymphocyte reaction EC_{50} (Table 1).

Flow cytometry

Graft-draining axillary and brachial lymph nodes were processed into single cell suspensions. Cells were surface stained for CD3, CD4, CD19, CD44, CXCR5, PD-1, ICOS, Thy1.1, IgD, CD95 and GL-7, and pulsed with LIVE/DEAD viability dye (Molecular Probes) prior to fixation. CXCR5 staining was performed by one-step and three-step (30) techniques. Intracellular staining for Bcl6, CTLA-4 and Foxp3 was performed using the Foxp3 Fixation/Permeabilization Buffer Kit (eBioscience). All antibodies were purchased from BioLegend and BD Biosciences. All samples were run on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo Software (Tree Star). CountBright Beads (Invitrogen) were used to determine absolute cell counts.

ELISA Anti-OVA antibody assessment

Flat-bottom 96 well Immulon microtiter plates (VWR) were coated with OVA protein (100µg/well, Sigma-Aldrich) in 0.05M carbonate-bicarbonate buffer overnight at 4°C. Plates were then blocked with 10% FBS in PBS-T for 1 hour at 37°C and then incubated with serum samples for 1.5 hours at 37°C. OVA-specific immunoglobulins were detected with biotinylated rat anti-mouse IgG_{2a} (RMG2a-62, BioLegend), and then incubated with streptavidin-HRP and developed using the TMB substrate system (Thermo Scientific). Reactions were stopped with H_2SO_4 and read at 450nm on a Spectra MAX 340PC Microplate reader (Molecular Devices).

Statistical Analysis

The Mann–Whitney U nonparametric t test was performed for analysis of unpaired groups, and the Holms Sidak method was used for grouped, multiple t test analyses. All analyses were performed using GraphPad Prism (GraphPad Software, Inc.). Statistical significance was attributed to p values < 0.05 (* < 0.05, ** < 0.01, *** < 0.001).

Results

TCR transgenic and endogenous, polyclonal donor-specific Tfh cells are detectable and expand following skin transplantation

Although previously identified (31), Tfh cell responses following transplantation have not been thoroughly characterized. Therefore, to interrogate the Tfh cell response to a transplant, we utilized our established TCR transgenic mOVA murine skin graft model (28) to identify donor-specific TCR transgenic Tfh cells. Thy1.1^{+} CD4^{+} OT-II and CD8^{+} OT-I T cells were adoptively transferred into naïve B6 mice that were then transplanted with skin from either syngeneic (B6) or minor antigen mismatched (mOVA) donors (Figure 1A). Mice were sacrificed 10 days post-transplant for flow cytometric draining lymph node (DLN) analysis. Adoptively transferred Thy1.1^{+} OVA-specific CD4^{+} T cells expanded in response to the mOVA graft but not the B6 isograft (Figures 1B, C). Examination of these OVA-specific, congenically labeled Thy1.1^{+} OT-II T cells by the canonical Tfh phenotypic markers CXCR5 and PD-1 (17) revealed robust expansion of transgenic donor-specific Tfh cells in response to an mOVA skin graft (Figures 1D, G). The identity of these CXCR5^{+}PD-1^{hi} T cells as bona fide Tfh cells was confirmed by their expression of the lineage-defining transcription factor Bcl6 (Figures 1D, F). Endogenous, polyclonal donor-reactive Tfh cells were also readily identifiable in response to an mOVA graft. Analysis of the activated, non-transgenic CD44^{+}Thy1.1^{-} T cell compartment showed robust expansion of CXCR5^{+}PD-1^{hi} Bcl6^{+} Tfh cells following an mOVA skin graft as compared to a syngeneic B6 graft (Figures 1E-G).

Further analysis of the TCR transgenic and endogenous donor-specific Tfh cell subsets revealed that a fraction of both express GL7 (Figures 2A, C), an activation marker that distinguishes less polarized $GL7^-$ Tfh cells from more polarized $GL7^+$ germinal center (GC) Tfh cells (32). $GL7^+$ GC Tfh cells expressed the highest levels of CXCR5, PD-1, Bcl6 and ICOS (Figures 2B, D), consistent with their reported more mature differentiation status and enhanced ability to provide B cell help (17). Thus, transgenic and endogenous, polyclonal donor-specific Tfh cells are detectable and accumulate in response to a skin graft, manifesting the characteristics typical of the multistage differentiation process Tfh cells undergo for GC formation and the provision of B cell help.

Selective CD28 pathway blockade results in superior inhibition of donor-reactive Tfh cells

Based on previous work from our group and others showing that selective CD28 blockade leads to improved graft survival and has a differential impact on certain T cell subsets compared to CTLA-4-Ig (22, 24), we sought to test the impact of a selective anti-CD28 dAb on Tfh cells in our murine mOVA skin transplant model. After adoptive transfer of $Thy1.1^+$ OT-I and OT-II T cells, naïve B6 recipient mice were transplanted mOVA skin grafts and left untreated, or treated with either CTLA-4-Ig or the anti-CD28 dAb (Figure 3A). All mice were sacrificed 10 days post-transplant for DLN analysis. Consistent with our prior observation, OVA-specific $Thy1.1^+$ $CD4^+$ T cells were significantly reduced in animals treated with the anti-CD28 dAb as compared to CTLA-4-Ig (Figures 3B, C). Further examination of the $Thy1.1^+$ compartment for donor-specific $Bcl6^+PD\text{-}1^{hi}$ Tfh cells revealed similar differential inhibition between treatment groups (Figure 3D). Both the frequency and number of donor-specific $Bcl6^+PD\text{-}1^{hi}$ Tfh cells were more greatly

inhibited by the selective CD28 dAb as compared to CTLA-4-Ig (Figure 3E). This improved inhibition of TCR transgenic Tfh cell accumulation with the anti-CD28 dAb was also observed within the endogenous, polyclonal Bcl6^{+}PD-1^{hi} Tfh cell response to the mOVA graft (Figure 3F). The accumulation of donor-specific Thy1.1^{-} Bcl6^{+}PD-1^{hi} Tfh cells as measured by frequency and number was more significantly inhibited by selective CD28 pathway blockade as compared to CTLA-4-Ig (Figure 3G). Hence, selective blockade of the CD28 pathway leads to improved inhibition of both TCR transgenic and endogenous, polyclonal donor-specific Tfh cell responses to a skin graft.

Donor-specific Tfh cells differentially upregulate CTLA-4

The coinhibitory molecule CTLA-4 has been recently implicated as a mediator of Tfh cell differentiation and function (33-35); however, its role on Tfh cells in transplantation is unknown. To begin to investigate whether intact CTLA-4 on donor-reactive Tfh cells could be a mechanism underlying the improved inhibition of Tfh cells with selective CD28 blockade, we examined the level of expression of CTLA-4 on Tfh cells in response to a donor skin graft. Mice were adoptively transferred Thy1.1^{+} OT-I and OT-II cells, transplanted mOVA skin, and sacrificed 10 days post-transplant for DLN analysis (Figure 1A). We observed that both TCR transgenic and endogenous, polyclonal CXCR5^{+}PD-1^{hi} donor-specific Tfh cells expressed greater amounts of CTLA-4 relative to naïve (CD44^{-}) and antigen-experienced (CD44^{+}), CXCR5^{-}PD-1^{-} and CXCR5^{-}PD-1^{+} non-Tfh CD4^{+} T cells (Figures 4A, C). The Tfh compartment contained the largest frequency of CTLA-4^{+} cells and exhibited the highest levels of CTLA-4, CXCR5 and PD-1 expression (Figures 4B, D). Furthermore, within the Tfh cell compartment, the more polarized GL7^{+} GC Tfh cells

expressed the highest levels of CTLA-4 as compared to $GL7^-$ Tfh cells. Thus Tfh cells exhibit greater CTLA-4 expression than antigen-experienced $CXCR5^-PD\text{-}1^+$ non-Tfh cells.

We next sought to determine whether the increased expression of CTLA-4 following a skin graft parallels the expression of Bcl6, CXCR5 and PD-1 by Tfh cells, and is commensurate with a more activated state in response to recognition of donor antigen. Thus, utilizing our same experimental model (Figure 1A), we evaluated the expression of CTLA-4 on endogenous, $CD44^+Thy1.1^-$ Tfh cells in response to an mOVA skin graft relative to a syngeneic B6 graft (Figure 5A). We found that the frequency of $CTLA\text{-}4^+$ cells and expression levels of CTLA-4, Bcl6, and CXCR5 did not change between $CD44^+$, $CXCR5^-PD\text{-}1^+$ non-Tfh $CD4^+$ T cells following either a B6 or mOVA skin graft (Figures 5B, C). The mOVA graft did induce a small increase in PD-1 expression on these non-Tfh cells. Conversely, graft-reactive $CXCR5^+PD\text{-}1^{hi}$ Tfh cells specifically exhibited a significant increase in the frequency of $CTLA\text{-}4^+$ cells in the mOVA but not the B6 grafted mice, and CTLA-4, Bcl6, CXCR5 and PD-1 expression were greatest following the mOVA graft as compared to the isograft (Figures 5D, E). Taken together, these data indicate that the differential expression of CTLA-4 on Tfh cells following a donor-mismatched graft parallels Bcl6 and CXCR5 expression and is a result of antigen exposure.

Selective CD28 pathway blockade leads to superior inhibition of donor-elicited GC B cells

The principle role of Tfh cells is to provide help to cognate B cells for the formation of GCs to generate mature antibody responses (17). Therefore we analyzed DLNs 10 days after skin grafting for GC B cell accumulation as a measure of the magnitude of the T cell dependent B cell

response and GC reaction. Utilizing the same murine skin transplant model (Figure 1A), naïve B6 mice were adoptively transferred OT-I and OT-II T cells, transplanted with syngeneic B6 or mOVA skin grafts and sacrificed 10 days post-transplant for DLN analysis. The frequency and number of $CD19^{+}IgD^{-}$ class-switched B cells were significantly increased following the mOVA skin graft (Figures 6A, B). GC B cells were identified as the $CD95^{+}GL7^{+}$ fraction of $CD19^{+}IgD^{-}$ B cells (Figure 6C) (36). Donor-elicited GC B cell frequency and number were significantly increased following an mOVA skin graft as compared to the accumulation of non-specific GC B cells in response to a B6 isograft (Figure 6D). GC B cell analysis of mice treated with CTLA-4-Ig or anti-CD28 dAb (Figure 3A) revealed that both agents significantly inhibited donor-elicited GC B cell accumulation as compared to untreated mice following an mOVA skin graft (Figure 6E); however, selective CD28 blockade led to significantly greater inhibition of donor-elicited GC B cells when compared to CTLA-4-Ig (Figure 6F). Thus, selective CD28 pathway blockade induces better control of not only donor-specific Tfh cell responses, but also the cognate, graft-elicited GC B cell response when compared to non-selective blockade with CTLA-4-Ig.

Selective CD28 blockade results in superior inhibition of DSA formation

GCs result from cognate Tfh and GC B cell interactions to mount mature, high affinity antibodies directed against specific antigenic challenges (17). Because selective CD28 pathway blockade led to superior inhibition of donor-reactive Tfh and GC B cell responses compared to CTLA-4-Ig, we performed serial serum collections of transplanted mice for DSA measurements. After adoptive transfer of TCR transgenic cells and syngeneic or mOVA skin grafting, mice were left untreated or treated with either CTLA-4-Ig or anti-CD28 dAb (Figure 7A). Serum from mice in all

treatment groups was measured via ELISA for anti-OVA IgG levels after transplant. DSA production was observed in the untreated mOVA-grafted mice while no anti-OVA antibodies were detected in the isograft recipients (Figure 7B). CTLA-4-Ig treated mice exhibited delayed but significant DSA formation relative to untreated and syngeneic controls, while anti-CD28 dAb-treated mice experienced no detectable DSA formation. Serum DSA titers 21 days post-transplant at the peak of the anti-OVA IgG response corroborated our finding that the selective CD28 blocker provides superior inhibition of DSA as compared to CTLA-4-Ig (Figure 7C). This differential inhibition of DSA was sustained beyond 56 days post-transplant (Figure 7D). Accordingly, better prevention of DSA formation by the anti-CD28 dAb compared to CTLA-4-Ig is consistent with the overall improved inhibition of Tfh-mediated responses observed with selective CD28 pathway blockade following transplantation.

Discussion

As knowledge of Tfh cell biology continues to rapidly grow, the role of this subset in mediating T cell dependent antibody responses (TDARs) becomes increasingly relevant to the field of transplantation (19, 20). Anti-HLA DSA arising from alloantigen-specific cognate Tfh:GC B cell interactions for the production of high affinity alloantibodies, and alloreactive memory B cells and long-lived plasma cells via the GC reaction, are now a well-recognized immunologic barrier to improved long-term outcomes in kidney transplantation as mediators of antibody mediated rejection (AMR) (1, 5, 6, 8). Current available therapies aimed at humoral allosensitization target B cells (e.g. rituximab), plasma cells (e.g. bortezomib), or DSA (e.g. plasmapheresis) after alloantibodies have developed with limited efficacy (4, 9, 37, 38), rendering current efforts "too little, too late." Shifting therapeutic focus to utilizing T cell-directed therapies against the initiation of the antibody response by targeting Tfh cells may prove to be the most efficacious and long lasting method of addressing the clinical problem of alloantibodies. Moreover, therapeutic strategies like costimulation blockade of the CD28 or CD40 pathway that are mechanistically appealing means of targeting Tfh cells have been translated and are clinically available (e.g. CTLA-4-Ig) (10), or are in the development pipeline (e.g. anti-CD28 dAb, anti-CD40 mAbs) (39) for potential translation to combat DSA.

Several transplant studies to date have reported on the favorable immunosuppressive impact of costimulatory blockade on the Tfh-GC axis responsible for anti-donor antibody formation. In a mouse model of chronic graft versus host disease (cGVHD) caused in part by alloantibody secretion, increased Tfh and GC B cells were required for cGVHD, and IL-21, ICOS and CD40

blockade all hindered GC formation and alloantibody-mediated cGVHD (40). Similarly, in a NHP kidney transplant model of AMR, costimulation blockade with belatacept or an anti-CD40 mAb prevented *de novo* DSA and was associated with reduced GC Tfh cells and GC reactivity as indicated by IL-21 production (41). Moreover, CTLA-4-Ig has been effective at controlling *de novo* DSA responses in mice (42), and delayed use of anti-CD154 and/or CTLA-4-Ig has been shown to dissolve nascent GCs, halt further development of alloantibody responses, established allo-B cell and memory B cell responses, and inhibit ongoing B cell responses in murine cardiac allograft models (43-45). Overall there is growing evidence that pursuing costimulation blockade strategies is a viable and promising method to combat humoral alloimmunity and the DSA burden in transplantation.

CD28 deficient mice exhibit significantly impaired immunoglobulin concentrations and class switching, and do not undergo the proliferative expansion necessary for GC formation (46, 47). As a result, CD28 is required for TDARs and the CD28 deficient phenotype is presumed to be due to a lack of Tfh cells. In contrast, CTLA-4 deficient mice exhibit lethal lymphoproliferative disease characterized by hypergammaglobulinemia (48, 49), and more recent data from the Sharpe and Sakaguchi labs have shown that CTLA-4 critically controls multiple aspects of the GC reaction (33, 35). In these studies, deletion of CTLA-4 on Tfh cells led to enhanced B cell class-switch recombination *in vitro*, and reduction of CTLA-4 on T regulatory and T follicular regulatory cells led to augmented humoral responses and defective suppression of germinal center development. Therefore, existing data on the influence of CD28 costimulation and CTLA-4 coinhibition on TDARs provide strong rationale for the use of selective CD28 blockade to tilt

the net balance of stimulatory and inhibitory signaling in favor of improved control of Tfh-mediated DSA production.

As previously mentioned, Treg and $CD8^+$ T cells have both been shown to exhibit differential responses to selective CD28 blockade versus CTLA-4-Ig as possible mechanisms responsible for the improved immunosuppressive effect and graft prolongation of selective CD28 blockade (22, 24). Ville et al recently tested an anti-CD28 antibody in a NHP model of kidney transplantation and showed less incidence of acute rejection as compared to belatacept (23). In exploring the mechanisms responsible for this observation, the authors identified an insignificant trend of higher Treg frequencies in anti-CD28-treated animals, and increased levels of the Tfh-associated cytokine IL-21 in belatacept-treated animals. The anti-CD28 antibody also resulted in better *in vitro* control of Tfh cell proliferation and improved control of secondary antibody responses to KLH antigen immunization in mice. These data circumstantially suggest that Tfh cells may in part mediate the improved results they observed with selective CD28 blockade. To directly determine the impact of selective CD28 blockade on the Tfh cell response and development of DSA, we utilized an *in vivo* preclinical transplant model in this study.

We show that selective CD28 blockade in a murine transplant model leads to greater inhibition of donor-reactive Tfh cells, GC B cells, and anti-donor antibody responses relative to CTLA-4-Ig. The use of TCR transgenic systems to facilitate examination of antigen-specific cells has met skepticism as a surrogate for natural, unfettered endogenous alloimmune responses. Model antigen T cell transgenic systems have been criticized to be non-representative of endogenous,

polyclonal responses and to alter aspects of endogenous alloimmunity (50). Here, we utilized an mOVA skin graft model to afford us the ability to examine the true donor-specific Tfh cell response by tracking OVA-specific TCR transgenic T cells, as well as the non-transgenic, OVA-reactive endogenous Tfh cell response. In this system, both transgenic and endogenous Tfh cells manifested the same phenotypic and differentiation characteristics in response to a donor skin graft (Figures 1, 2, 4), and were equally inhibited by CD28 pathway blockade with either CTLA-4-Ig or anti-CD28 dAb (Figure 3). While we expect the introduction of TCR transgenic T cells to impact the immune response by altering donor-reactive precursor frequency and the balance of competition for antigen between transgenic and endogenous cells, our results indicate that both populations of donor-reactive Tfh cells manifest characteristics consistent with the literature and respond equally to the immunosuppressive reagents tested here.

CTLA-4-Ig is known to be less effective in more immunologically stringent mouse and NHP models (29, 51, 52), and has been associated with higher rates of acute rejection after kidney transplantation in humans (53). The exact mechanisms mediating this CTLA-4-Ig-resistance have not been entirely elucidated, but ironically the mechanism of action of CTLA-4-Ig may underlie this reduced efficacy (54). Because CTLA-4-Ig binds to CD80/86 on APCs and blocks their engagement with both CD28 and CTLA-4, it is likely that blocking CTLA-4 may detract from the overall goal of attenuating alloreactive T cell responses, in that CTLA-4 is inhibitory and activates regulatory T cells to abrogate immune responses (48, 49, 55). In support of this hypothesis, we previously demonstrated that improved inhibition of donor-reactive $CD8^+$ T cells with selective CD28 blockade is CTLA-4 dependent (22). Our current observation that CTLA-4 is

differentially upregulated on donor-reactive Tfh cells in response to a skin graft (Figures 4, 5) strongly suggests that CTLA-4 also plays an important inhibitory or regulatory role in Tfh cell-mediated DSA responses, and that the superior anti-CD28 dAb-mediated inhibition of the donor-reactive Tfh cell response may be CTLA-4-dependent. We postulate that blockade of CTLA-mediated inhibition of Tfh cells underlies the inability of CTLA-4-Ig to completely prevent DSA formation in both humans (10, 13) (unpublished data, IR Badell) and our experimental model. However, whether the observed impact of CD28-specific antagonism on the Tfh cell response and DSA is CTLA-4-dependent remains to be determined and is currently under investigation in our laboratory.

While we found that selective CD28 blockade preferentially inhibited the donor-reactive Tfh cell response relative to CTLA-4-Ig in this study, the anti-CD28 dAb was systemically delivered and free to interact with CD28 on a variety of immune cell types with a multitude of antigen specificities. Considering the ubiquitous roles of CD28 and CTLA-4, it is possible that the observed superior anti-humoral effect of the anti-CD28 dAb is not exclusive to Tfh cells. In fact, the early extrafollicular antibody response is known to be GC-independent (56, 57) and has been shown to also depend on $CD4^+$ T cells (58, 59). Thus improved inhibition of DSA with selective CD28 blockade may not be solely secondary to Tfh-dependent antibody production immediately after transplant. Moreover, because CD28 costimulation blockade has been clinically associated with concerns regarding virus-specific immunity (14, 60), the impact of more potent selective CD28 blockade on pathogen-specific T cells warrants close attention. Overall, the direct effect of the anti-CD28 dAb on protective immune responses and whether

our findings directly result from an intrinsic effect on the $CD4^+$ Tfh cell lineage remain to be studied.

In summary, this study introduces Tfh cells and the Tfh-mediated response as additional components of the anti-donor adaptive immune response that experience superior inhibition with the use of selective CD28 costimulation blockade as compared to CTLA-4-Ig in transplantation. Our data highlight that targeting CD28 costimulation while leaving the coinhibitor CTLA-4 free to engage the B7 ligands results in better inhibition of Tfh cells, GC B cells and DSA in response to a donor skin graft, and that the expression of CTLA-4 is differentially elevated on the Tfh cell lineage, suggesting a CTLA-4-dependent, Tfh cell intrinsic mechanism of improved inhibition with selective CD28 blockade that warrants further study. These findings, along with continued work clarifying the net cell intrinsic influence of costimulatory and coinhibitory signaling on Tfh cell differentiation and function, and Tfh-dependent antibody production promises to guide therapeutic strategies aimed at controlling deleterious anti-HLA antibodies and improving long-term outcomes in kidney transplantation.

Figures and Figure Legends

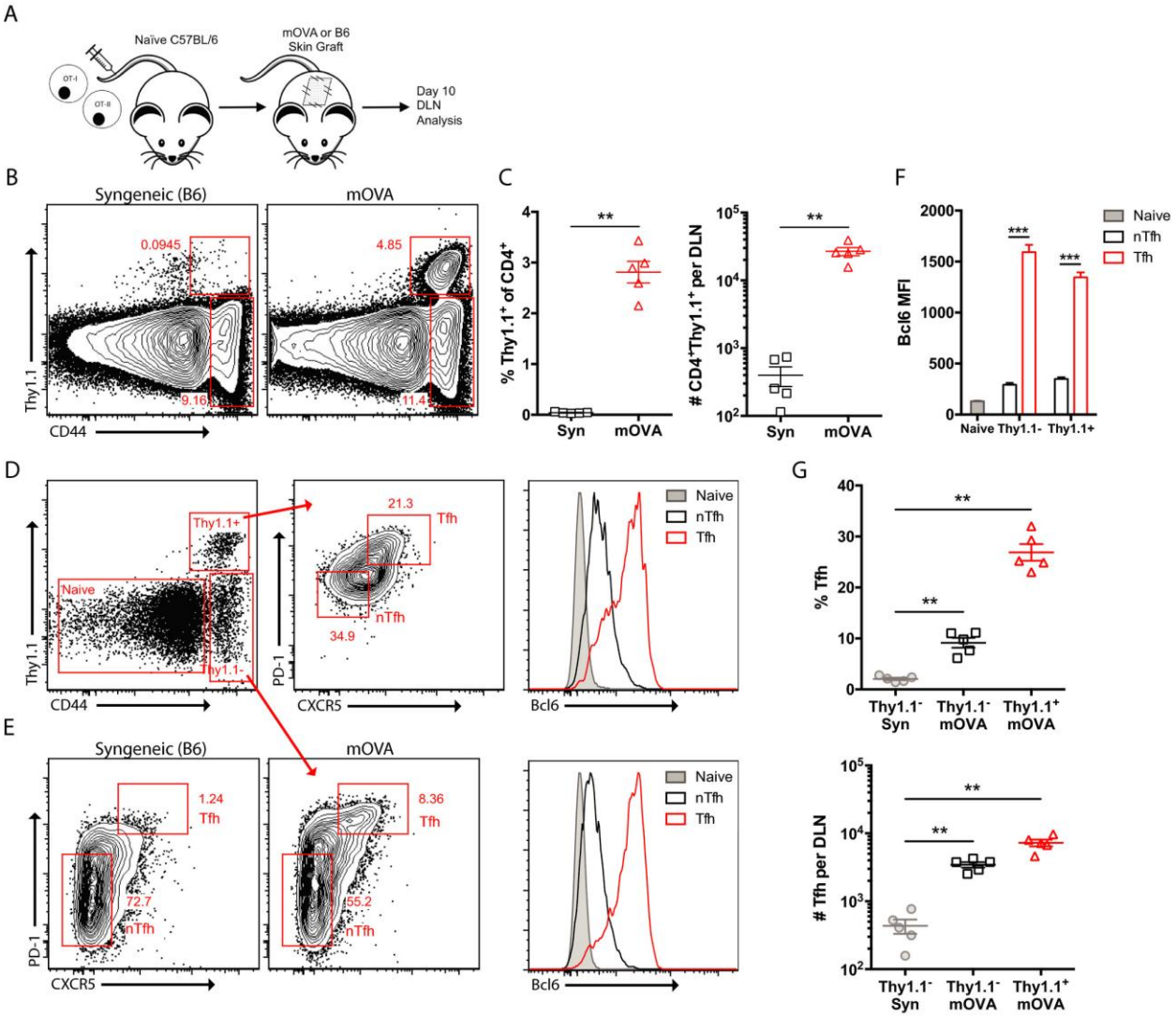


Figure 1. CXCR5^{+}PD-1^{hi} Bcl6^{+} donor-specific Tfh cells expand following transplantation.

A: Naïve B6 mice were adoptively transferred 10^{6} of each Thy1.1^{+} CD4^{+} OT-II and CD8^{+} OT-I T cells, transplanted skin from B6 or mOVA donors, and sacrificed 10 days post-transplant for DLN analysis. B: Flow cytometry plots (gated on CD4^{+}Foxp3^{-} T cells) displaying the frequencies of donor-specific Thy1.1^{+} and endogenous CD44^{+}Thy1.1^{-} T cells. C: Summary data of the frequencies and numbers of OVA-specific CD4^{+}Thy1.1^{+} T cells (n=5 per group). D: Flow plot (first panel, gated on CD4^{+}Foxp3^{-} T cells) depicts naïve (CD44^{-}Thy1.1^{-}), activated endogenous

(CD44^{+}Thy1.1^{-}), and OVA-specific TCR transgenic (CD44^{+}Thy1.1^{+}) CD4^{+} T cell populations. Flow plot (second panel, gated on CD44^{+}Thy1.1^{+} T cells) displays the frequencies of CXCR5^{+}PD-1^{hi} Tfh and CXCR5^{-}PD-1$^{-/lo}$ non-Tfh (nTfh) donor-specific CD4^{+} T cells. Histogram depicts Bcl6 expression by the designated T cell populations. E: Flow plots (gated on CD44^{+}Thy1.1^{-} T cells) displaying the frequencies of CXCR5^{+}PD-1^{hi} Tfh and CXCR5^{-}PD-1$^{-/lo}$ nTfh within the endogenous, polyclonal Thy1.1^{-} compartment. Histogram depicts Bcl6 expression by the designated T cell populations. F: Summary data of Bcl6 expression on Tfh and nTfh within the Thy1.1^{+} and Thy1.1^{-} T cell compartments (n=5). G: Summary data of the frequencies and numbers of transgenic Thy1.1^{+} Tfh and endogenous, polyclonal Thy1.1^{-} Tfh cells (n=5 per group). Summary data represent mean (SE) and are representative of 4 independent experiments with a total of 15-20 mice per group.

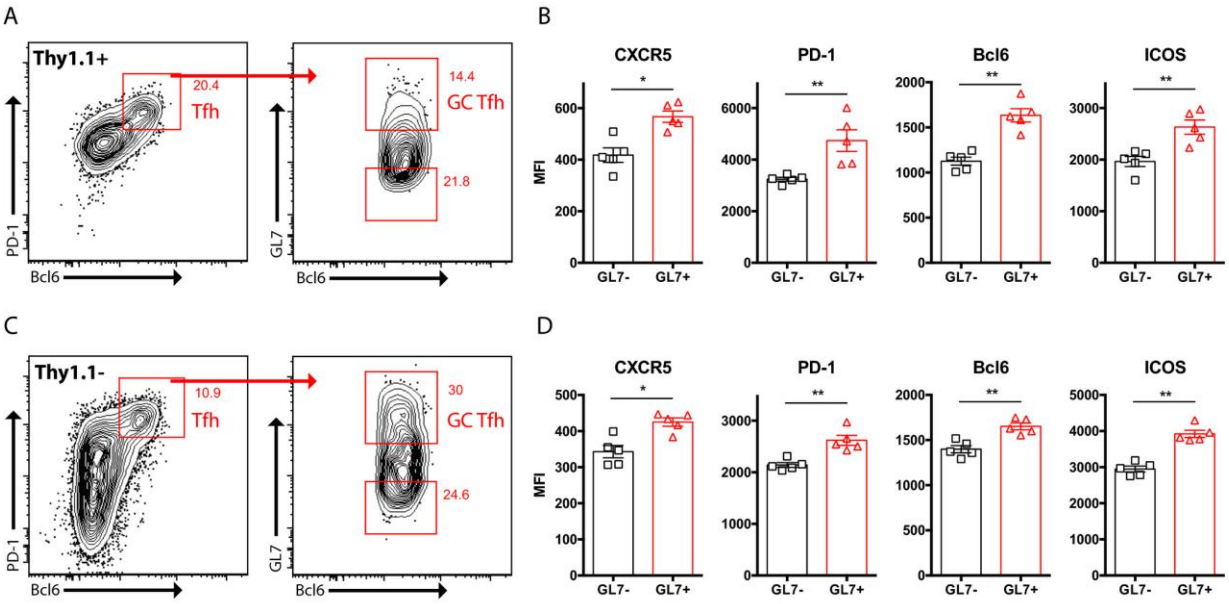


Figure 2. Tfh cells differentiate into more polarized GL-7^{+} GC Tfh cells following transplantation.

A: Flow cytometry plots (gated on CD4^{+}Foxp3^{-}Thy1.1^{+} T cells) display TCR transgenic Bcl6^{+}PD-1^{hi} Tfh cells and GL7^{+} GC Tfh cells. B: Summary data of CXCR5, PD-1, Bcl6 and ICOS expression by GL7^{-} and GL7^{+} Thy1.1^{+} Tfh cells (n=5). C: Flow plots (gated on CD4^{+}Foxp3^{-}CD44^{+}Thy1.1^{-} T cells) display endogenous, polyclonal Bcl6^{+}PD-1^{hi} Tfh cells and GL7^{+} GC Tfh cells. D: Summary data of CXCR5, PD-1, Bcl6 and ICOS expression by GL7^{-} and GL7^{+} Thy1.1^{-} Tfh cells (n=5). Summary data represent mean (SE) and are representative of 4 independent experiments with a total of 15-20 mice.

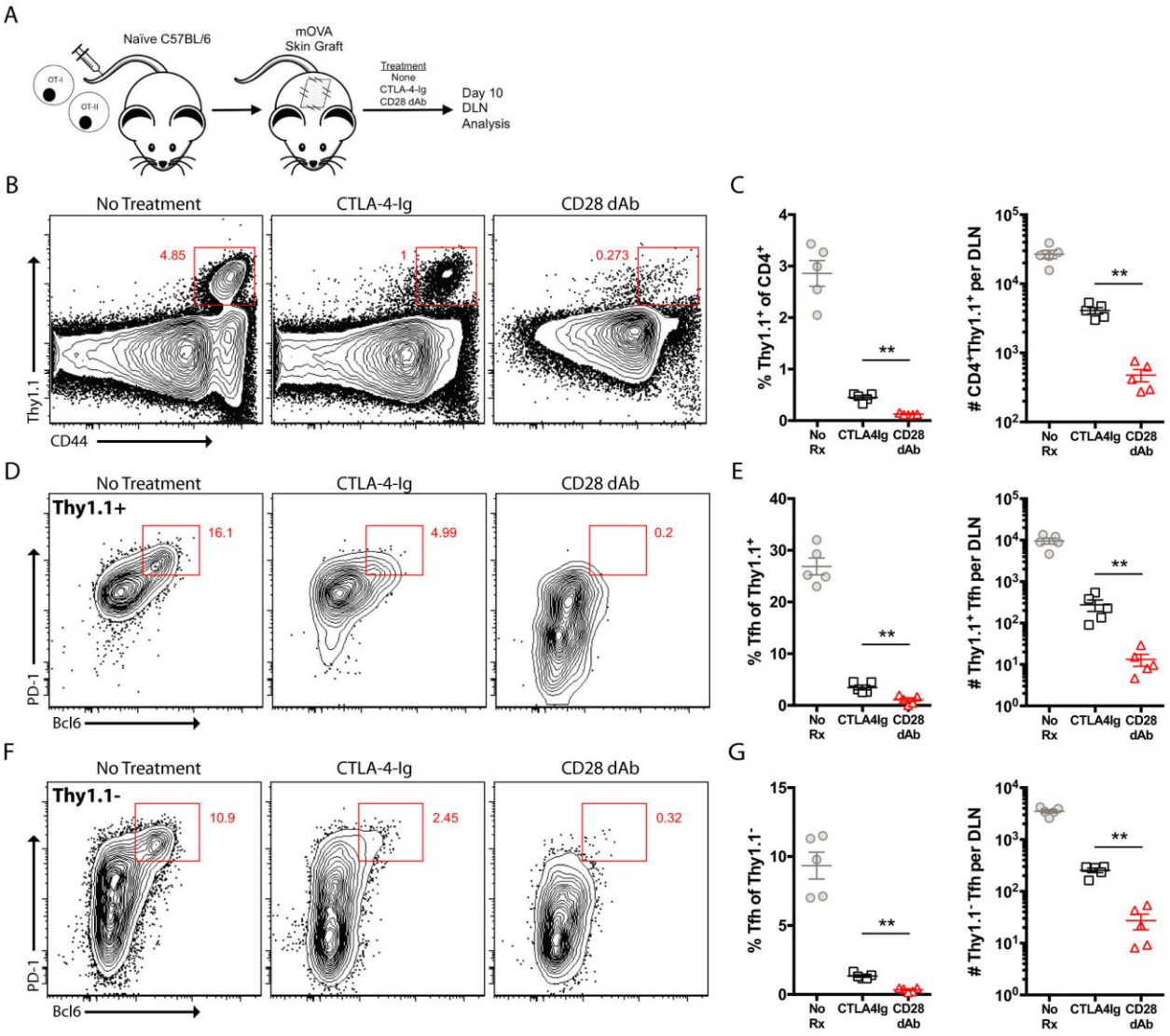


Figure 3. Selective CD28 blockade leads to superior inhibition of donor-reactive Tfh cells following transplantation.

A: Naïve B6 mice were adoptively transferred 10^6 of each Thy1.1$^+$ CD4$^+$ OT-II and CD8$^+$ OT-I T cells, transplanted mOVA skin grafts, left untreated or treated with CTLA-4-Ig or anti-CD28 dAb, and sacrificed 10 days post-transplant for DLN analysis. B: Flow cytometry plots (gated on CD4$^+$Foxp3$^-$ T cells) display the frequencies of donor-specific Thy1.1$^+$ T cells amongst the treatment groups. C: Summary data of the frequencies and numbers of OVA-specific

$CD4^+Thy1.1^+$ T cells (n=5 per group). D: Flow plots (gated on $CD4^+Foxp3^-Thy1.1^+$ T cells) display the frequencies of donor-specific $Thy1.1^+$ $Bcl6^+PD\text{-}1^{hi}$ Tfh cells amongst the treatment groups. E: Summary data of the frequencies and numbers of TCR transgenic Tfh cells (n=5 per group). F: Flow plots (gated on $CD4^+Foxp3^-CD44^+Thy1.1^-$ T cells) display the frequencies of donor-reactive $CD44^+Thy1.1^-$ $Bcl6^+PD\text{-}1^{hi}$ Tfh cells amongst the treatment groups. G: Summary data of the frequencies and numbers of endogenous, polyclonal $CD44^+Thy1.1^-$ Tfh cells (n=5 per group). Summary data represent mean (SE) and are representative of 4 independent experiments with a total of 15-20 mice per group.

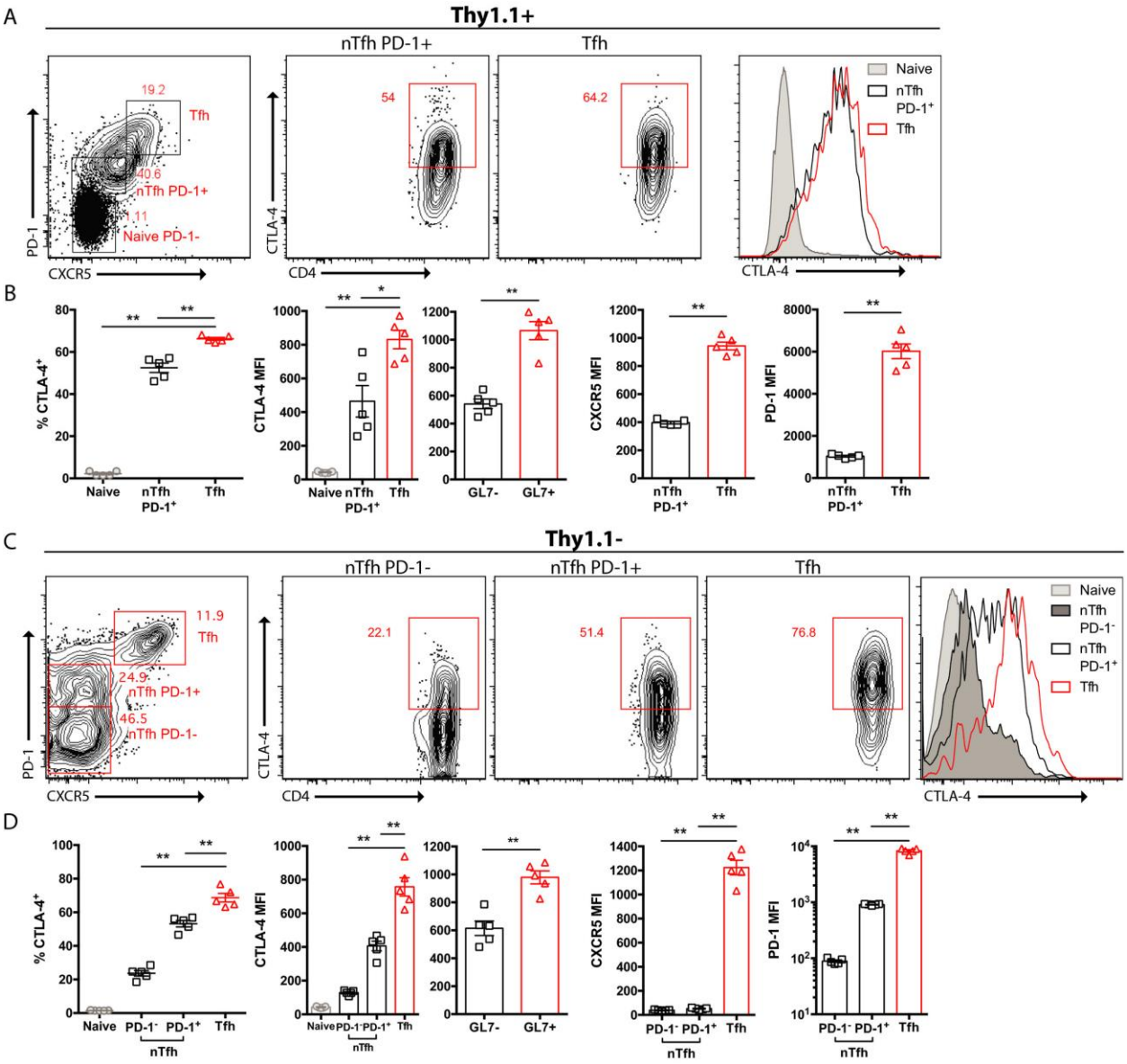


Figure 4. Donor-reactive Tfh cells differentially express high levels of CTLA-4.

Naïve B6 mice were adoptively transferred Thy1.1^{+} OT-I and OT-II cells, transplanted mOVA skin grafts, and sacrificed 10 days after transplant for DLN analysis. A: Flow cytometry plots (gated on CD4^{+}Foxp3^{-}CD44^{+}Thy1.1^{+} T cells) display the frequencies of CTLA-4^{+} Thy1.1^{+} non-Tfh (nTfh PD-1^{+}, CXCR5^{-}PD-1^{+}) and Tfh (CXCR5^{+}PD-1^{hi}) cells. Histogram depicts CTLA-4 expression by the indicated T cell subsets. B: Summary data of the frequencies of CTLA-4^{+} cells and CTLA-4, CXCR5

and PD-1 expression (n=5). C: Flow plots (gated on $CD4^+Foxp3^-CD44^+Thy1.1^-$ T cells) display the frequencies of CTLA-4^+ $CD44^+Thy1.1^-$ nTfh PD-1^- ($CXCR5^-PD\text{-}1^-$), nTfh PD-1^+ ($CXCR5^-PD\text{-}1^+$) and Tfh ($CXCR5^+PD\text{-}1^{hi}$) cells. Histogram depicts CTLA-4 expression by the indicated T cell subsets. D: Summary data of the frequencies of CTLA-4^+ cells and CTLA-4, CXCR5 and PD-1 expression (n=5). Summary data represent mean (SE) and are representative of 4 independent experiments with a total of 15-20 mice.

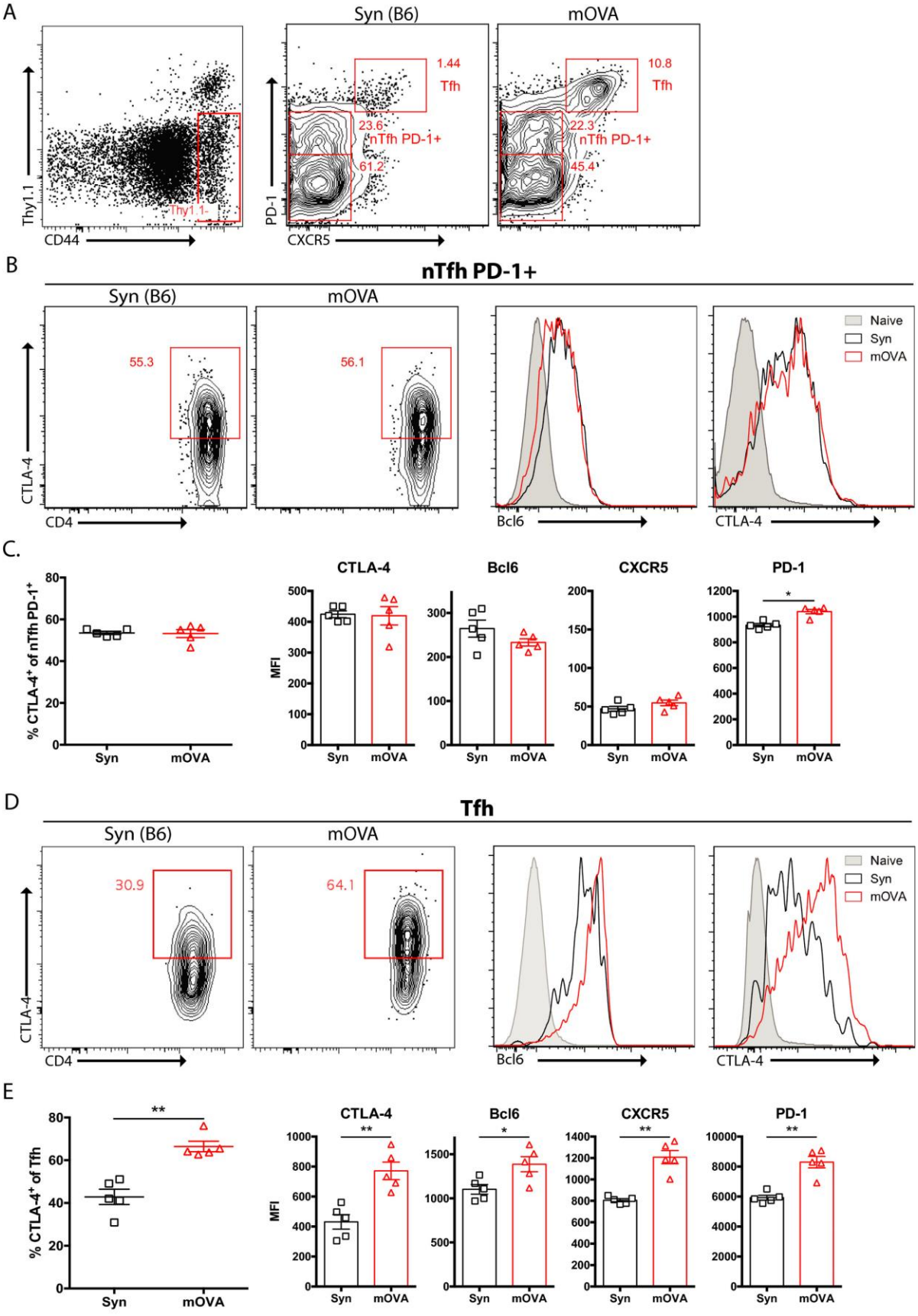

A
Syn (B6)
mOVA
Thy1.1
CD44
Thy1.1-
PD-1
CXCR5
1.44
Tfh
23.6
nTfh PD-1+
61.2
10.8
Tfh
22.3
nTfh PD-1+
45.4
B
nTfh PD-1+
Syn (B6)
mOVA
55.3
56.1
CTLA-4
CD4
Bcl6
CTLA-4
Naive
Syn
mOVA
C.
% CTLA-4+ of nTfh PD-1+
Syn
mOVA
CTLA-4
Bcl6
CXCR5
PD-1
MFI
*
D
Tfh
Syn (B6)
mOVA
30.9
64.1
CTLA-4
CD4
Bcl6
CTLA-4
Naive
Syn
mOVA
E
% CTLA-4+ of Tfh
**
CTLA-4
Bcl6
CXCR5
PD-1
MFI
**
*
**
**
Syn
mOVA

Figure 5. Donor-reactive Tfh cells upregulate CTLA-4 expression in response to donor antigen. Naïve B6 mice were adoptively transferred Thy1.1^{+} OT-I and OT-II cells, transplanted B6 or mOVA skin grafts, and sacrificed 10 days after transplant for DLN analysis. A: Flow cytometry plots (gated on CD4^{+}Foxp3^{-}CD44^{+}Thy1.1^{-} T cells) display the endogenous, polyclonal Tfh (CXCR5^{+}PD-1^{hi}) and non-Tfh (nTfh PD-1^{+}, CXCR5^{-}PD-1^{+}) cell responses to syngeneic and mOVA skin grafts. B: Flow plots (gated on CXCR5^{-}PD-1^{+} nTfh PD-1^{+} cells) display the frequencies of CTLA-4^{+} cells. Histograms depict Bcl6 and CTLA-4 expression on nTfh PD-1^{+} cells. C: Summary data of the frequencies of CTLA-4^{+} nTfh PD-1^{+} cells and their CTLA-4, Bcl6, CXCR5 and PD-1 expression levels (n=5 per group). D: Flow plots (gated on CXCR5^{+}PD-1^{hi} Tfh cells) display the frequencies of CTLA-4^{+} cells. Histograms depict Bcl6 and CTLA-4 expression on Tfh cells. E: Summary data of the frequencies of CTLA-4^{+} Tfh cells and their CTLA-4, Bcl6, CXCR5 and PD-1 expression levels (n=5 per group). Summary data represent mean (SE) and are representative of 4 independent experiments with a total of 15-20 mice per group.

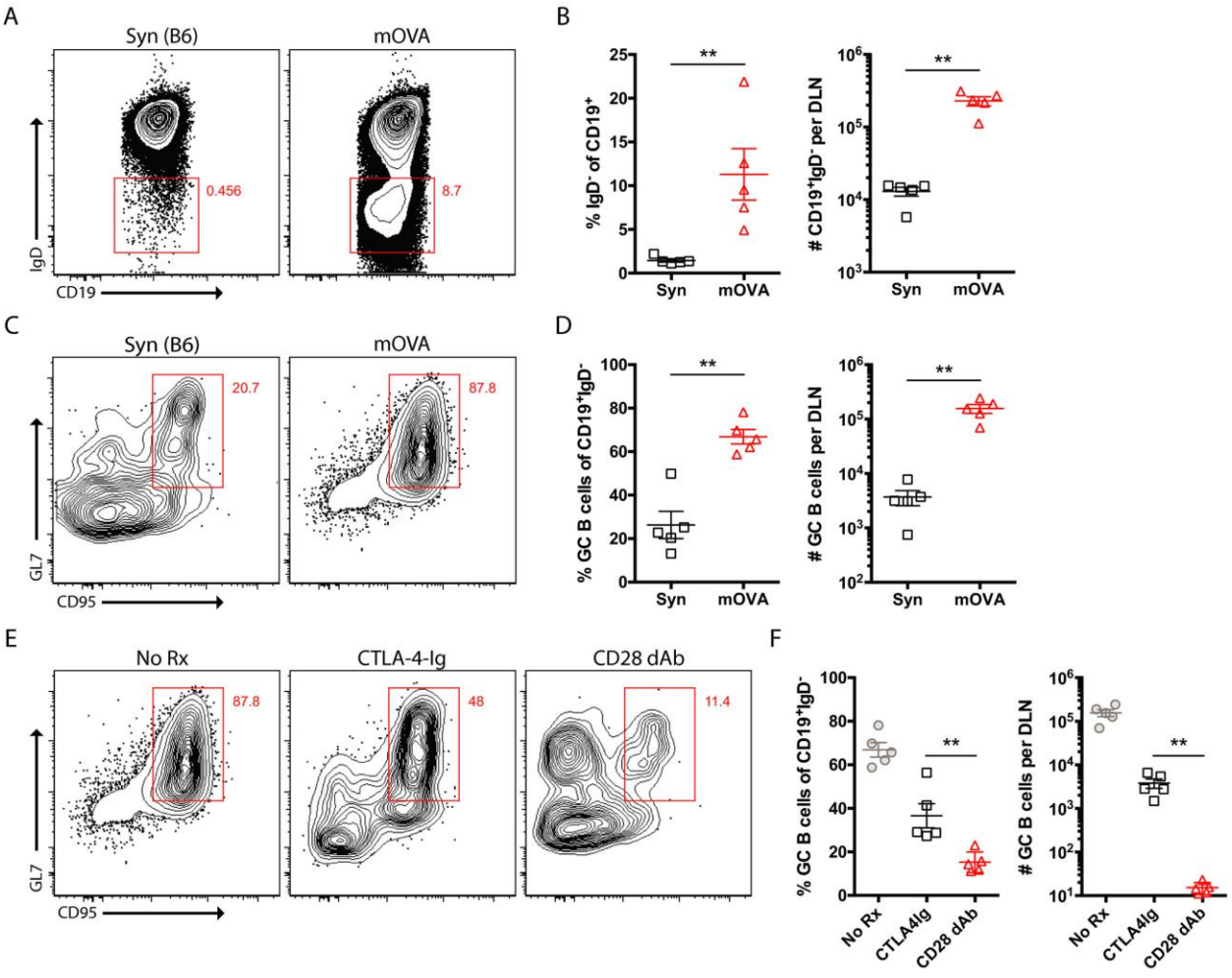
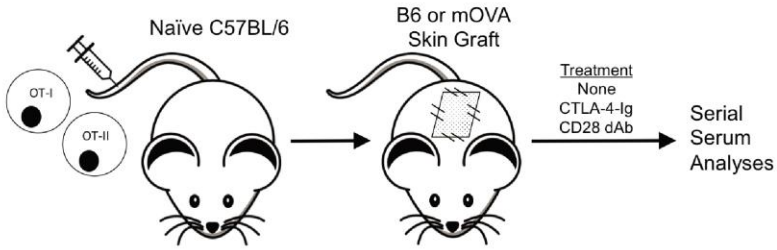


Figure 6. Selective CD28 blockade results in superior inhibition of CD95$^+$GL7$^+$ GC B cells following transplantation.

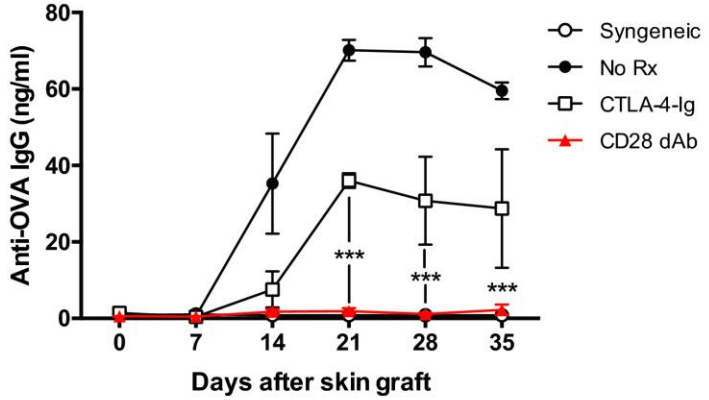
Naïve B6 mice were adoptively transferred Thy1.1$^+$ OT-I and OT-II cells, transplanted B6 or mOVA skin grafts, left untreated or treated with CTLA-4-Ig or anti-CD28 dAb, and sacrificed 10 days after transplant for DLN analysis. A: Flow cytometry plots (gated on CD3$^-$CD19$^+$ lymphocytes) display the frequencies of CD19$^+$IgD$^-$ B cells. B: Summary data of the frequencies and numbers of CD19$^+$IgD$^-$ B cells (n=5 per group). C: Flow plots (gated on CD19$^+$IgD$^-$ B cells) display the frequencies of CD95$^+$GL7$^+$ GC B cells. D: Summary data of the frequencies and numbers of GC B cells (n=5 per group). E: Flow plots (gated on CD19$^+$IgD$^-$ B cells) display the

frequencies of donor-elicited GC B cells amongst the treatment groups. F: Summary data of the frequencies and numbers of GC B cells (n=5 per group). Summary data represent mean (SE) and are representative of 4 independent experiments with a total of 15-20 mice per group.

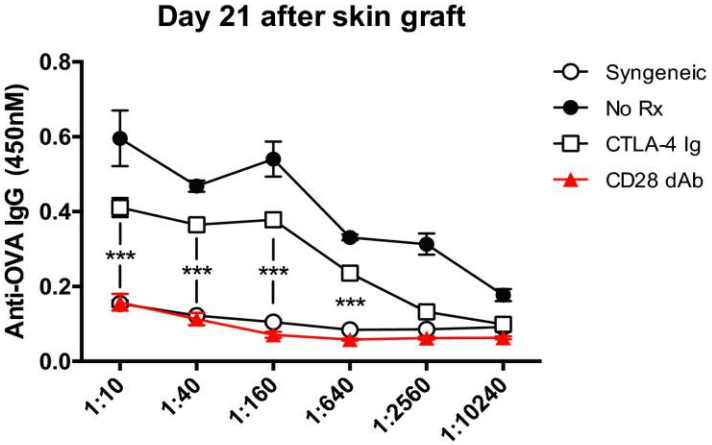
A


Naïve C57BL/6
B6 or mOVA
Skin Graft
OT-I
OT-II
Treatment
None
CTLA-4-Ig
CD28 dAb
Serial
Serum
Analyses

B


Anti-OVA IgG (ng/ml)
80
60
40
20
0
0
7
14
21
28
35
Days after skin graft
***
***
***
Syngeneic
No Rx
CTLA-4-Ig
CD28 dAb

C


Day 21 after skin graft
Anti-OVA IgG (450nM)
0.8
0.6
0.4
0.2
0.0
1:10
1:40
1:160
1:640
1:2560
1:10240
***
***
***
***
Syngeneic
No Rx
CTLA-4 Ig
CD28 dAb

D

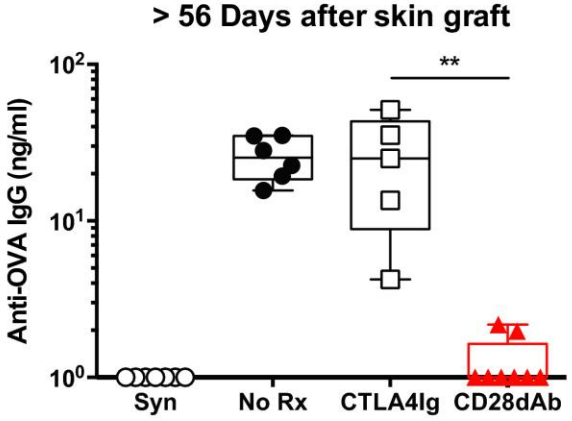

> 56 Days after skin graft
Anti-OVA IgG (ng/ml)
10²
10¹
10⁰
**
Syn
No Rx
CTLA4Ig
CD28dAb

Figure 7. Selective CD28 blockade results in superior inhibition of DSA following transplantation.

A: Naïve B6 mice were adoptively transferred 10^6 of each Thy1.1$^+$ CD4$^+$ OT-II and CD8$^+$ OT-I T cells, transplanted mOVA skin grafts, left untreated or treated with CTLA-4-Ig or anti-CD28 dAb, and serum serially collected up to 84 days post-transplant for weekly DSA assessment. B: Anti-OVA IgG production as measured by ELISA at the indicated time points (n=5 per group). C: Serial serum dilutions of anti-OVA IgG production by animals in the treatment groups 21 days after skin graft (n=5 per group). D: Anti-OVA IgG levels between 56- and 84-days post-transplant (n=5-8 per group). Data represent mean (SE) and are representative of 3 independent experiments with a total of 15 mice per group.

References

1. Sellares J, de Freitas DG, Mengel M, Reeve J, Einecke G, Sis B, et al. Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. Am J Transplant. 2012;12(2):388-99.

2. Wiebe C, Gibson IW, Blydt-Hansen TD, Pochinco D, Birk PE, Ho J, et al. Rates and determinants of progression to graft failure in kidney allograft recipients with de novo donor-specific antibody. Am J Transplant. 2015;15(11):2921-30.

3. Lefaucheur C, Loupy A, Hill GS, Andrade J, Nochy D, Antoine C, et al. Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation. J Am Soc Nephrol. 2010;21(8):1398-406.

4. Loupy A, Hill GS, Jordan SC. The impact of donor-specific anti-HLA antibodies on late kidney allograft failure. Nat Rev Nephrol. 2012;8(6):348-57.

5. Mohan S, Palanisamy A, Tsapepas D, Tanriover B, Crew RJ, Dube G, et al. Donor-specific antibodies adversely affect kidney allograft outcomes. J Am Soc Nephrol. 2012;23(12):2061-71.

6. Wiebe C, Gibson IW, Blydt-Hansen TD, Karpinski M, Ho J, Storsley LJ, et al. Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant. Am J Transplant. 2012;12(5):1157-67.

7. Hart A, Smith JM, Skeans MA, Gustafson SK, Stewart DE, Cherikh WS, et al. OPTN/SRTR 2015 Annual Data Report: Kidney. American Journal of Transplantation. 2017;17:21-116.

8. Everly MJ, Rebellato LM, Haisch CE, Ozawa M, Parker K, Briley KP, et al. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. Transplantation. 2013;95(3):410-7.

9. Djamali A, Kaufman DB, Ellis TM, Zhong W, Matas A, Samaniego M. Diagnosis and management of antibody-mediated rejection: current status and novel approaches. Am J Transplant. 2014;14(2):255-71.

10. Vincenti F, Rostaing L, Grinyo J, Rice K, Steinberg S, Gaite L, et al. Belatacept and Long-Term Outcomes in Kidney Transplantation. N Engl J Med. 2016;374(4):333-43.

11. Vanrenterghem Y, Bresnahan B, Campistol J, Durrbach A, Grinyo J, Neumayer HH, et al. Belatacept-based regimens are associated with improved cardiovascular and metabolic risk factors compared with cyclosporine in kidney transplant recipients (BENEFIT and BENEFIT-EXT studies). Transplantation. 2011;91(9):976-83.

12. Rostaing L, Vincenti F, Grinyo J, Rice KM, Bresnahan B, Steinberg S, et al. Long-term belatacept exposure maintains efficacy and safety at 5 years: results from the long-term extension of the BENEFIT study. Am J Transplant. 2013;13(11):2875-83.

13. Vincenti F, Larsen CP, Alberu J, Bresnahan B, Garcia VD, Kothari J, et al. Three-year outcomes from BENEFIT, a randomized, active-controlled, parallel-group study in adult kidney transplant recipients. Am J Transplant. 2012;12(1):210-7.

14. Heher E, Markmann JF. The Clearer BENEFITS of Belatacept. N Engl J Med. 2016;374(4):388-9.

15. Linsley PS, Wallace PM, Johnson J, Gibson MG, Greene JL, Ledbetter JA, et al. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. Science. 1992;257(5071):792-5.

16. Larsen CP, Pearson TC, Adams AB, Tso P, Shirasugi N, Strobert E, et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. Am J Transplant. 2005;5(3):443-53.

17. Crotty S. Follicular helper CD4 T cells (TFH). Annu Rev Immunol. 2011;29:621-63.

18. Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity. 2014;41(4):529-42.

19. Badell IR, Ford ML. T follicular helper cells in the generation of alloantibody and graft rejection. Curr Opin Organ Transplant. 2016;21(1):1-6.

20. Walters GD, Vinuesa CG. T Follicular Helper Cells in Transplantation. Transplantation. 2016;100(8):1650-5.

21. Poirier N, Mary C, Dilek N, Hervouet J, Minault D, Blancho G, et al. Preclinical efficacy and immunological safety of FR104, an antagonist anti-CD28 monovalent Fab' antibody. Am J Transplant. 2012;12(10):2630-40.

22. Liu D, Krummey SM, Badell IR, Wagener M, Schneeweis LA, Stetsko DK, et al. 2B4 (CD244) induced by selective CD28 blockade functionally regulates allograft-specific CD8+ T cell responses. J Exp Med. 2014;211(2):297-311.

23. Ville S, Poirier N, Branchereau J, Charpy V, Pengam S, Nerriere-Daguin V, et al. Anti-CD28 Antibody and Belatacept Exert Differential Effects on Mechanisms of Renal Allograft Rejection. J Am Soc Nephrol. 2016;27(12):3577-88.

24. Poirier N, Azimzadeh AM, Zhang T, Dilek N, Mary C, Nguyen B, et al. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. Sci Transl Med. 2010;2(17):17ra0.

25. Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. Cell. 1994;76(1):17-27.

26. Barnden MJ, Allison J, Heath WR, Carbone FR. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol Cell Biol. 1998;76(1):34-40.

27. Ehst BD, Ingulli E, Jenkins MK. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. Am J Transplant. 2003;3(11):1355-62.

28. Ford ML, Koehn BH, Wagener ME, Jiang W, Gangappa S, Pearson TC, et al. Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. J Exp Med. 2007;204(2):299-309.

29. Trambley J, Bingaman AW, Lin A, Elwood ET, Waitze SY, Ha J, et al. Asialo GM1(+) CD8(+) T cells play a critical role in costimulation blockade-resistant allograft rejection. J Clin Invest. 1999;104(12):1715-22.

30. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science. 2009;325(5943):1006-10.

31. Conlon TM, Saeb-Parsy K, Cole JL, Motallebzadeh R, Qureshi MS, Rehakova S, et al. Germinal Center Alloantibody Responses Are Mediated Exclusively by Indirect-Pathway CD4 T Follicular Helper Cells. Journal of Immunology. 2012;188(6):2643-52.

32. Yusuf I, Kageyama R, Monticelli L, Johnston RJ, Ditoro D, Hansen K, et al. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). J Immunol. 2010;185(1):190-202.

33. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. Immunity. 2014;41(6):1026-39.

34. Wang CJ, Heuts F, Ovcinnikovs V, Wardzinski L, Bowers C, Schmidt EM, et al. CTLA-4 controls follicular helper T-cell differentiation by regulating the strength of CD28 engagement. Proc Natl Acad Sci U S A. 2015;112(2):524-9.

35. Wing JB, Ise W, Kurosaki T, Sakaguchi S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. Immunity. 2014;41(6):1013-25.

36. Victora GD, Nussenzweig MC. Germinal centers. Annu Rev Immunol. 2012;30:429-57.

37. Woodle ES, Alloway RR, Girnita A. Proteasome inhibitor treatment of antibody-mediated allograft rejection. Curr Opin Organ Transplant. 2011;16(4):434-8.

38. Vo AA, Lukovsky M, Toyoda M, Wang J, Reinsmoen NL, Lai CH, et al. Rituximab and intravenous immune globulin for desensitization during renal transplantation. N Engl J Med. 2008;359(3):242-51.

39. Wojciechowski D, Vincenti F. Current status of costimulatory blockade in renal transplantation. Curr Opin Nephrol Hypertens. 2016;25(6):583-90.

40. Flynn R, Du J, Veenstra RG, Reichenbach DK, Panoskaltsis-Mortari A, Taylor PA, et al. Increased T follicular helper cells and germinal center B cells are required for cGVHD and bronchiolitis obliterans. Blood. 2014;123(25):3988-98.

41. Kim EJ, Kwun J, Gibby AC, Hong JJ, Farris AB, 3rd, Iwakoshi NN, et al. Costimulation blockade alters germinal center responses and prevents antibody-mediated rejection. Am J Transplant. 2014;14(1):59-69.

42. Kim I, Wu G, Chai NN, Klein AS, Jordan SC. Immunological characterization of de novo and recall alloantibody suppression by CTLA4Ig in a mouse model of allosensitization. Transpl Immunol. 2016;38:84-92.

43. Chen J, Yin H, Xu J, Wang Q, Edelblum KL, Sciammas R, et al. Reversing endogenous alloreactive B cell GC responses with anti-CD154 or CTLA-4Ig. Am J Transplant. 2013;13(9):2280-92.

44. Chen J, Wang Q, Yin D, Vu V, Sciammas R, Chong AS. Cutting Edge: CTLA-4Ig Inhibits Memory B Cell Responses and Promotes Allograft Survival in Sensitized Recipients. J Immunol. 2015;195(9):4069-73.

45. Young JS, Chen J, Miller ML, Vu V, Tian C, Moon JJ, et al. Delayed Cytotoxic T Lymphocyte-Associated Protein 4-Immunoglobulin Treatment Reverses Ongoing Alloantibody Responses and Rescues Allografts From Acute Rejection. Am J Transplant. 2016;16(8):2312-23.

46. Shahinian A, Pfeffer K, Lee KP, Kundig TM, Kishihara K, Wakeham A, et al. Differential T cell costimulatory requirements in CD28-deficient mice. Science. 1993;261(5121):609-12.

47. Ferguson SE, Han S, Kelsoe G, Thompson CB. CD28 is required for germinal center formation. J Immunol. 1996;156(12):4576-81.

48. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 1995;3(5):541-7.

49. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science. 2008;322(5899):271-5.

50. Miller ML, Chen J, Daniels MD, McKeague MG, Wang Y, Yin D, et al. Adoptive Transfer of Tracer-Alloreactive CD4(+) T Cell Receptor Transgenic T Cells Alters the Endogenous Immune Response to an Allograft. Am J Transplant. 2016;16(10):2842-53.

51. Adams AB, Williams MA, Jones TR, Shirasugi N, Durham MM, Kaech SM, et al. Heterologous immunity provides a potent barrier to transplantation tolerance. J Clin Invest. 2003;111(12):1887-95.

52. Nadazdin O, Boskovic S, Murakami T, Tocco G, Smith RN, Colvin RB, et al. Host alloreactive memory T cells influence tolerance to kidney allografts in nonhuman primates. Sci Transl Med. 2011;3(86):86ra51.

53. Vincenti F, Charpentier B, Vanrenterghem Y, Rostaing L, Bresnahan B, Darji P, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). Am J Transplant. 2010;10(3):535-46.

54. Ford ML, Adams AB, Pearson TC. Targeting co-stimulatory pathways: transplantation and autoimmunity. Nature Reviews Nephrology. 2014;10(1):14-24.

55. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science. 1995;270(5238):985-8.

56. Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. SAP is required for generating long-term humoral immunity. Nature. 2003;421(6920):282-7.

57. MacLennan IC, Toellner KM, Cunningham AF, Serre K, Sze DM, Zuniga E, et al. Extrafollicular antibody responses. Immunol Rev. 2003;194:8-18.

58. Odegard JM, Marks BR, DiPlacido LD, Poholek AC, Kono DH, Dong C, et al. ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. J Exp Med. 2008;205(12):2873-86.

59. Lee SK, Rigby RJ, Zotos D, Tsai LM, Kawamoto S, Marshall JL, et al. B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. J Exp Med. 2011;208(7):1377-88.

60. Grinyo J, Charpentier B, Pestana JM, Vanrenterghem Y, Vincenti F, Reyes-Acevedo R, et al. An integrated safety profile analysis of belatacept in kidney transplant recipients. Transplantation. 2010;90(12):1521-7.

Chapter 3:

Superior Inhibition of Germinal Center and Alloantibody Responses with Selective CD28 Blockade is CTLA-4-Dependent and T Follicular Helper Cell-specific

Abstract

Anti-donor antibodies cause immunologic injury in transplantation. CD28 blockade with CTLA-4-Ig has the ability to reduce the incidence of these donor-specific antibodies (DSA), but its mechanism is suboptimal for the inhibition of alloimmunity in that CTLA-4-Ig blocks both CD28 costimulation and CTLA-4 coinhibition. Thus selective CD28 blockade that spares CTLA-4 has potential to result in improved inhibition of humoral alloimmunity. To test this possibility, we utilized a full allogeneic mismatch murine transplant model and T follicular helper (Tfh):B cell co-culture system. We observed that selective blockade with an anti-CD28 domain antibody (dAb) compared to CTLA-4-Ig led to superior inhibition of Tfh cell, germinal center and DSA responses *in vivo*, and better control of B cell responses *in vitro*. CTLA-4 blockade enhanced the humoral alloresponse, and in combination with anti-CD28 dAb abrogated the effects of selective blockade. This CTLA-4-dependent inhibition was Tfh cell-specific in that CTLA-4 expression by Tfh cells was necessary and sufficient for the improved humoral inhibition observed with selective blockade. As CD28 blockade attracts interest for the control of alloantibodies in the clinic, these data support selective blockade as a superior strategy to address DSA via the sparing of CTLA-4 and more potent targeting of Tfh cells.

Introduction

Kidney transplantation is the treatment modality of choice for the majority of patients suffering from end-stage renal disease, with widely accepted benefits in patient survival and quality of life (1, 2). Advances in solid organ transplantation have significantly reduced acute rejection rates, leading to significant improvements in short-term kidney allograft survival (3). However, long-term outcomes following kidney transplantation remain suboptimal due in part to allograft injury resulting from HLA antibodies directed against the donor (4). These donor-specific antibodies (DSA) have been increasingly recognized to cause early and late immunologic injury of kidney allografts, and therefore present an immunologic barrier to the improvement of long-term kidney transplant outcomes. Despite the deleterious role of DSA following transplantation, large knowledge deficits exist regarding the mechanisms underlying their development and maintenance, and robust therapeutic strategies to control DSA-mediated allograft dysfunction have yet to be developed (5-7).

Targeting of the CD28 costimulation pathway with belatacept, a second generation CTLA-4-Ig, has achieved clinical translation and improved long-term recipient outcomes following kidney transplantation (8). However, de novo use of belatacept has been limited by increased acute rejection rates and uncertainty in immunologically high risk patients (9). This has partially been attributed to its mechanism of action. CTLA-4-Ig acts to inhibit T cell activation through binding of the ligands CD80 and CD86, thereby preventing their engagement with the T cell co-stimulator CD28 and inhibiting T cell priming and activation (10). Undesirably, this mechanism

of action also indiscriminately blocks CD80 and CD86 ligation of the co-inhibitor CTLA-4, depriving T cells of potentially important inhibitory signals and regulatory interactions. A wealth of evidence exists regarding the immune stimulatory impact associated with the loss of CTLA-4 activity (11, 12), suggesting that the secondary effect of CTLA-4 blockade may significantly detract from the overall goal of attenuating alloreactivity. Accordingly, others and we have demonstrated that next generation costimulation blockade that selectively antagonizes CD28 costimulatory signaling and preserves CTLA-4 coinhibition leads to superior control of alloimmune responses (13-15). Therefore, selective CD28 blockade holds great promise to further improve upon the encouraging long-term renal transplant outcomes realized with belatacept.

Preclinical and clinical studies have consistently shown that CTLA-4-Ig relative to conventional immunosuppression is more effective at reducing de novo alloantibody formation (8, 16), but the efficacy of belatacept on pre-formed antibodies in sensitized recipients and DSA over the long-term is unknown. Results from an ongoing clinical trial designed to evaluate the ability of belatacept monotherapy to prevent DSA formation in kidney transplant recipients with failed allografts indicate that belatacept alone is not sufficient to completely prevent DSA in this setting (17). Moreover, we have shown that selective CD28 blockade is superior to CTLA-4-Ig at preventing alloantibodies in a minor antigen mismatch murine transplant model (18). Thus, optimization of current methods of costimulation blockade (i.e. CTLA-4-Ig) is a promising and intriguing strategy to address the problem of HLA antibodies in the clinic.

Although the mechanisms of antibody inhibition by CD28 costimulation blockade have not been entirely elucidated, it is likely due in large part to the inhibition of T follicular helper (Tfh) cell differentiation and function (19, 20). Tfh cells are a lineage of $CD4^+$ T cells required for the provision of B cell help to generate mature antibody responses that also heavily depend on CD28 (21). Tfh cells have been implicated in many immune processes and their inhibition has been associated with the prevention of anti-donor antibody responses in transplantation (18, 22, 23). Importantly, several studies have implicated CTLA-4 as a key regulator of Tfh cells primarily through regulatory T cell-mediated mechanisms (24, 25). Therefore, improved inhibition of alloantibody responses with selective CD28 blockade relative to CTLA-4-Ig may directly result from the preservation of CTLA-4 co-inhibitory capacity following transplantation.

In this study, we utilized a clinically relevant BALB/c to B6 full MHC mismatch skin allograft model to examine the role of CTLA-4 coinhibition in the setting of selective CD28 blockade. We demonstrate that selective CD28 blockade results in superior inhibition of the Tfh cell, germinal center (GC) and DSA responses compared to CTLA-4-Ig. Donor-elicited Tfh cells differentially upregulated the coinhibitor CTLA-4, and CTLA-4 blockade augmented the Tfh cell and GC alloresponses. Anti-CTLA-4 treatment in conjunction with selective CD28 blockade reversed the superior inhibition observed with CD28-specific blockade *in vivo* after allotransplantation and in *in vitro* Tfh:B cell co-cultures. This CTLA-4 dependence was Tfh cell-specific in that CTLA-4 expression by Tfh cells was necessary and sufficient for the improved humoral inhibition observed with selective CD28 blockade. These findings support the development of next

generation costimulation blockers that selectively target CD28 and preserve the inhibitory and regulatory functions of CTLA-4 as a more potent immunosuppressive strategy to combat HLA antibodies in clinical transplantation.

Materials and Methods

Mice

B6-Ly5.1/Cr (H2-K^b) and BALB/c (H-$2K^d$) mice were obtained from the National Cancer Institute and housed in pathogen-free facilities.

Skin Transplants and Immunosuppression

Bilateral dorsal full-thickness tail and ear skin were transplanted onto recipient mice. Skin graft recipients received no treatment, anti-CD28 dAb (100 µg, Bristol-Myers Squibb), CTLA-4-Ig (200 µg, Bristol-Myers Squibb), or anti-CTLA-4 mAb (9H10, 250 µg, BioXcell). Treatments were administered intraperitoneally on post-transplant days 0, 2, 4, 6 and 8, and then weekly thereafter. Anti-CD28 dAb and CTLA-4-Ig dosing was based on molecular weight, serum half-life, and murine mixed lymphocyte reaction EC_{50} (14, 18).

Flow Cytometry

Graft-draining axillary and brachial lymph nodes were processed into single-cell suspensions. Cells were surface stained for indicated markers and pulsed with LIVE/DEAD viability dye (Molecular Probes) before fixation. Intracellular staining was performed with Foxp3 Fixation/Permeabilization Buffer Kit (eBioscience). All antibodies were from BioLegend and BD Biosciences. All samples were run on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed by using FlowJo Software (Flowjo, LLC). CountBright Beads (Invitrogen) were used to determine absolute cell counts.

Tfh:B Cell Co-Culture

T and B cells from graft-DLNs were enriched, flow sorted and co-cultured as previously described (44). Briefly, magnetic bead negative selection (Miltenyi Biotec) was used to enrich $CD4^+$ T and $CD19^+$ B cells. T cells from DLNs were flow sorted into Tfh ($CD19^-CD4^+PD\text{-}1^{hi}CXCR5^+GITR^-$) cells on a FACSAria II (BD Biosciences). For proliferation assessment, B cells were stained with eFluor670 proliferation dye (eBioscience). $2x10^4$ T cells were cultured with $5x10^4$ B cells in 96-well plates in anti-CD3ε (2C11, 2 μg/mL, BioXcell) and anti-IgM (5 μg/mL, Jackson Immunoresearch) containing media for 5 days. Culture media was supplemented with immunosuppression where indicated at the following concentrations: CTLA-4-Ig (50 μg/mL), anti-CD28 dAb (25 μg/mL), anti-CTLA-4 mAb (50 μg/mL). As in the *in vivo* experiments, anti-CD28 dAb and CTLA-4-Ig dosing was based on molecular weight, serum half-life, and murine mixed lymphocyte reaction EC_{50} (14, 18). Cultured cells were analyzed by flow cytometry, and supernatants analyzed for total IgG levels by ELISA.

Antibody Assessments

Serum was collected from transplanted animals to test for anti-donor antibodies. For flow cytometric crossmatch, BALB/c splenocytes were processed into single-cell suspensions and pre-treated with Fc Block (BioLegend), followed by incubation with recipient serum at 4°C. Splenocytes were then washed and labeled with surface markers and anti-mouse IgG (BioLegend) for quantification of anti-BALB/c IgG by flow cytometry. For ELISA total IgG antibody measurements from co-culture supernatant, flat-bottom 96-well Immulon 4HBX microtiter plates (VWR) were coated with anti-mouse Ig (5 μg/well; Sigma-Aldrich) overnight at

4°C. Coated plates were blocked with 10% FBS in PBS-T for 1 hour at 37°C, and then incubated with culture supernatant samples for 1.5 hours at 37°C. Total IgG was detected with HRP goat anti-mouse IgG (Poly4053, BioLegend), developed by using the TMB substrate system (Thermo Scientific), and read at 450 nm on a Spectra MAX 340PC Microplate reader (Molecular Devices).

Statistics

The Mann–Whitney U nonparametric t test was performed for analysis of unpaired groups, and the Holms–Sidak method was used for grouped, multiple t test analyses. All analyses were performed by using GraphPad Prism (GraphPad Software, Inc.). Statistical significance was attributed to p values <0.05 (*<0.05, **<0.01, ***<0.001).

Study Approval

All animal studies were approved by the Emory University Institutional Animal Care and Use Committee and conducted in accordance with their guidelines.

Results

Selective CD28 blockade exhibits superior inhibition of T cell dependent antibody responses compared to CTLA-4-Ig in a full MHC mismatch allotransplantation model

Our group has previously demonstrated that selective CD28 blockade results in superior inhibition of both cellular and humoral alloresponses compared to CTLA-4-Ig in a surrogate minor antigen (OVA) mismatch model (18). Based on observed similarities between the alloreactive transgenic and endogenous polyclonal Tfh cell responses in the OVA model, we sought to test the impact of selective CD28 blockade on the Tfh cell-mediated alloantibody response in a full MHC mismatch murine skin transplant model for enhanced clinical applicability. As such, naïve B6 recipients were transplanted with skin grafts from syngeneic (B6) or allogeneic (BALB/c) donor mice and were left untreated or treated with either CTLA-4-Ig or an anti-CD28 domain antibody (dAb) (Figure 1A). Recipients were serially bled for serum collection and flow crossmatch analysis to test for the development of anti-donor antibodies or euthanized for cellular draining lymph node (DLN) analysis. Untreated allogeneic graft recipients formed anti-BALB/c alloantibodies by posttransplant day 14, while DSA emerged by day 21 in CTLA-4-Ig treated mice (Figure 1B). Conversely and similar to the syngeneic graft recipients, anti-CD28 dAb treated mice did not develop DSA. To interrogate the cellular response, graft-DLNs were analyzed 10 days post-transplant by flow cytometry. Donor-reactive Tfh ($CXCR5^{+}PD\text{-}1^{hi}$) and GC Tfh ($Bcl6^{hi}PD\text{-}1^{hi}$ of Tfh cells) cells were readily identifiable in untreated animals receiving allogeneic grafts as compared to syngeneic grafts (Figures 1C, D). While CTLA-4-Ig resulted in reduced frequencies and absolute numbers of Tfh and GC Tfh cells, the anti-CD28 dAb completely eliminated this cellular alloresponse. Examination of B cell

subsets within the graft-DLNs also revealed greater inhibition of the GC B cell ($GL7^+CD95^+$), plasmablast ($CD19^+B220^-CD138^+$), and plasma cell ($CD19^-B220^-CD138^+$) responses by the CD28-specific dAb relative to CTLA-4-Ig (Figures 1E, F). Taken together, these findings demonstrate that selective CD28 blockade provides superior inhibition of the GC response compared to CTLA-4-Ig in a clinically relevant full allogeneic mismatch model, thereby resulting in improved inhibition of DSA.

CTLA-4 blockade augments the Tfh and GC B cell alloresponses after transplantation

The coinhibitory receptor CTLA-4 has been implicated as a critical mediator of Tfh cell differentiation and function (24-26). In order to investigate whether intact CTLA-4 on Tfh cells could be a potential mechanism underlying the improved inhibition of Tfh cells with selective CD28 blockade, we examined the level of expression of CTLA-4 on Tfh cells in response to an allogeneic skin graft. Naïve B6 recipients were transplanted with syngeneic (B6) or BALB/c donor skin grafts and sacrificed 10 days post-transplant for graft-DLN analysis. Donor-reactive Tfh cells exhibited greater expression of CTLA-4 relative to naïve ($CD62L^+CD44^-$) and antigen-experienced non-Tfh ($CD44^+CXCR5^-$) $CD4^+$ T cells (Figure 2A). The Tfh cell compartment contained the largest frequency of CTLA-4^+ cells and exhibited the highest levels of CTLA-4 expression (Figure 2B). Furthermore, we found that polarized GC Tfh (Bcl-6^{hi}PD-1^{hi}) cells within the Tfh cell compartment expressed the highest levels of CTLA-4 and contained the greatest frequency of CTLA-4^+ Tfh cells (Figures 2A, B). This differential expression of CTLA-4 on donor-reactive Tfh and GC Tfh cells suggests that CTLA-4-mediated coinhibition may play a critical role in Tfh cell-mediated alloreactivity. To test this possibility, skin-grafted mice were treated with a

blocking anti-CTLA-4 mAb and the graft-DLNs examined 10 days after transplant (Figure 2C). We observed that CTLA-4 antagonism alone significantly increased the frequency and absolute numbers of graft-reactive Tfh and GC B cells compared to untreated controls (Figures 2D, E), indicating that blockade of CTLA-4 coinhibition leads to augmentation of the Tfh cell-mediated GC response following allotransplantation. These data support the hypothesis that CTLA-4 is an important functional inhibitor of Tfh cell-mediated alloreactivity and may underlie the superior humoral inhibition observed with selective CD28 blockade (Figure 1).

Selective CD28 blockade inhibition of Tfh-mediated alloantibody formation is CTLA-4-dependent

In order to determine the role of preserved CTLA-4 coinhibitory capacity in enhancing the immunosuppressive effects of selective CD28 blockade over that of CTLA-4-Ig, we utilized the same full MHC mismatch BALB/c to B6 skin allograft model. Naïve B6 mice were transplanted with BALB/c skin and left untreated or administered either anti-CD28 dAb monotherapy or anti-CD28 dAb in combination with anti-CTLA-4 mAb (Figure 3A). Flow crossmatch analysis of serially collected serum for anti-BALB/c IgG levels posttransplant showed DSA formation in untreated animals and complete inhibition of anti-donor antibodies in the anti-CD28 dAb treated mice (Figures 3B, C). Interestingly, the addition of anti-CTLA-4 mAb to anti-CD28 dAb treated skin graft recipients resulted in the development of DSA relative to anti-CD28 dAb alone. Thus, CTLA-4 antagonism reversed selective CD28 blockade-mediated inhibition of alloantibody formation.

We next sought to test the effect of anti-CTLA-4 on the cellular GC response. Based on our prior observation that the peak alloreactive Tfh response in secondary lymphoid organs posttransplant precedes antibody formation by 4 days (27), we examined the GC response in graft-DLNs 24 days after transplant (4 days prior to antibody detection on day 28, Figures 3B, C). Analysis of the Tfh cell compartment showed that the addition of anti-CTLA-4 mAb significantly increased the frequency and absolute numbers of both $CXCR5^{+}PD\text{-}1^{hi}$ Tfh and $BCL\text{-}6^{hi}PD\text{-}1^{hi}$ GC Tfh cells over that observed in anti-CD28 dAb alone treated animals (Figures 3D, E). Examination of B cell subsets revealed similar findings, with CTLA-4 blockade abrogating the inhibition of GC B cells, plasmablasts and plasma cells by anti-CD28 monotherapy (Figures 3F, G). Overall, blockade of the coinhibitor CTLA-4 reversed selective CD28 blockade-mediated inhibition of alloreactive Tfh cells, B cell subsets, and DSA following transplantation.

CTLA-4 is critical for the superior inhibition of selective CD28 blockade at the level of Tfh:B cell cognate interactions

To further test the hypothesis that CTLA-4 coinhibition of cognate Tfh:B cell interactions is responsible for the improved efficacy of selective CD28 blockade to inhibit DSA, we performed alloreactive Tfh:B cell co-cultures. Naïve B6 mice with full MHC mismatched BALB/c skin grafts were left untreated and sacrificed 10 days post-transplant (Figure 4A). Sorted Tfh and enriched B cells from graft-DLNs were co-cultured for 5 days to assess B cell proliferation, class switch recombination (CSR) and antibody production. Tfh:B cell co-cultures were left untreated or treated with CTLA-4-Ig, anti-CD28 dAb, or anti-CD28 dAb plus anti-CTLA-4 mAb. Untreated co-cultures exhibited robust B cell proliferation as measured by proliferation dye and Ki-67

expression (Figures 4B-D), while CTLA-4-Ig partially inhibited and anti-CD28 dAb completely inhibited this B cell proliferative burst representative of GC B cell responses. Intriguingly and consistent with our *in vivo* data, the addition of anti-CTLA-4 mAb to selective CD28 blockade resulted in reversal of the inhibition of B cell proliferation observed with the anti-CD28 dAb alone (Figures 4B-D). CTLA-4 blockade had the same impact on the differentiation of B cells into class-switched GC-like GL-7^{+}IgG1^{+} B cells and their proliferation as compared to anti-CD28 dAb alone (Figures 4E, F). Interestingly, Tfh cell proliferation also exhibited the same pattern of inhibition as the B cells, with anti-CTLA-4 partially reversing the immunosuppressive effects of the anti-CD28 dAb to levels similar to CTLA-4-Ig (Figure 4G). Furthermore, analysis of culture supernatants for IgG production showed significantly increased levels of total IgG when CTLA-4 blockade was combined with selective CD28 blockade relative to anti-CD28 dAb alone (Figure 4H). Hence these data indicate that the co-inhibitor CTLA-4 plays a critical role in the superior inhibition observed with selective CD28 blockade compared to non-selective costimulation blockade with CTLA-4-Ig (Figure 1), and that CTLA-4 is acting at the level of Tfh:B cell cognate interaction.

CTLA-4-dependent superior inhibition of selective CD28 Blockade is Tfh cell-specific

Because CD28 and CTLA-4 have been reported to be expressed and functional on B cells (28-30), we next investigated whether the CTLA-dependent superior inhibition observed with selective CD28 blockade is Tfh cell-specific. Utilizing the same Tfh:B cell co-culture system, either sorted Tfh or enriched B cells from graft-DLNs were individually pre-treated with anti-CTLA-4 mAb and then co-cultured in the presence of anti-CD28 dAb for 5 days (Figure 5A). As

such, this approach enabled us to determine whether Tfh cell- or B cell-derived CTLA-4 is driving improved inhibition with selective CD28 blockade. As expected, selective blockade with the anti-CD28 dAb alone inhibited B cell proliferation, and this inhibition was reversed when both Tfh and B cells were exposed to anti-CTLA-4 mAb (Figures 5B-D). Interestingly, anti-CTLA-4 pre-treated Tfh cells, but not pre-treated B cells, abrogated the inhibition of B cell proliferation with selective CD28 blockade alone. Similarly, only anti-CTLA-4 pre-treated Tfh cells and not pre-treated B cells exhibited the ability to reverse anti-CD28 dAb-mediated inhibition of B cell differentiation into GL-7^{+}IgG1^{+} class-switched B cells, Tfh cell proliferation, and antibody production (Figures 5E-H). Thus CTLA-4 antagonism reverses the increased efficacy of selective CD28 blockade to control humoral alloresponses relative to CTLA-4-Ig in a Tfh cell-specific manner. Namely, CTLA-4 expression by Tfh cells is necessary and sufficient for the improved inhibition observed with selective CD28 blockade.

Discussion

In the last decade, it has become well recognized that alloantibodies are an important immunologic cause of allograft injury and premature kidney graft loss (4, 31). Rapid advances in our understanding of Tfh cell biology, the important role this subset plays in mediating T cell dependent antibody responses, and their dependence on CD28 and CTLA-4 has made them an increasingly relevant therapeutic target to combat DSA (21, 32). CD28 costimulation blockade with belatacept has reduced the incidence of de novo DSA (8), but its mechanism of action is suboptimal for the purpose of inhibiting alloimmunity in that CTLA-4-Ig not only blocks CD28 costimulation but also prevents critical coinhibitory and regulatory activity mediated via CTLA-4 (10). Here, we demonstrate that selective CD28 blockade is better than CTLA-4-Ig at inhibiting the humoral alloresponse in a clinically relevant full allogeneic mismatch model, and that this superior inhibition is mechanistically CTLA-4-dependent and Tfh cell-specific. As CD28 costimulation blockade attracts growing interest as a method of controlling alloantibodies in the clinic (6), these data support next generation selective CD28 blockade as a more efficacious strategy of addressing the clinical burden of DSA via the sparing of CTLA-4 and more potent targeting of Tfh cells.

Given the ubiquitous roles CD28 and CTLA-4 have on distinct T cell subsets, Treg, Th17, and $CD8^+$ T cells have not surprisingly all been shown to exhibit differential responses to selective CD28 blockade versus CTLA-4-Ig in experimental models of transplantation (13-15, 33, 34). Our group has also demonstrated that selective blockade results in better inhibition of alloantibody responses in a TCR transgenic minor antigen mismatch model (18), but whether the superior

impact of selective CD28 blockade on DSA is CTLA-4-dependent or Tfh cell-specific has not been previously reported. In this study, we provide *in vivo* data indicating that anti-CD28 dAb-mediated alloantibody inhibition is CTLA-4-dependent, and *in vitro* Tfh:B cell co-culture data demonstrating that CTLA-4 expression by Tfh cells is necessary and sufficient for the superior humoral inhibition observed with selective CD28 blockade. While the global effects of CD28-specific blockade may partially contribute to the superior anti-humoral results observed with the anti-CD28 dAb, our data strongly support that selective blockade is functioning primarily on Tfh cells at the cognate Tfh:B cell level to enhance DSA inhibition through the coinhibitor CTLA-4.

In considering the determinants of T cell dependent antibody production, Tregs and T follicular regulatory (Tfr) cells have been elegantly shown to regulate Tfh cell and humoral immune responses (24, 25). Because they, like effector T cells, depend on CD28 signals for their activation and differentiation, not unexpectedly examination of the endogenous, graft-elicited Treg and Tfr cell populations revealed reductions in the quantity of both subsets that was not reversed by CTLA-4 antagonism (data not shown). Additionally, CTLA-4 antagonism abrogated anti-CD28 dAb-mediated inhibition of B cell proliferation, CSR and antibody production *in vitro* independent of Treg and Tfr cells (Figure 4). Together, these observations make it unlikely that regulatory T cells are driving the improved humoral inhibition observed in our transplant model. Alternatively, sustained antibody responses have been reported to depend on CD28 function in plasma cells (30), and rare reports have identified CTLA-4 on B cells (28, 29). As such, we were able to isolate the functional role of CTLA-4 during selective CD28 blockade to the Tfh

cell and not B cells utilizing the *in vitro* Tfh:B cell co-culture system (Figure 5). Therefore, preserving CTLA-4 coinhibitory capacity on Tfh cells is likely the chief mechanism conferring improved control of Tfh cell, GC and DSA responses under selective CD28 blockade in this preclinical transplant model.

GCs do not develop in the absence of CD28, presumably due to a lack of Tfh cells (35, 36). Tfh differentiation and function has been modeled to be a multistep process that depends on CD28-B7 interactions at both the priming T:DC stage and subsequent Tfh:B cell stage to form GCs (21). Prevailing paradigms have put forth that CD80/86 ligation of CD28 are critical for both DC and B cell interactions with Tfh cells. However, recent work by Watanabe et al. utilizing conditional knockout systems in mice showed that B7 molecule expression was required on DCs for initial T:DC interactions but not on B cells for Tfh cell differentiation, GC formation and class-switched antibody responses following vaccination (37). These data suggest that CD28 blockade of Tfh-driven antibody production would effectively impair early stage Tfh cell responses resulting from T:DC priming but not ongoing or later stage Tfh-driven alloreactivity as Tfh:B cell conjugate interactions that lead to antibody formation were not CD28-B7 dependent. Furthermore, delayed costimulation blockade with CTLA-4-Ig has been shown to reverse ongoing alloantibody responses mostly independent of graft-specific $CD4^+$ T cells and Tfh cells in a murine heart transplant model (38). While CD28 blockade is indeed excellent at inhibiting early Tfh cell responses (18, 27), our findings demonstrating the ability of selective CD28 blockade to inhibit maturely differentiated effector Tfh:B cell conjugate interactions in a CTLA-4-dependent manner present an alternative perspective on the significance of CD28-B7

engagement between Tfh and B cells in the context of a GC response. These conflicting observations could result from differences in animal model (vaccination vs. transplant), type of allograft (heart vs. skin), or mode of CD28 costimulation blockade (selective vs. non-selective). Nonetheless, our data indicate that effector Tfh cell-driven cognate B cell interactions and the alloantibody responses that result from them do in fact depend on CD28.

The susceptibility of mature effector Tfh cells engaged in cognate B cell interactions that drive GCs, and not just naïve pre-Tfh $CD4^+$ T cells to CD28 blockade has significant therapeutic clinical implications. There has been resurgent clinical interest in the use of CD28 costimulation blockade to attenuate alloantibody responses (6). Several recent studies have reported a modest ability of belatacept to control nascent or pre-existing HLA antibodies. Post-hoc analyses of the BENEFIT and BENEFIT-EXT clinical trials (39, 40), as well as the BELACOR clinical trial examining kidney transplant outcomes in recipients with preformed DSAs (41), have all demonstrated decreases in HLA antibodies with belatacept. In a sensitized nonhuman primate kidney transplant model, belatacept in combination with bortezomib desensitization and anti-CD40 therapy mediated reductions in DSA and the cellular components of the humoral response (42). These preclinical and clinical studies represent the growing concept of utilizing costimulation blockade to target CD28-mediated humoral immunity beyond initial $CD4^+$ T cell priming and licensing of Tfh cell differentiation. Our findings provide mechanistic rationale for the continued extended application of CD28 blockade to combat pre-existing humoral sensitization in transplantation with CD28 costimulation blockers. Moreover, the ability of selective CD28 blockade to better inhibit effector Tfh:B cell interactions via CTLA-4 suggests

that selective agents may be clinically more potent at inhibiting memory Tfh cell-driven or more terminal GC alloreactivity due to prior HLA humoral sensitization. With CD28-specific agents in the development pipeline (43), next generation selective CD28 blockade is poised to offer a new therapeutic option to address the large unmet need of managing alloantibodies in sensitized transplant recipients.

In summary, this study provides evidence that superior inhibition of Tfh-mediated DSA responses through selective CD28 blockade therapy is CTLA-4-dependent. Our data highlight the potential for next generation selective CD28 costimulation blockade to provide superior control of GC and alloantibody responses through the preservation of CTLA-4 on Tfh cells. The observed susceptibility of effector Tfh cells to anti-CD28 therapy supports a shift in therapeutic focus to targeting Tfh cells as a means of controlling ongoing or pre-existing humoral alloresponses. Overall, these findings promise to inform therapeutic efforts aimed at controlling both *de novo* DSA responses and pre-existing alloantibodies to improve long-term outcomes in kidney transplantation.

Figures and Figure Legends

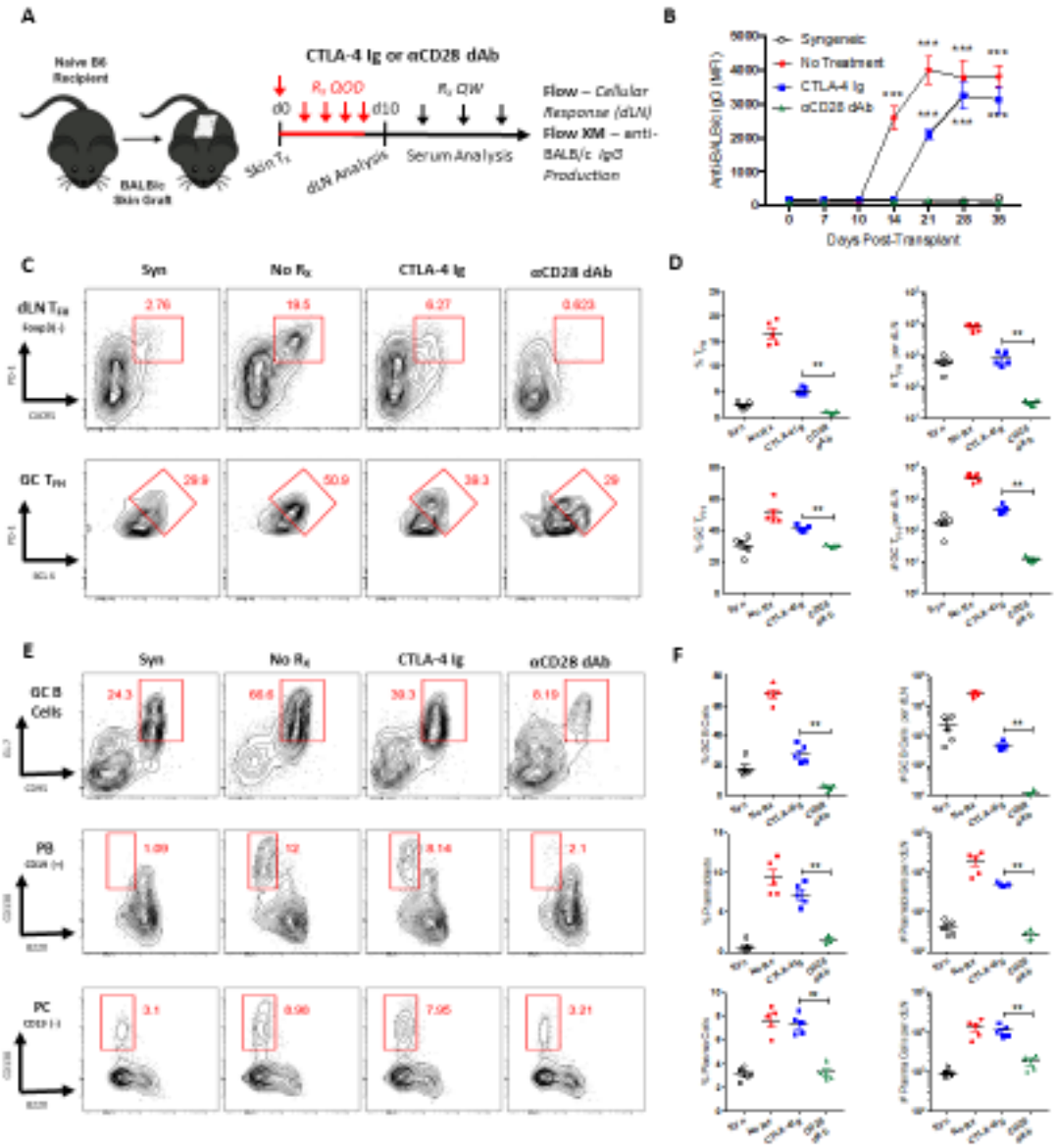


Figure 1. Selective CD28 blockade exhibits superior inhibition of T cell dependent antibody responses compared to CTLA-4-Ig

(A) Naïve B6 mice were transplanted with skin from syngeneic (B6) or allogeneic (BALB/c) donors and sacrificed 10 days post-transplant for graft-DLN analysis or serially bled for serum analysis. Transplanted mice were left untreated or treated with either CTLA-4-Ig or anti-CD28 dAb every other day (QOD, red) the first week and then weekly (QW, black) thereafter. (B)

Summary data of anti-donor total serum IgG over time (n=5 per group). (C) Representative flow cytometric plots displaying the frequencies of DLN Tfh ($CXCR5^{+}PD\text{-}1^{hi}$, gated on $CD4^{+}CD44^{hi}Foxp3^{-}$ T cells) and GC Tfh ($Bcl6^{hi}PD\text{-}1^{hi}$, gated on $CXCR5^{+}PD\text{-}1^{hi}$ Tfh cells) cells under each treatment condition. (D) Summary data of the frequencies and numbers of Tfh and GC Tfh cells (n=5 per group). (E) Representative flow plots displaying the frequencies of DLN GC B cells ($GL7^{+}CD95^{+}$, gated on $IgD^{-}CD19^{+}B220^{+}CD138^{-}$ B cells), plasmablasts ($B220^{-}CD138^{+}$, gated on $IgD^{-}CD19^{+}$ B cells), and plasma cells ($B220^{-}CD138^{+}$, gated on $IgD^{-}CD19^{-}$ B cells). (F) Summary data of the frequencies and numbers of GC B cells, plasmablasts, and plasma cells (n=5 per group). Summary data represent mean (SE) and are representative of at least 2 independent experiments with a total of at least 10 mice per group. $^{**}p < 0.01$, $^{***}p< 0.001$.

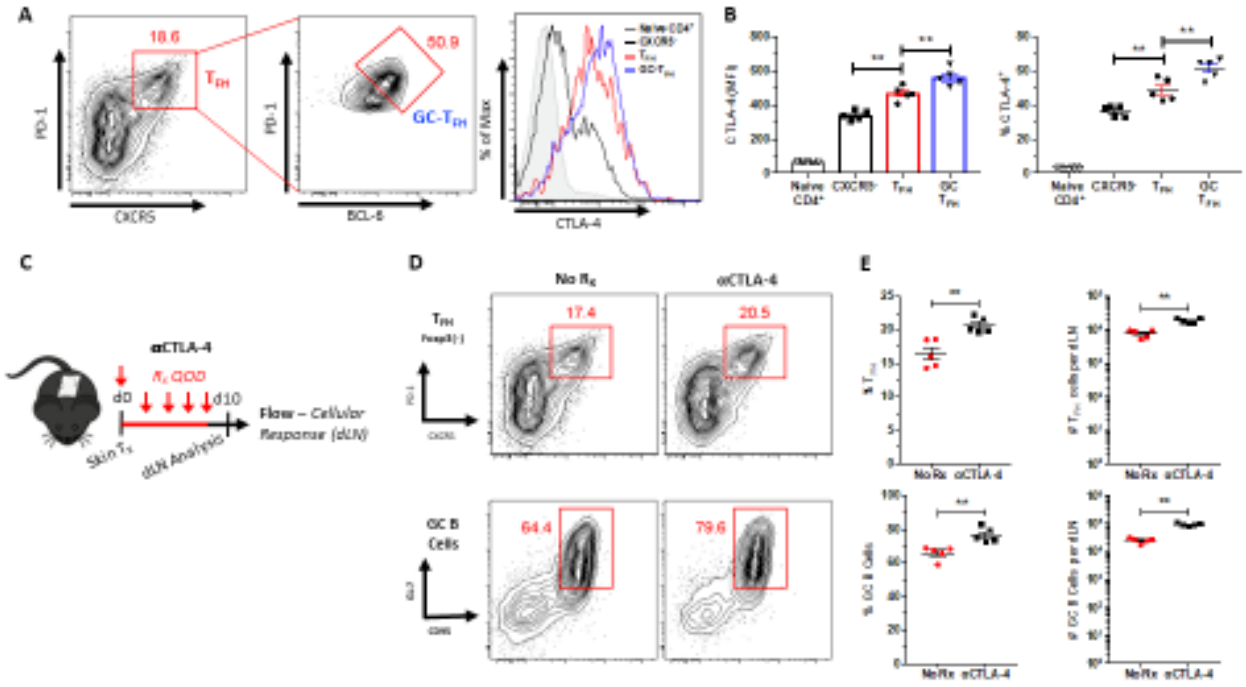


Figure 2. CTLA-4 blockade augments the Tfh and GC B cell alloresponses

Naïve B6 mice were transplanted with skin from BALB/c donors and sacrificed 10 days post-transplant for graft-DLN analysis. (A) Representative flow plots of Tfh ($CXCR5^+PD\text{-}1^{hi}$, gated on $CD4^+CD44^{hi}Foxp3^-$ T cells) and GC Tfh ($Bcl6^{hi}PD\text{-}1^{hi}$, gated on $CXCR5^+PD\text{-}1^{hi}$ Tfh cells) cells, and representative histograms depicting CTLA-4 expression on naïve $CD4^+$ T cells ($CD44^{lo}CD62L^+$), $CD4^+CD44^{hi}CXCR5^-$ T cells, Tfh cells and GC Tfh cells. (B) Summary data of CTLA-4 expression levels and frequencies of $CTLA\text{-}4^+$ cells amongst the indicated subsets (n=5 per group). (C) Naïve B6 mice were transplanted BALB/c skin, either left untreated or treated with anti-CTLA-4 mAb every other day (QOD, red), and graft-DLNs examined 10 days posttransplant. (D) Representative flow plots displaying the frequencies of Tfh and GC B cells ($GL7^+CD95^+$, gated on $IgD^-CD19^+B220^+CD138^-$ B cells) from untreated and anti-CTLA-4-treated mice. (E) Summary data of the frequencies and numbers of Tfh and GC B cells (n=5 per group). Summary data represent mean (SE). $^{**}p < 0.01$.

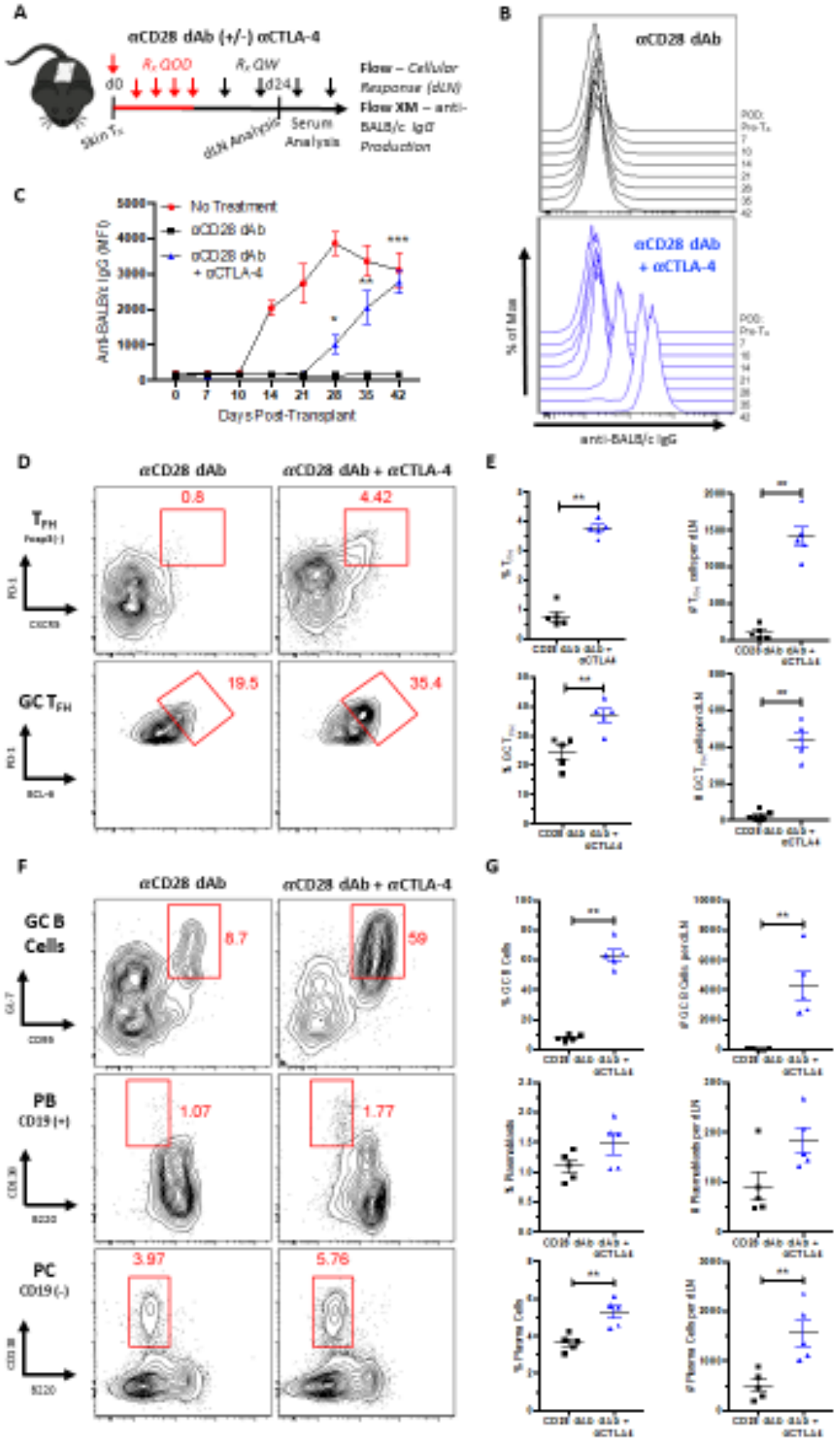

A
αCD28 dAb (+/-) αCTLA-4
d0
d24
Flow – Cellular Response (dLN)
Flow XM – anti-BALB/c IgG Production
Serum Analysis
B
αCD28 dAb
αCD28 dAb + αCTLA-4
% of Max
anti-BALB/c IgG
C
No Treatment
αCD28 dAb
αCD28 dAb + αCTLA-4
Anti-BALB/c IgG (MFI)
Days Post-Transplant
D
αCD28 dAb
αCD28 dAb + αCTLA-4
0.8
4.42
19.5
35.4
E
F
GC B Cells
PB
PC
1.07
1.77
3.97
5.76
G

Figure 3. Selective CD28 blockade inhibition of Tfh-mediated alloantibody formation is CTLA-4-dependent

(A) Naïve B6 mice were transplanted with skin from BALB/c donors and sacrificed 24 days post-transplant for graft-DLN analysis or serially bled for serum analysis. Transplanted mice were left untreated or treated with anti-CD28 dAb alone or anti-CD28 dAb plus anti-CTLA-4 mAb every other day (QOD, red) the first week and then weekly (QW, black) thereafter. (B) Representative histograms depicting anti-donor total serum IgG over time. (C) Summary data of anti-donor total serum IgG over time (n=5 per group). (D) Representative flow plots displaying the frequencies of DLN Tfh ($CXCR5^{+}PD\text{-}1^{hi}$, gated on $CD4^{+}CD44^{hi}Foxp3^{-}$ T cells) and GC Tfh ($Bcl6^{hi}PD\text{-}1^{hi}$, gated on $CXCR5^{+}PD\text{-}1^{hi}$ Tfh cells) cells under each treatment condition. (E) Summary data of the frequencies and numbers of Tfh and GC Tfh cells (n=5 per group). (F) Representative flow plots displaying the frequencies of DLN GC B cells ($GL7^{+}CD95^{+}$, gated on $IgD^{-}CD19^{+}B220^{+}CD138^{-}$ B cells), plasmablasts ($B220^{-}CD138^{+}$, gated on $IgD^{-}CD19^{+}$ B cells), and plasma cells ($B220^{-}CD138^{+}$, gated on $IgD^{-}CD19^{-}$ B cells). (G) Summary data of the frequencies and numbers of GC B cells, plasmablasts, and plasma cells (n=5 per group). Summary data represent mean (SE) and are representative of at least 2 independent experiments with a total of at least 10 mice per group. $*p < 0.05$, $**p < 0.01$.

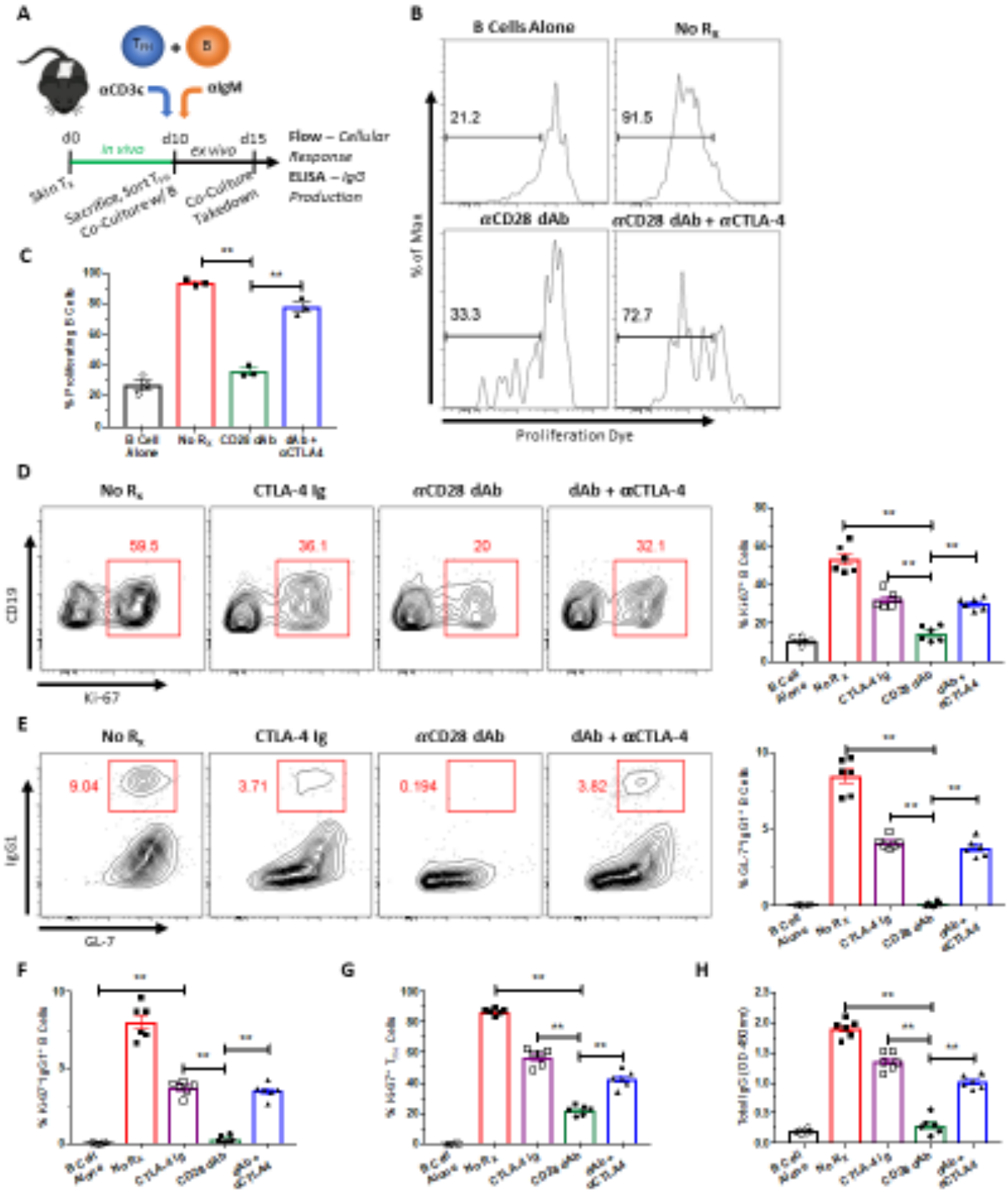


Figure 4. CTLA-4 is critical for the superior inhibition of selective CD28 blockade at the level of Tfh:B cell cognate interactions

(A) Naïve B6 mice were transplanted with BALB/c skin, left untreated, and sacrificed 10 days post-transplant. Tfh and B cells from graft-DLNs were isolated and co-cultured for 5 days. Tfh:B

cell co-cultures were left untreated, or treated with CTLA-4-Ig, anti-CD28 dAb, or anti-CD28 dAb plus anti-CTLA-4 mAb. (B) Representative histograms depicting B cell proliferation by eFluor670 proliferation dye under the indicated conditions. (C) Summary data of the frequencies of proliferating B cells (n=3 per group). (D) Representative flow plots of B cell proliferation as measured by Ki-67 and summary data of the frequencies of proliferating Ki-67$^+$ B cells (n=6 per group). (E) Representative flow plots of class-switched GC-like GL7$^+$IgG1$^+$ B cells and summary data of the frequencies of these class-switched B cells (n=6 per group). Summary data of the frequencies of (F) proliferating class-switched B cells (n=6 per group), (G) proliferating Tfh cells (n=6 per group), and (H) supernatant total IgG levels (n=6 per group). Summary data represent mean (SE) and are representative of at least 2 independent experiments, with cells pooled from 10 mice per experiment and 3-6 co-culture wells per treatment group. **$p < 0.01$.

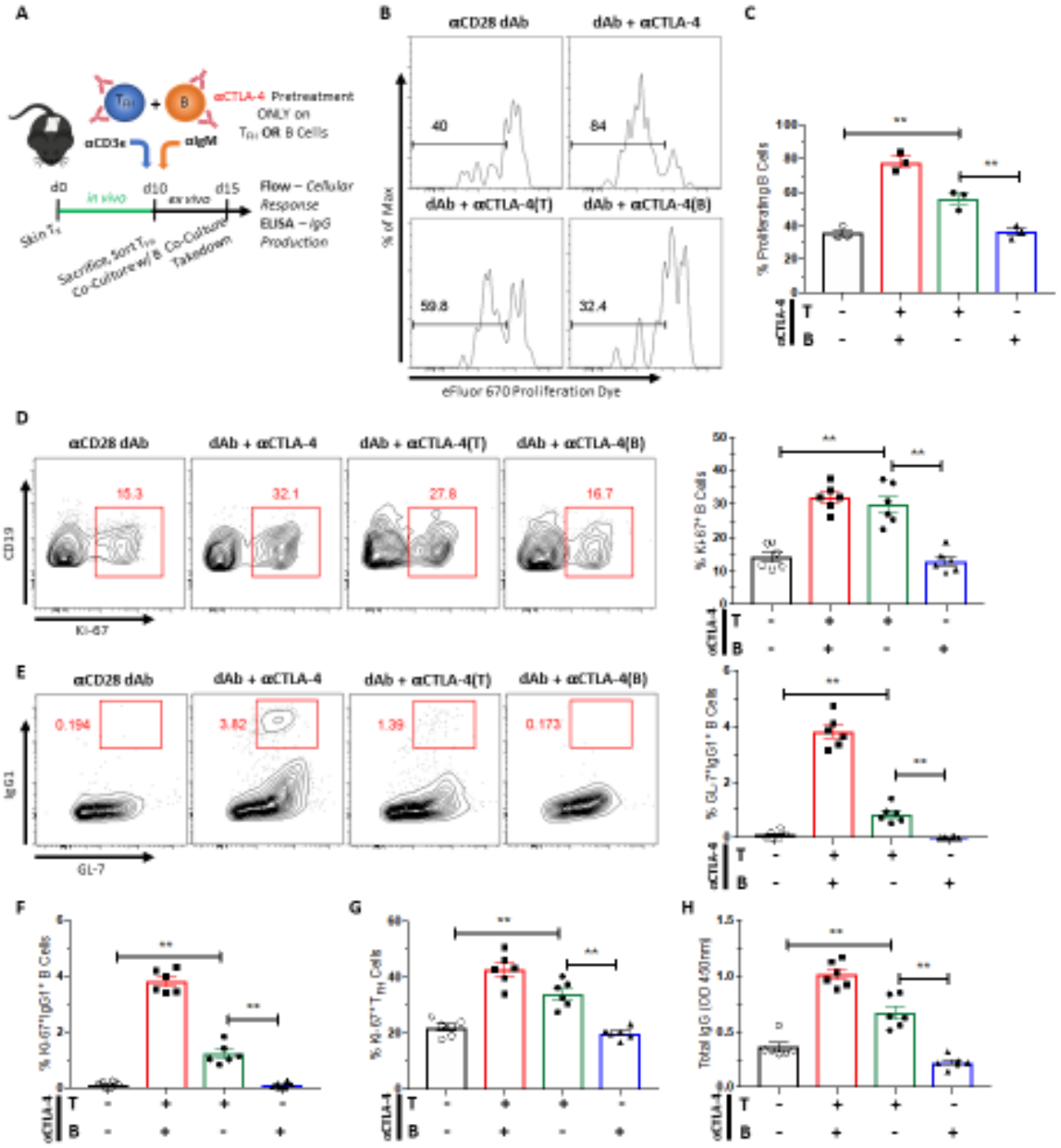


Figure 5. CTLA-4-dependent superior inhibition of selective CD28 Blockade is Tfh cell-specific

(A) Naïve B6 mice were transplanted with BALB/c skin, left untreated, and sacrificed 10 days post-transplant. Tfh and B cells from graft-DLNs were isolated and co-cultured for 5 days. Tfh:B cell co-cultures were treated with anti-CD28 dAb alone, anti-CD28 dAb plus anti-CTLA-4 mAb, or

anti-CD28 dAb plus individual pre-treatment of either Tfh or B cells with anti-CTLA-4 mAb. (B) Representative histograms depicting B cell proliferation by eFluor670 proliferation dye under the indicated conditions. (C) Summary data of the frequencies of proliferating B cells (n=3 per group). (D) Representative flow plots of B cell proliferation as measured by Ki-67 and summary data of the frequencies of proliferating Ki-67$^+$ B cells (n=6 per group). (E) Representative flow plots of class-switched GC-like GL7$^+$IgG1$^+$ B cells and summary data of the frequencies of these class-switched B cells (n=6 per group). Summary data of the frequencies of (F) proliferating class-switched B cells (n=6 per group), (G) proliferating Tfh cells (n=6 per group), and (H) supernatant total IgG levels (n=6 per group). Summary data represent mean (SE) and are representative of at least 2 independent experiments, with cells pooled from 10 mice per experiment and 3-6 co-culture wells per treatment group. **$p < 0.01$.

References

1. Wolfe RA, Ashby VB, Milford EL, Ojo AO, Ettenger RE, Agodoa LY, et al. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. *N Engl J Med.* 1999;341(23):1725-30.
2. Evans RW, Manninen DL, Garrison LP, Jr., Hart LG, Blagg CR, Gutman RA, et al. The quality of life of patients with end-stage renal disease. *N Engl J Med.* 1985;312(9):553-9.
3. Hart A, Smith JM, Skeans MA, Gustafson SK, Wilk AR, Castro S, et al. OPTN/SRTR 2017 Annual Data Report: Kidney. *Am J Transplant.* 2019;19 Suppl 2:19-123.
4. Loupy A, Hill GS, and Jordan SC. The impact of donor-specific anti-HLA antibodies on late kidney allograft failure. *Nat Rev Nephrol.* 2012;8(6):348-57.
5. Djamali A, Kaufman DB, Ellis TM, Zhong W, Matas A, and Samaniego M. Diagnosis and management of antibody-mediated rejection: current status and novel approaches. *Am J Transplant.* 2014;14(2):255-71.
6. Jordan SC, Ammerman N, Choi J, Huang E, Peng A, Sethi S, et al. Novel Therapeutic Approaches to Allosensitization and Antibody-mediated Rejection. *Transplantation.* 2019;103(2):262-72.
7. Chong AS, Rothstein DM, Safa K, and Riella LV. Outstanding questions in transplantation: B cells, alloantibodies, and humoral rejection. *Am J Transplant.* 2019;19(8):2155-63.
8. Vincenti F, Rostaing L, Grinyo J, Rice K, Steinberg S, Gaite L, et al. Belatacept and Long-Term Outcomes in Kidney Transplantation. *N Engl J Med.* 2016;374(4):333-43.
9. Heher E, and Markmann JF. The Clearer BENEFITS of Belatacept. *N Engl J Med.* 2016;374(4):388-9.

10. Ford ML, Adams AB, and Pearson TC. Targeting co-stimulatory pathways: transplantation and autoimmunity. *Nature Reviews Nephrology.* 2014;10(1):14-24.

11. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, and Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity.* 1995;3(5):541-7.

12. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science.* 1995;270(5238):985-8.

13. Poirier N, Azimzadeh AM, Zhang T, Dilek N, Mary C, Nguyen B, et al. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. *Sci Transl Med.* 2010;2(17):17ra0.

14. Liu D, Krummey SM, Badell IR, Wagener M, Schneeweis LA, Stetsko DK, et al. 2B4 (CD244) induced by selective CD28 blockade functionally regulates allograft-specific CD8+ T cell responses. *J Exp Med.* 2014;211(2):297-311.

15. Zaitsu M, Issa F, Hester J, Vanhove B, and Wood KJ. Selective blockade of CD28 on human T cells facilitates regulation of alloimmune responses. *Jci Insight.* 2017;2(19).

16. Larsen CP, Pearson TC, Adams AB, Tso P, Shirasugi N, Strobert E, et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant.* 2005;5(3):443-53.

17. Badell IR, Elbein R, Bray RA, Gebel HM, Adams AB, and Larsen CP. Belatacept Monotherapy in Kidney Transplant Recipients with Failed Allografts Reduces Humoral

Sensitization in a Single Center Randomized Controlled Trial. *Am J Transplant.* 2019;19(S3):450.

18. Badell IR, La Muraglia GM, 2nd, Liu D, Wagener ME, Ding G, and Ford ML. Selective CD28 Blockade Results in Superior Inhibition of Donor-Specific T Follicular Helper Cell and Antibody Responses Relative to CTLA4-Ig. *Am J Transplant.* 2018;18(1):89-101.
19. Badell IR, and Ford ML. T follicular helper cells in the generation of alloantibody and graft rejection. *Curr Opin Organ Transplant.* 2016;21(1):1-6.
20. Walters GD, and Vinuesa CG. T Follicular Helper Cells in Transplantation. *Transplantation.* 2016;100(8):1650-5.
21. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol.* 2011;29:621-63.
22. Conlon TM, Saeb-Parsy K, Cole JL, Motallebzadeh R, Qureshi MS, Rehakova S, et al. Germinal Center Alloantibody Responses Are Mediated Exclusively by Indirect-Pathway CD4 T Follicular Helper Cells. *Journal of Immunology.* 2012;188(6):2643-52.
23. Kim EJ, Kwun J, Gibby AC, Hong JJ, Farris AB, 3rd, Iwakoshi NN, et al. Costimulation blockade alters germinal center responses and prevents antibody-mediated rejection. *Am J Transplant.* 2014;14(1):59-69.
24. Sage PT, Paterson AM, Lovitch SB, and Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity.* 2014;41(6):1026-39.
25. Wing JB, Ise W, Kurosaki T, and Sakaguchi S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity.* 2014;41(6):1013-25.

26. Wang CJ, Heuts F, Ovcinnikovs V, Wardzinski L, Bowers C, Schmidt EM, et al. CTLA-4 controls follicular helper T-cell differentiation by regulating the strength of CD28 engagement. *Proc Natl Acad Sci U S A.* 2015;112(2):524-9.

27. La Muraglia GM, 2nd, Wagener ME, Ford ML, and Badell IR. Circulating T follicular helper cells are a biomarker of humoral alloreactivity and predict donor-specific antibody formation after transplantation. *Am J Transplant.* 2019.

28. Kuiper HM, Brouwer M, Linsley PS, and van Lier RA. Activated T cells can induce high levels of CTLA-4 expression on B cells. *J Immunol.* 1995;155(4):1776-83.

29. Quandt D, Hoff H, Rudolph M, Fillatreau S, and Brunner-Weinzierl MC. A new role of CTLA-4 on B cells in thymus-dependent immune responses in vivo. *J Immunol.* 2007;179(11):7316-24.

30. Rozanski CH, Arens R, Carlson LM, Nair J, Boise LH, Chanan-Khan AA, et al. Sustained antibody responses depend on CD28 function in bone marrow-resident plasma cells. *J Exp Med.* 2011;208(7):1435-46.

31. Sellares J, de Freitas DG, Mengel M, Reeve J, Einecke G, Sis B, et al. Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. *Am J Transplant.* 2012;12(2):388-99.

32. Crotty S. T Follicular Helper Cell Biology: A Decade of Discovery and Diseases. *Immunity.* 2019;50(5):1132-48.

33. Krummey SM, Cheeseman JA, Conger JA, Jang PS, Mehta AK, Kirk AD, et al. High CTLA-4 expression on Th17 cells results in increased sensitivity to CTLA-4 coinhibition and resistance to belatacept. *Am J Transplant.* 2014;14(3):607-14.

34. Watkins BK, Tkachev V, Furlan SN, Hunt DJ, Betz K, Yu A, et al. CD28 blockade controls T cell activation to prevent graft-versus-host disease in primates. *J Clin Invest.* 2018;128(9):3991-4007.

35. Shahinian A, Pfeffer K, Lee KP, Kundig TM, Kishihara K, Wakeham A, et al. Differential T cell costimulatory requirements in CD28-deficient mice. *Science.* 1993;261(5121):609-12.

36. Ferguson SE, Han S, Kelsoe G, and Thompson CB. CD28 is required for germinal center formation. *J Immunol.* 1996;156(12):4576-81.

37. Watanabe M, Fujihara C, Radtke AJ, Chiang YJ, Bhatia S, Germain RN, et al. Co-stimulatory function in primary germinal center responses: CD40 and B7 are required on distinct antigen-presenting cells. *J Exp Med.* 2017;214(9):2795-810.

38. Young JS, Chen J, Miller ML, Vu V, Tian C, Moon JJ, et al. Delayed Cytotoxic T Lymphocyte-Associated Protein 4-Immunoglobulin Treatment Reverses Ongoing Alloantibody Responses and Rescues Allografts From Acute Rejection. *Am J Transplant.* 2016;16(8):2312-23.

39. Bray RA, Gebel HM, Townsend R, Roberts ME, Polinsky M, Yang L, et al. De novo donor-specific antibodies in belatacept-treated vs cyclosporine-treated kidney-transplant recipients: Post hoc analyses of the randomized phase III BENEFIT and BENEFIT-EXT studies. *Am J Transplant.* 2018;18(7):1783-9.

40. Bray RA, Gebel HM, Townsend R, Roberts ME, Polinsky M, Yang L, et al. Posttransplant reduction in preexisting donor-specific antibody levels after belatacept- versus

cyclosporine-based immunosuppression: Post hoc analyses of BENEFIT and BENEFIT-EXT. *Am J Transplant.* 2018;18(7):1774-82.

41. Leibler C, Matignon M, Moktefi A, Samson C, Zarour A, Malard S, et al. Belatacept in renal transplant recipient with mild immunologic risk factor: A pilot prospective study (BELACOR). *Am J Transplant.* 2019;19(3):894-906.

42. Burghuber CK, Manook M, Ezekian B, Gibby AC, Leopardi FV, Song M, et al. Dual targeting: Combining costimulation blockade and bortezomib to permit kidney transplantation in sensitized recipients. *Am J Transplant.* 2019;19(3):724-36.

43. Vanhove B, Poirier N, Soulillou JP, and Blancho G. Selective Costimulation Blockade With Antagonist Anti-CD28 Therapeutics in Transplantation. *Transplantation.* 2019.

44. Sage PT, and Sharpe AH. In vitro assay to sensitively measure T(FR) suppressive capacity and T(FH) stimulation of B cell responses. *Methods Mol Biol.* 2015;1291:151-60.

Chapter 4:

Circulating T Follicular Helper Cells are a Biomarker of Humoral Alloreactivity and Predict Donor-Specific Antibody Formation after Transplantation

Figure 4.1. Circulating $CXCR5^+$ $CD4^+$ T cells expand following transplantation and display phenotypic and functional characteristics of cTfh cells.

Figure 4.2. Circulating Tfh cell kinetics parallel graft-dLN GC reactivity following transplantation.

Figure 4.3. Donor-reactive cTfh cells exhibit an activated $ICOS^+PD\text{-}1^+$ phenotype after transplantation.

Figure 4.4. Circulating Tfh cells precede DSA formation following transplantation.

Figure 4.5. Antigen-specific TCR transgenic cTfh cells display similar phenotypic characteristics and kinetics to endogenous alloreactive cTfh cells following transplantation.

Figure 4.6. Circulating Tfh cells predict breakthrough alloantibodies despite immunosuppression.

Figure 4.7. Delayed anti-CD28 CoB initiated after detection of $ICOS^+PD\text{-}1^+$ cTfh cells prevents DSA formation.

Abstract

Donor-specific antibodies (DSAs) contribute to renal allograft loss. However, biomarkers to guide clinical management of DSA posttransplant or detect humoral alloimmune responses before alloantibodies develop are not available. Circulating T follicular helper (cTfh) cells are $CD4^+CXCR5^+$ Tfh-like cells in the blood that have been associated with alloantibodies in transplant recipients, but whether they precede antibody formation for their evaluation as a predictive biomarker in transplant is unknown. To evaluate the ability of cTfh cells to predict DSA, we used murine transplant models to determine the temporal relationship between cTfh cells, germinal center formation, and DSA development. We observed that donor reactive $CD4^+CXCR5^+$ cTfh cells expand after allotransplant. These cTfh cells were equivalent to graft-draining lymph node-derived Tfh cells in their ability to provide B cell help for antibody production. cTfh cell expansion and differentiation into $ICOS^+PD\text{-}1^+$ cells temporally correlated with germinal center alloreactivity and preceded the generation of DSAs in instances of modified and unmodified alloantibody formation. Importantly, delayed costimulation blockade initiated after the detection of $ICOS^+PD\text{-}1^+$ cTfh cells prevented DSAs. These findings suggest that cTfh cells could serve as a biomarker for humoral alloreactivity before the detection of alloantibodies and inform therapeutic approaches to prevent DSAs.

Introduction

Over the last several decades, advances in solid organ transplantation have significantly reduced acute rejection rates leading to great improvements in short-term kidney allograft survival (1). However, long-term outcomes following kidney transplantation remain suboptimal. Historically, late kidney allograft failure was chiefly attributed to calcineurin inhibitor toxicity and chronic allograft nephropathy (2), but it is now well recognized that donor-specific HLA antibodies are an important immunologic cause of acute and chronic allograft injury that shorten renal allograft survival (3). These anti-HLA donor-specific antibodies (DSA) exist either pre-transplant or arise *de novo* after transplant and are associated with inferior transplant outcomes (4, 5). Despite the prevalence and deleterious impact of HLA antibodies in kidney transplantation, diagnostic biomarkers to reliably guide clinical management of pathologic DSA have not been developed (6).

While the availability of sensitive assays for the identification of HLA antibodies has greatly enhanced pre-transplant donor-recipient matching to reduce the incidence of early antibody-mediated rejection (7), their utility in the post-transplant setting has been limited (8, 9). Once anti-donor antibodies are detected, reproducible therapeutic options to curtail the natural history of antibody-mediated kidney allograft injury are not clinically available (3, 6). Therefore, the value of detecting DSA to either direct the initiation of treatment strategies aimed at inhibiting alloantibody-mediated injury or measure the effectiveness of such strategies is lacking. As such, there is great need for the development of alternative biomarkers that can diagnose the

presence of humoral alloimmunity in kidney transplant recipients and facilitate the initiation of therapeutic interventions to prevent premature allograft loss.

T follicular helper (Tfh) cells are a lineage of $CD4^+$ T cells distinguishable by their unique expression of the chemokine receptor CXCR5 and are required for the provision of B cell help to generate class-switched, high affinity antibody responses for an effective humoral immune response (10). Tfh cells have now been implicated in many immune processes during health and disease (11), and inhibition of their differentiation and function prevents the development of anti-donor antibody responses in transplantation (12-14). Hence the detection of Tfh cells as a new biomarker for alloantibodies in transplantation is theoretically appealing, but access to Tfh cells is limited in humans because they are primarily present in secondary lymphoid organs (SLO) and are therefore not accessible via non-invasive methods for diagnostic purposes. However, newly identified $CD4^+CXCR5^+$ circulating Tfh (cTfh) cells in peripheral blood have been recently shown to correlate with vaccine responses and autoimmune disease activity in experimental animal models and humans (15, 16), and thus may overcome this barrier to accessibility. Although there have been a few observational reports associating cTfh cells with alloantibodies in transplant recipients (17-19), the temporal relationship of cTfh cells to DSA and their ability to function as a predictive biomarker in transplantation is unknown.

In this study, we used murine transplant models to test the ability of cTfh cells to predict the development of alloantibodies by determining the temporal relationship between both polyclonal endogenous and antigen-specific cTfh cells, germinal center (GC) reactivity and DSA

formation. We demonstrate that alloreactive $CXCR5^+$ $CD4^+$ T cells detected in the blood after transplant are phenotypically and functionally cTfh cells. The expansion kinetics of donor-reactive cTfh cells and their phenotypic differentiation into $ICOS^+PD\text{-}1^+$ cells temporally correlated with GC alloreactivity and predicted the generation of DSA. Delayed treatment with costimulation blockade initiated after transplant upon the detection of $ICOS^+PD\text{-}1^+$ cTfh cells successfully inhibited alloantibody formation. Thus alloreactive cTfh cells could potentially serve as a clinical biomarker for nascent or ongoing humoral alloimmune responses before the detection of anti-donor antibodies and inform therapeutic approaches to prevent DSA.

Materials and Methods

2.1 *Mice*

B6-Ly5.1/Cr (H2-K^b) and BALB/c (H-2K^d) mice were obtained from the National Cancer Institute. Ovalbumin (OVA)-specific TCR transgenic OT-I (20) and OT-II (21) mice purchased from Taconic Farms were bred to Thy1.1$^+$ background from The Jackson Laboratory at Emory University. C57BL/6 mice that constitutively express membrane-bound OVA (mOVA) under the β-actin promoter (22) were a gift from M. Jenkins (University of Minnesota). All animals were housed in pathogen-free facilities and maintained in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

2.2 *T cell adoptive transfers, skin transplantation, and immunosuppression*

For adoptive transfers of OVA-specific T cells, splenocytes isolated from Thy1.1$^+$ OT-I and Thy1.1$^+$ OT-II mice were quantified via TruCount bead analysis (BD Biosciences) and 1.0x10^6 of each Thy1.1$^+$ OT-I and Thy1.1$^+$ OT-II T cells was injected intravenously into naïve B6-Ly5.1/Cr mice 24-48 hours prior to skin transplantation. Bilateral dorsal full-thickness tail and ear skin were transplanted onto recipient mice (23). Skin graft recipients received no treatment, anti-CD28 domain antibody (dAb) (100 μg, Bristol-Myers Squibb), CTLA-4-Ig (200 μg, Bristol-Myers Squibb) or tacrolimus (5 mg/kg, Astellas). Anti-CD28 dAb and CTLA-4-Ig were administered intraperitoneally on post-transplant days 0, 2, 4, 6 and 8 and then weekly. Delayed anti-CD28 dAb was administered on days 7, 9, 11, 13 and 15 and then weekly. Tacrolimus was administered s.c. daily with mean trough levels of X.

2.3 *Flow cytometry*

Graft-draining axillary and brachial lymph nodes were processed into single-cell suspensions. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation of whole blood preparations. Cells were surface stained for indicated markers and pulsed with LIVE/DEAD viability dye (Molecular Probes) before fixation. Intracellular staining was performed with Foxp3 Fixation/Permeabilization Buffer Kit (eBioscience). All antibodies were from BioLegend and BD Biosciences. All samples were run on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed by using FlowJo Software (Flowjo, LLC). CountBright Beads (Invitrogen) were used to determine absolute cell counts.

2.4 *T:B cell co-culture*

T and B cells from blood and graft-dLNs were enriched, flow sorted and co-cultured as previously described (24). Briefly, magnetic bead negative selection (Miltenyi Biotec) was used to enrich $CD4^+$ T and $CD19^+$ B cells. T cells from dLNs were flow sorted into $CXCR5^-$ ($CD19^-CD4^+PD\text{-}1^{int}CXCR5^-GITR^-$) and Tfh ($CD19^-CD4^+PD\text{-}1^{hi}CXCR5^+GITR^-$) cells, and from blood into $CXCR5^-$ ($CD19^-CD4^+CXCR5^-GITR^-$) and cTfh ($CD19^-CD4^+CXCR5^+GITR^-$) cells on a FACSAria II (BD Biosciences). $3x10^4$ T cells were cultured with $5x10^4$ B cells in 96-well plates in anti-CD3ε (2C11, 2 μg/mL, BioXcell) and anti-IgM (5 μg/mL, Jackson Immunoresearch) containing media for 6 days. Cultured cells were analyzed by flow cytometry and supernatants for total IgG levels by ELISA.

2.5 *Anti-donor antibody assessment*

For flow cytometric crossmatch, BALB/c splenocytes were processed into single-cell suspensions and pre-treated with Fc Block (BioLegend), followed by incubation with recipient serum at 4°C. Splenocytes were then washed and labeled with surface markers and anti-mouse IgG (BioLegend) for quantification of anti-BALB/c IgG by flow cytometry. For ELISA anti-OVA antibody measurements, flat-bottom 96-well Immulon 4HBX microtiter plates (VWR) were coated with OVA protein (100 μg/well; Sigma-Aldrich) overnight at 4°C, blocked with 10% FBS in PBS-T for 1 hour at 37°C, and then incubated with serum samples for 1.5 hours at 37°C. OVA-specific immunoglobulins were detected with HRP goat anti-mouse IgG (Poly4053, BioLegend), developed by using the TMB substrate system (Thermo Scientific), and read at 450 nm on a Spectra MAX 340PC Microplate reader (Molecular Devices).

2.6 *Statistical analysis*

The Mann–Whitney U nonparametric t test was performed for analysis of unpaired groups, and the Holms–Sidak method was used for grouped, multiple t test analyses. All analyses were performed by using GraphPad Prism (GraphPad Software, Inc.). Statistical significance was attributed to p values <0.05 (*<0.05, **<0.01, ***<0.001).

Results

3.1 *$CXCR5^+$ $CD4^+$ T cells are detectable in the blood, expand after transplant, and exhibit phenotypic and functional characteristics of cTfh cells*

To interrogate the $CXCR5^+$ $CD4^+$ T cell response in the blood following transplantation we utilized a full MHC mismatch BALB/c to B6 murine skin transplant model (Figure 1A). Naïve B6 recipients were transplanted bilateral full thickness skin grafts from either syngeneic (B6) or allogeneic (BALB/c) donor mice and sacrificed 10 days post-transplant for PBMC and graft-draining lymph node (dLN) analysis. $CXCR5^+$ $CD4^+$ T cells were identified in the blood posttransplant (Figure 1B) and exhibited robust expansion in response to the BALB/c allograft as compared to the syngeneic B6 graft (Figure 1C). Analysis of these $CXCR5^+$ $CD4^+Foxp3^-$ T cells revealed a less polarized but similar phenotype to graft-dLN $CXCR5^+PD\text{-}1^{hi}$ Tfh cells (Figures 1D, E), and a distinct phenotype from other circulating naïve and antigen-experienced $CD4^+$ T cells (Figure 1F). They exhibited greater expression of the Tfh markers CXCR5, PD-1, TCF-1, TIGIT and GL-7 relative to antigen-experienced ($CD44^{hi}CXCR5^-$) $CD4^+$ T cells (10, 25), as well as increased expression of the costimulatory molecules CD28 and ICOS, and the proliferation marker Ki-67.

To test the ability of these circulating $CXCR5^+$ T cells to provide B cell help, we performed in vitro T:B cell co-cultures of donor-reactive $CXCR5^-$ and $CXCR5^+$ $CD4^+CD44^+$ T cells from the blood and dLNs of transplanted mice. In contrast to $CXCR5^-$ cells, circulating $CXCR5^+$ cells induced GC-like B cell differentiation, class switching and IgG antibody production equivalent to their $CXCR5^+$ Tfh cell DLN counterparts (Figures 1G, H). Taken together, these data indicate that alloreactive

CXCR5$^+$ CD4$^+$ T cells in the peripheral blood are indeed cTfh cells that expand in response to an allograft and display the unique phenotypic and functional characteristics of Tfh cells (16, 26-28).

3.2 *Circulating Tfh cell kinetics parallel graft-dLN GC reactivity after transplantation*

The idea of utilizing cTfh cells as a surrogate marker for GC activity and protective antibody formation in SLOs has been introduced in HIV and influenza vaccination (28, 29). To begin to investigate whether cTfh cells could serve as a potential indicator of GC activity in transplantation, we transplanted B6 recipient mice with syngeneic (B6) or allogeneic (BALB/c) skin grafts and measured cTfh cell kinetics in relation to GC Tfh and B cell reactivity in graft-DLNs. We detected a rise in the frequency and number of cTfh cells that peaked on day 10 and contracted by day 21 (Figures 2A, B). Similarly, GC alloreactivity as measured by Tfh cell differentiation and GC B cell expansion in the DLNs also peaked 10 days after transplant with contraction beginning by day 14 (Figures 2A, C, D). Graft-elicited antibody secreting cells (CD19$^-$ and CD19$^+$ IgD$^-$B220$^-$CD138$^+$ B cells) peaked 10 days post-transplant, while memory B cells (IgG$^-$ and IgG$^+$ IgD$^-$B220$^+$CD38$^+$CD80$^+$) did not exhibit any significant changes over time (data not shown). Collectively, these data show that cTfh cell kinetics reliably reflect GC kinetics in SLOs (Figure 2E) and therefore could potentially be used as a biomarker for GC reactivity in the peripheral blood.

3.3 *Donor-reactive cTfh cells exhibit an activated ICOS$^+$PD-1$^+$ phenotype after transplantation*

Due to previously described heterogeneity of CXCR5$^+$ cTfh cells in relation to antigen exposure or disease activity (11, 30), we sought to determine whether phenotypic differences developed

within the cTfh cell subset after transplantation. Thus, we examined the cTfh cell phenotype in naïve and syngeneic or allogeneic skin-grafted mice 10 days post-transplantation. Phenotypic analysis revealed significant differences in the expression of the Tfh activation markers PD-1 and ICOS on cTfh cells in response to an allograft as compared to an isograft or in naïve mice (Figure 3A). This observation prompted the assessment of PD-1 and ICOS co-expression on these cTfh cells (Figure 3B). Interestingly, we observed that the alloreactive cTfh cells were composed of a significantly greater fraction of $ICOS^{+}PD\text{-}1^{+}$ cells (Figures 3C, D), and that the increase in frequency and number of cTfh cells with this polarized phenotype resulted from alloantigen exposure, as the phenotype did not manifest in syngeneic-grafted or naïve mice. This $ICOS^{+}PD\text{-}1^{+}$ cTfh cell population showed greater expression of CXCR5, TCF-1, CD154, Ki-67, GL-7 and other markers of Tfh cell activation as compared to less polarized $ICOS^{-}PD\text{-}1^{-}$ cTfh cells (Figure 3E). Intriguingly, this $ICOS^{+}PD\text{-}1^{+}$ cTfh cell subset began to manifest as early as 7 days after transplant, with a 3-fold increase over baseline (day 0) by day 7 and 4-fold increase at the peak of the GC response on day 10 (Figures 3F-H). These findings indicate that cTfh cells develop a distinguishable activated phenotype after allotransplantation that emerges earlier (day 7) than the discernible rise in the quantity of cTfh cells (day 10, Figure 2) and could predict alloantibody formation.

3.4 *Circulating Tfh cells precede DSA formation after transplantation*

Based on the observation that detectable changes in the quantity and quality of cTfh cells correspond with GC alloreactivity (Figures 2, 3), we next sought to determine the kinetics of DSA formation in our model. Naïve B6 recipients received either B6 or BALB/c skin grafts and were serially bled for serum collection and flow crossmatch analysis to test for the development of

anti-donor antibodies. As expected, the syngeneic graft recipients did not develop DSA, while the allogeneic graft recipients formed anti-BALB/c alloantibodies by posttransplant day 14 with peak titers by day 28 (Figures 4A, B). Comparison of our observed cTfh cell kinetics to the onset of DSA formation demonstrates that the rise in frequency and number of cTfh cells in the blood begins and peaks before DSAs are first detectable in the serum 14 days after transplant (Figure 4C). Interestingly, manifestation of the activated $ICOS^+PD\text{-}1^+$ phenotype within the cTfh cell population occurs even earlier (day 7, Figure 3) than the increase in total alloreactive cTfh cells (day 10, Figure 2) and precedes the generation of DSA by 7 days (Figure 4D). Thus, changes in the quantity of cTfh cells and their phenotype could provide a non-invasive means of predicting *de novo* DSA formation following transplantation.

3.5 *Antigen-specific TCR transgenic cTfh cells display similar phenotypic characteristics and kinetics to endogenous alloreactive cTfh cells following transplantation*

We previously reported that donor-reactive endogenous Tfh cells in graft-dLNs exhibited the same phenotypic and differentiation characteristics as transgenic antigen-specific Tfh cells in response to a donor skin graft (14). To characterize the antigen-specific cTfh cell response, we utilized our established TCR transgenic mOVA murine skin transplant model (31) to track donor-specific TCR transgenic cTfh cells. $Thy1.1^+$ $CD4^+$ OT-II and $CD8^+$ OT-I T cells were adoptively transferred into naive B6 mice, which were then transplanted with skin from either syngeneic (B6) or minor antigen-mismatched (mOVA) donors (Figure 5A). Mice were then serially sacrificed for PBMC, dLN and serum analyses. OVA-specific $Thy1.1^+CXCR5^+$ cTfh cells were detected in the peripheral blood in response to the mOVA grafts but not the B6 isografts (Figures 5B, C), peaking

in frequency and number 10 days after transplant. They exhibited a Tfh-like phenotype with increased expression of CXCR5, PD-1, and ICOS relative to Thy1.1^{+}CXCR5^{-} and naïve CD4^{+} OT-II T cells (Figure 5D). Notably, these antigen-specific OT-II cTfh cells were composed of a substantial proportion of ICOS^{+}PD-1^{+} cells (Figures 5E, F) that were detectable 7 days after transplant (Figure 5G). Examination of the OT-II Tfh cell response in the graft-dLNs correlated with Thy1.1^{+} cTfh cell kinetics (data not shown), and most importantly, the rise in antigen-specific cTfh cells in the blood preceded the development of anti-OVA DSA in mOVA graft recipients (Figures 5H, I). As such, TCR transgenic donor-specific cTfh cells manifest similar phenotypic features and expansion kinetics to endogenous, alloreactive cTfh cells in relation to the generation of DSA.

3.6 *Circulating Tfh cells predict breakthrough alloantibodies despite immunosuppression*

Preventing *de novo* DSA in our mouse skin graft models with immunomodulatory therapy correlates with GC inhibition in the dLNs (14), but the impact of immunosuppression on alloreactive cTfh cells and their ability to predict DSA is not known. Hence, we sought to determine the relationship between cTfh cells, immunosuppression, and DSA inhibition. Naïve skin-grafted mice were treated with CD28 costimulation blockade (CoB, CTLA-4-Ig or anti-CD28 dAb), tacrolimus, or, left untreated. Peripheral blood analysis on post-transplant day 10 showed that immunosuppression resulted in significant reductions in the frequency and number of total cTfh cells and the ICOS^{+}PD-1^{+} cTfh cell subset observed in response to an allogeneic graft as compared to untreated controls (Figures 6A-D). Tfh and GC B cells in the graft-dLNs were also reduced (Figures 6E, F), along with complete inhibition of DSA formation with the more potent anti-CD28 dAb (Figure 6G). Importantly, cTfh cell quantity and phenotype positively correlated

with breakthrough GC reactivity and preceded DSA under less potent immunosuppression with tacrolimus, and CTLA-4-Ig (Figures 6A-G). Therefore, alloreactive cTfh cell expansion and phenotypic differentiation predicted DSA in instances of both modified and unmodified alloantibody formation.

3.7 *Delayed anti-CD28 CoB initiated after detection of ICOS$^+$PD-1$^+$ cTfh cells prevents DSA formation*

Having determined that cTfh cells predict DSA in our model, we next tested whether initiating immunosuppression after transplant upon the detection of alloreactive cTfh cells could prevent the generation of alloantibodies. Naïve B6 mice were transplanted BALB/c skin and their blood examined for the development of ICOS$^+$PD-1$^+$ cTfh cells. Once detected on posttransplant day 7 (Figures 7A-D), immunosuppression was initiated with anti-CD28 dAb. Strikingly, delayed treatment with anti-CD28 CoB abrogated continued expansion and differentiation of donor-reactive cTfh cells and inhibited the development of DSA compared to untreated controls (Figures 7E, F). As with the initiation of treatment at the time of transplant (Figure 6), the rise and fall of the cTfh cell response was related to the formation of anti-donor antibodies. As such, immunosuppression initiated after the detection of alloreactive ICOS$^+$PD-1$^+$ cTfh cells in the blood successfully inhibited DSA formation.

Discussion

Circulating Tfh cells have been shown to correlate with autoimmune disease, vaccine responses and broadly neutralizing antibodies against HIV (26-29, 32). Their presence in the blood, relation to GC Tfh cells in SLOs, and ability to indicate GC reactivity has introduced the possibility of utilizing them as a biomarker for alloantibody responses in transplantation (11, 16). While Tfh cells have generated considerable interest in transplantation and have been implicated in anti-donor antibody responses (12-14, 33), little is known regarding the cTfh cell response to an allograft. Two observational studies have reported that $CXCR5^+$ $CD4^+$ T cells in the blood of kidney transplant recipients are present at higher frequencies in cases of chronic rejection and *de novo* DSA (17, 18), and a more recent study observed higher frequencies of cTfh cells in sensitized transplant recipients with greater expansion post-transplant in patients that developed *de novo* HLA antibodies (19). Conversely, reduced fractions of activated cTfh cells have been reported in operationally tolerant (34) and belatacept-treated (35) renal transplant recipients. While these studies suggest an association between cTfh cells and humoral alloimmune phenotype in human transplant recipients, experimental data examining the temporal relationship between cTfh cells and alloantibody formation for their evaluation as a biomarker in transplantation are needed.

In this study, we provide experimental evidence that alloreactive, antigen-specific cTfh cells functionally capable of providing B cell help for antibody production are detectable in the blood and expand after transplant, correlate with ongoing GC alloreactivity in graft-dLNs and precede the manifestation of anti-donor antibodies. Intriguingly, we also observed temporal polarization of cTfh cells towards an activated $ICOS^+PD\text{-}1^+$ phenotype in an allo-dependent manner post-

transplant that preceded DSA formation earlier than the quantitative rise in cTfh cells. Notably, the development of cTfh cells occurred in the context of modified and unmodified DSA formation (Figure 6), and the detection of $ICOS^+PD\text{-}1^+$ cTfh cells in the blood successfully prompted initiation of immunosuppression to prevent DSA (Figure 7).

Blood Tfh cells have been primarily characterized as antigen-experienced $CXCR5^+$ $CD4^+$ memory T cells that accumulate after repeated antigen exposures and have the ability to surveil SLOs with the purpose of accelerating memory antibody responses (36, 37). However, considerable phenotypic and functional heterogeneity has been observed within cTfh cells to suggest that the total pool of $CXCR5^+$ $CD4^+$ T cells is comprised of a variety of different subsets with varying degrees of differentiation that include both quiescent, memory-like subsets and more activated, effector-like subsets indicative of active Tfh cell differentiation in lymphoid organs (16, 30, 38). Thus, fluctuations in the composition of the cTfh cell pool may reflect periods of active humoral immune reactivity.

Several studies have demonstrated that the expression of ICOS and PD-1 on cTfh cells correlates with autoimmune disease activity and protective antibody responses following vaccination (27-29), and that the absence or low levels of ICOS or PD-1 on cTfh cells are indicative of a resting, inactive state. In clinical transplantation, allograft recipients with complex immune histories are likely to have heterogenous pools of polyreactive $CXCR5^+$ cTfh cell memory, where detecting acute shifts in the phenotypic composition of these blood Tfh cells may specifically indicate active humoral alloreactivity. While the observed changes in cTfh cells in this study are alloantigen-

elicited, similar changes may occur to any antigen (i.e. infectious pathogens) that stimulates a GC-dependent antibody response. As such, continued investigation of activated cTfh cell subsets like the $ICOS^{+}PD\text{-}1^{+}$ subset recognized in this study may facilitate identification and tracking of allo-specific T cells responsible for alloantibody responses. Alternatively, examining the role of alloreactive B cell lineage cells in the blood may also aid in the identification of donor-specific cellular biomarkers of alloantibodies. In light of these considerations, our data on the manifestation of an alloreactive $ICOS^{+}PD\text{-}1^{+}$ cTfh cell population with increased expression of Ki-67, CD154 and other Tfh program-specific markers in response to an allogeneic skin graft is noteworthy and consistent with published observations, further supporting their potential as a biomarker for humoral alloimmunity.

Our findings are proof of concept that quantitative and qualitative changes in blood Tfh cells are a cellular indicator of alloantibody formation and could guide clinical management strategies to mitigate the negative impact of DSA following transplantation. While the period between the detection of phenotypic changes in cTfh cells and the generation of DSA in our experimental mouse model of unmodified antibody formation is seven days and may afford enough of a clinical window to prevent alloantibodies in certain scenarios, the window of opportunity for intervention is very likely to be months or years in clinical transplantation under standard immunosuppression (3, 39).

Antibody-mediated injury and the emergence of *de novo* DSA in the clinic most often occur several years after transplant and have been associated with prior episodes of cellular rejection

and subclinical histopathologic changes months to years before the serologic detection of DSA (5, 40). Thus, in the presence of immunosuppression, a cTfh cell signature in the blood may be present and detectable during these protracted clinical periods of occult germinal center alloreactivity. In support of this hypothesis, the development of breakthrough DSA under modified conditions with tacrolimus or CTLA-4-Ig in this study was in fact preceded by cTfh cell changes in the blood more than 7 days prior to the detection of DSA (Figure 6). This observation supports the notion that changes in cTfh cells during episodes of allograft dysfunction or periodically over the course of a transplant could be used as an indicator of developing humoral alloimmunity before the serologic detection of alloantibodies. It is probable that the rate of Tfh cell differentiation, GC reactivity and the development of de novo DSA is a net result of the balance between the nature of alloantigen along with the immune environment, and the quantity and quality of immunosuppression.

Given the smoldering nature and lack of effective clinical therapies to combat alloantibody formation and DSA-mediated injury (3, 6, 40), the ability of delayed immunosuppression initiated upon identification of alloreactive $ICOS^{+}PD\text{-}1^{+}$ cTfh cells before the development of DSA to inhibit the humoral response is of clinical interest. Young et al. have previously shown that delayed treatment with a higher dose and greater frequency of CTLA-4-Ig administration also reversed ongoing alloantibody responses in a mouse heterotopic heart transplant model (41), but delayed treatment was arbitrarily initiated and not prompted by the identification of a potential biomarker as in this study. These findings are crucial to considering the clinical applicability and

potential translation of cTfh cells as a biomarker for humoral alloimmune monitoring in transplant recipients, and also supports the use of CoB as rescue therapy to prevent DSA.

In summary, this study provides evidence that quantitative and phenotypic changes in alloreactive, donor-specific cTfh cells in the blood of transplanted mice indicate GC alloreactivity and precede *de novo* DSA formation. Our data highlight the potential for cTfh cells to be utilized as a predictive biomarker for humoral alloreactivity and the generation of DSA in clinical transplantation that could trigger the initiation of therapy to prevent alloantibody-mediated allograft injury. These findings support additional investigation of cTfh cells in vascularized transplant models and humans to enhance their applicability as a clinically relevant cellular biomarker for the diagnosis or prediction of HLA antibody-mediated processes in transplantation.

Figures and Figure Legends

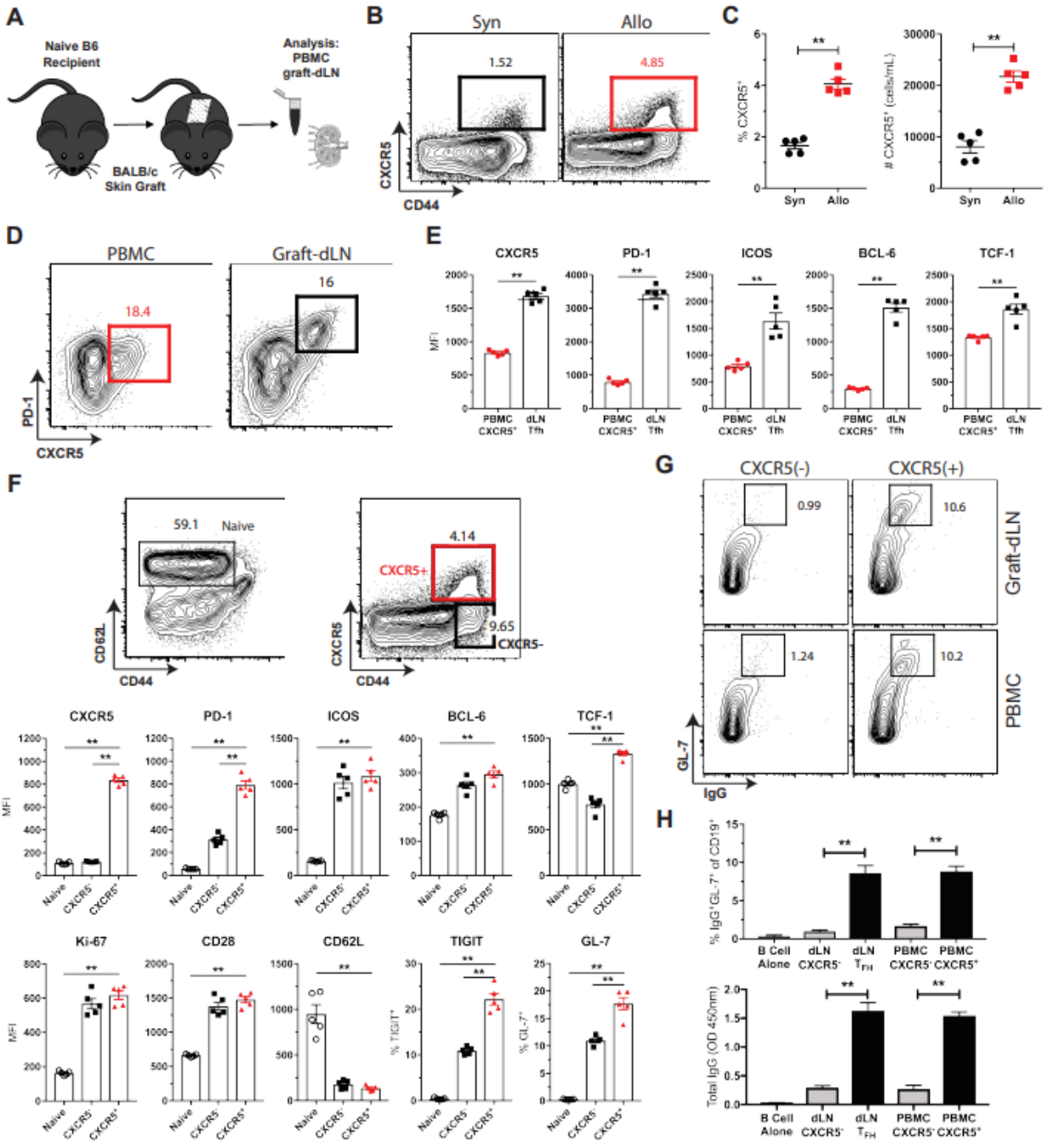


Figure 1. Circulating CXCR5$^+$ CD4$^+$ T cells expand following transplantation and display phenotypic and functional characteristics of cTfh cells.

(A) Naïve B6 mice were transplanted with skin from either B6 (Syn) or BALB/c (Allo) donors and sacrificed 10 days post-transplantation for PBMC and graft-dLN analysis. (B) Flow cytometric plots

(gated on $CD4^+Foxp3^-$ T cells) displaying the frequencies of $CXCR5^+$ T cells. (C) Summary data of the frequencies and numbers of cTfh cells (n=5 per group). (D) Representative flow plots (gated on $CD4^+Foxp3^-$ T cells) of blood-derived $CXCR5^+$ (PBMC) and graft-dLN Tfh cells as defined by CXCR5 and PD-1. (E) Summary data of phenotypic marker expression by $CXCR5^+$ PBMCs and graft-dLN Tfh cells (n=5 per group). (F) Flow plots (gated on $CD4^+Foxp3^-$ T cells) depict gating strategy for naïve ($CD44^{lo}CD62L^+$), antigen-experienced ($CD44^{hi}CXCR5^-$) and $CXCR5^+$ T cell populations with summary data of phenotypic marker expression by each subset, respectively (n=5 per group). (G) Representative flow plots (gated on $CD19^+$IA-IE^+ B Cells) displaying the frequencies of GC-like B cells following 6-day *in vitro* T:B cell co-culture from BALB/c transplanted mice. (H) Summary data of the frequencies of $IgG^+GL\text{-}7^+$ B cells and total IgG after T:B cell co-culture (n=3 with cells pooled from 5-10 mice). Summary data represent mean (SE) and are representative of 2-4 independent experiments with a total of 10-20 mice per group. *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

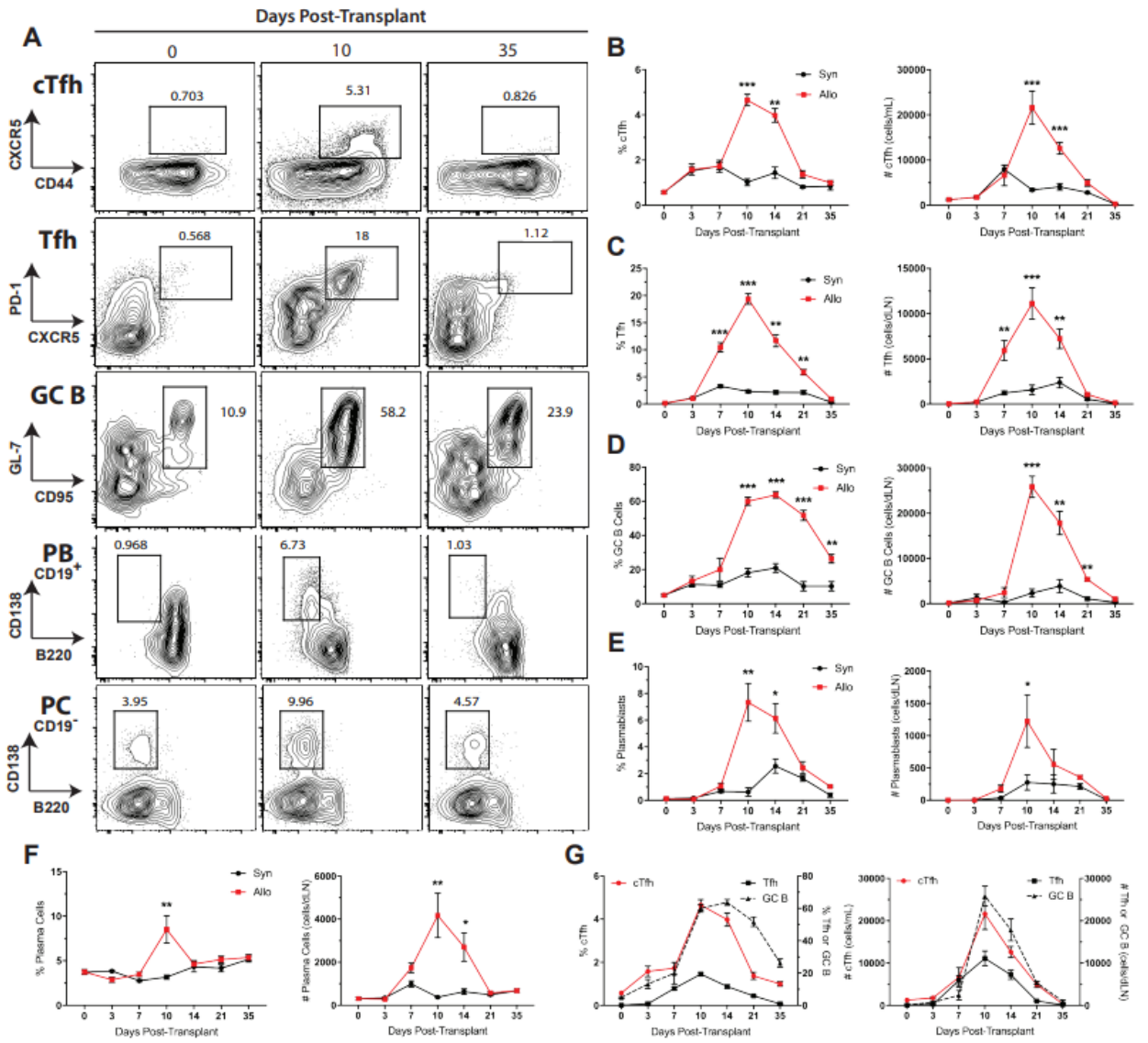


Figure 2. Circulating Tfh cell kinetics parallel graft-dLN GC reactivity following transplantation.

Naïve B6 mice were transplanted with skin from either B6 (Syn) or BALB/c (Allo) donors and serially sacrificed post-transplantation for PBMC and graft-dLN analysis. (A) Representative flow plots displaying the frequencies of blood cTfh (gated on $CD4^{+}Foxp3^{-}$ T cells), graft-dLN Tfh (gated on $CD4^{+}Foxp3^{-}CD44^{hi}$ T cells) and GC B (gated on $CD19^{+}IgD^{-}B220^{+}CD138^{-}$ B cells) cells over time. Summary data of (B) cTfh cell, (C) graft-dLN Tfh cell and (D) GC B cell frequencies and numbers over time (n=5 per group). (E) Summary data of cTfh cell frequencies and numbers relative to

graft-dLN Tfh and GC B cell frequencies and numbers, respectively (n=5 per group). Summary data represent mean (SE) and are representative of three independent experiments with a total of 15 mice per group. *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

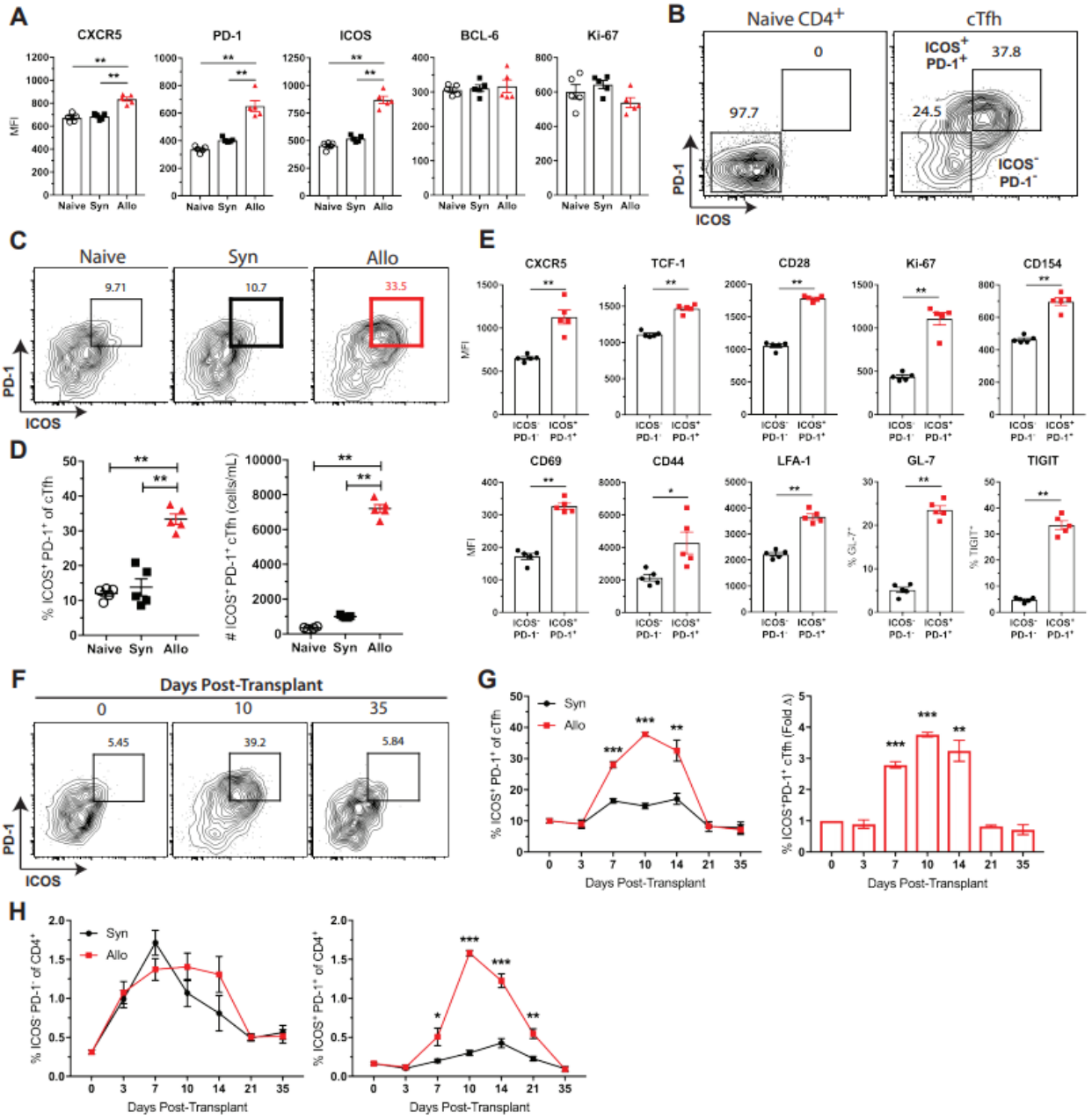


Figure 3. Donor-reactive cTfh cells exhibit an activated ICOS$^+$PD-1$^+$ phenotype after transplantation.

Naïve B6 mice were transplanted with skin from either B6 (Syn) or BALB/c (Allo) donors and sacrificed for PBMC analysis. (A) Summary data of phenotypic marker expression on cTfh cells from naïve, syngeneic and allogeneic skin-grafted mice ten days after transplant (n=5 per group).

(B) Representative flow plots depicting ICOS and PD-1 expression on naïve ($CD44^{lo}CD62L^{+}$) $CD4^{+}$ T cells and $CXCR5^{+}$ cTfh cells. (C) Representative flow plots (gated on $CD4^{+}Foxp3^{-}CXCR5^{+}$ T cells) displaying the frequencies of $ICOS^{+}PD\text{-}1^{+}$ cTfh cells. (D) Summary data of the frequencies and numbers of $ICOS^{+}PD\text{-}1^{+}$ cTfh cells (n=5 per group). (E) Summary data of phenotypic marker expression on $ICOS^{-}PD\text{-}1^{-}$ and $ICOS^{+}PD\text{-}1^{+}$ cTfh cells (n=5 per group). (F) Representative flow plots (gated on $CD4^{+}CXCR5^{+}$ T cells) displaying the frequencies of $ICOS^{+}PD\text{-}1^{+}$ cTfh cells over time. (G) Summary data of $ICOS^{+}PD\text{-}1^{+}$ cTfh cell frequencies and fold change over time (n=5 per group). (H) Summary data of $ICOS^{-}PD\text{-}1^{-}$ and $ICOS^{+}PD\text{-}1^{+}$ cTfh cell frequencies of $CD4^{+}$ T cells over time. Summary data represent mean (SE) and are representative of 3-4 independent experiments with a total of 15-20 mice per group. *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

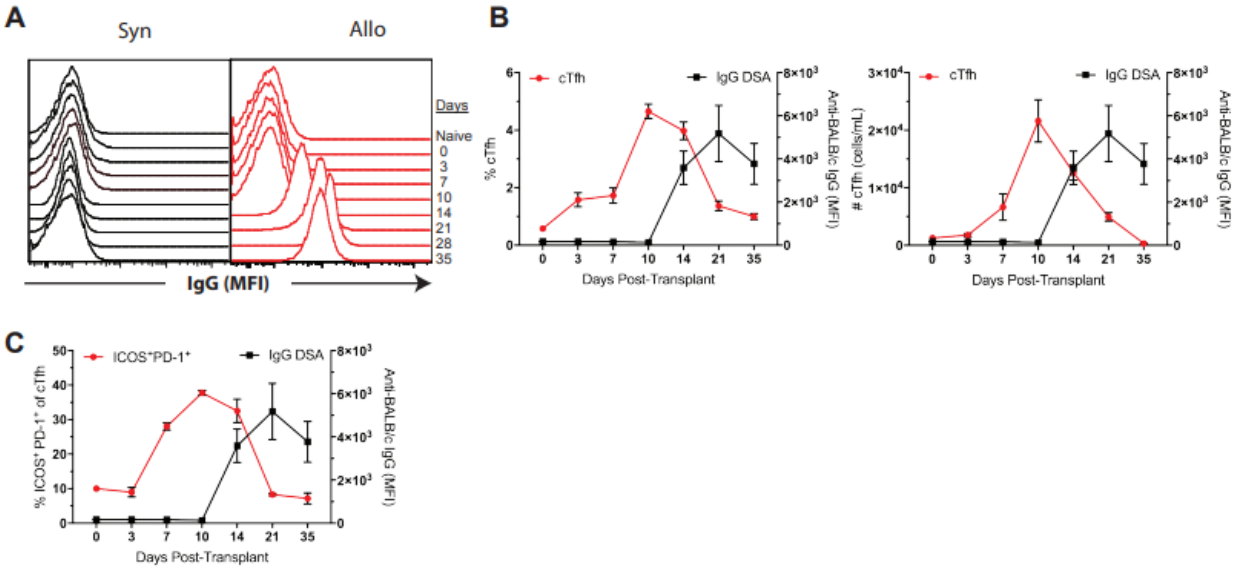


Figure 4. Circulating Tfh cells precede DSA formation following transplantation.

Naïve B6 mice were transplanted with skin from either B6 (Syn) or BALB/c (Allo) donors and serially bled for serum collection and flow crossmatch analysis. (A) Representative histograms of anti-donor IgG in syngeneic and allogeneic skin-grafted mice over time. (B) Summary data of cTfh cell frequencies and numbers relative to anti-donor IgG over time (n=5 per group). (C) Summary data of $ICOS^+PD\text{-}1^+$ cTfh cell frequencies relative to DSA formation over time (n=5 per group). Summary data represent mean (SE) and are representative of three independent experiments with a total of 15 mice per group. *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

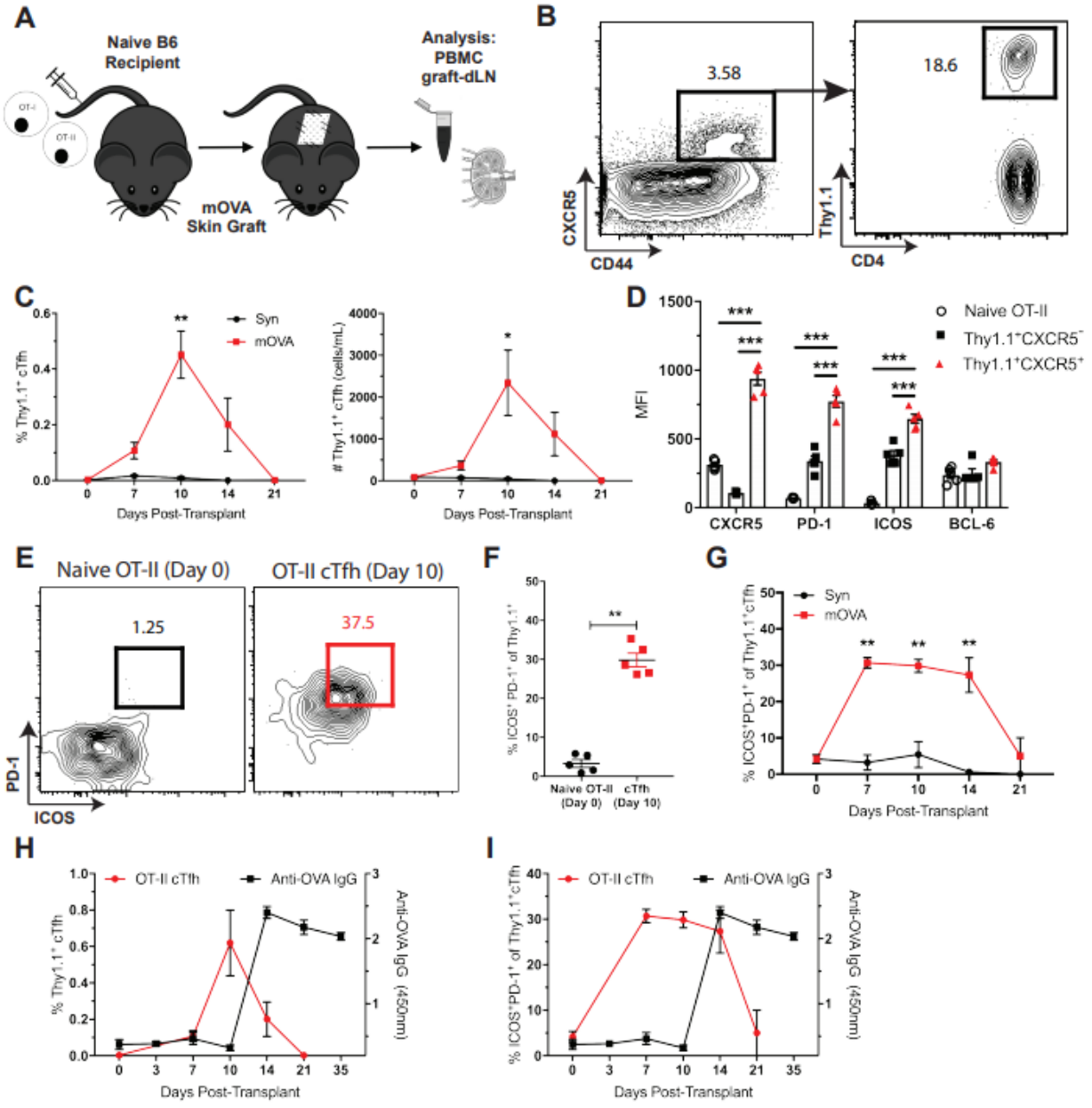


Figure 5. Antigen-specific TCR transgenic cTfh cells display similar phenotypic characteristics and kinetics to endogenous alloreactive cTfh cells following transplantation.

(A) Naïve B6 mice were adoptively transferred 10^6 of each Thy1.1$^+$ CD4$^+$ OT-II and CD8$^+$ OT-I T cells, transplanted skin from B6 (Syn) or mOVA donors and sacrificed at indicated time points post-transplantation for PBMC, graft-dLN and serum analyses. (B) Flow plots (gated on

$CD4^+Foxp3^-$ T cells) depict gating strategy for $CXCR5^+Thy1.1^+$ OVA-specific cTfh cells. (C) Summary data of $Thy1.1^+$ cTfh cell frequencies and numbers following B6 (Syn) or mOVA skin transplantation over time (n=5 per group). (D) Summary data of phenotypic marker expression in naïve ($CD44^{lo}CD62L^+$) OT-II cells, and $Thy1.1^+$ $CXCR5^-$ or $CXCR5^+$ OT-II cells from the peripheral blood 10 days post-transplantation (n=5 per group). (E) Flow plots depicting ICOS and PD-1 expression on naive OT-II cells on day 0 and $Thy1.1^+CXCR5^+$ cTfh cells 10 days post-transplantation. (F) Summary data of the frequencies of $ICOS^+PD\text{-}1^+$ of naïve OT-II cells on day 0 and $Thy1.1^+$ cTfh cells 10 days after transplant (n=5 per group). (G) Summary data of $ICOS^+PD\text{-}1^+$ $Thy1.1^+$ cTfh cell frequencies over time (n=5 per group). Summary data of (H) $Thy1.1^+$ cTfh cell and (I) $ICOS^+PD\text{-}1^+$ $Thy1.1^+$ cTfh cell frequencies relative to anti-OVA IgG formation over time (n=5 per group). Summary data represent mean (SE) and are representative of three independent experiments with a total of 10-15 mice per group. $*p < 0.05$, $**p < 0.01$, $***p< 0.001$.

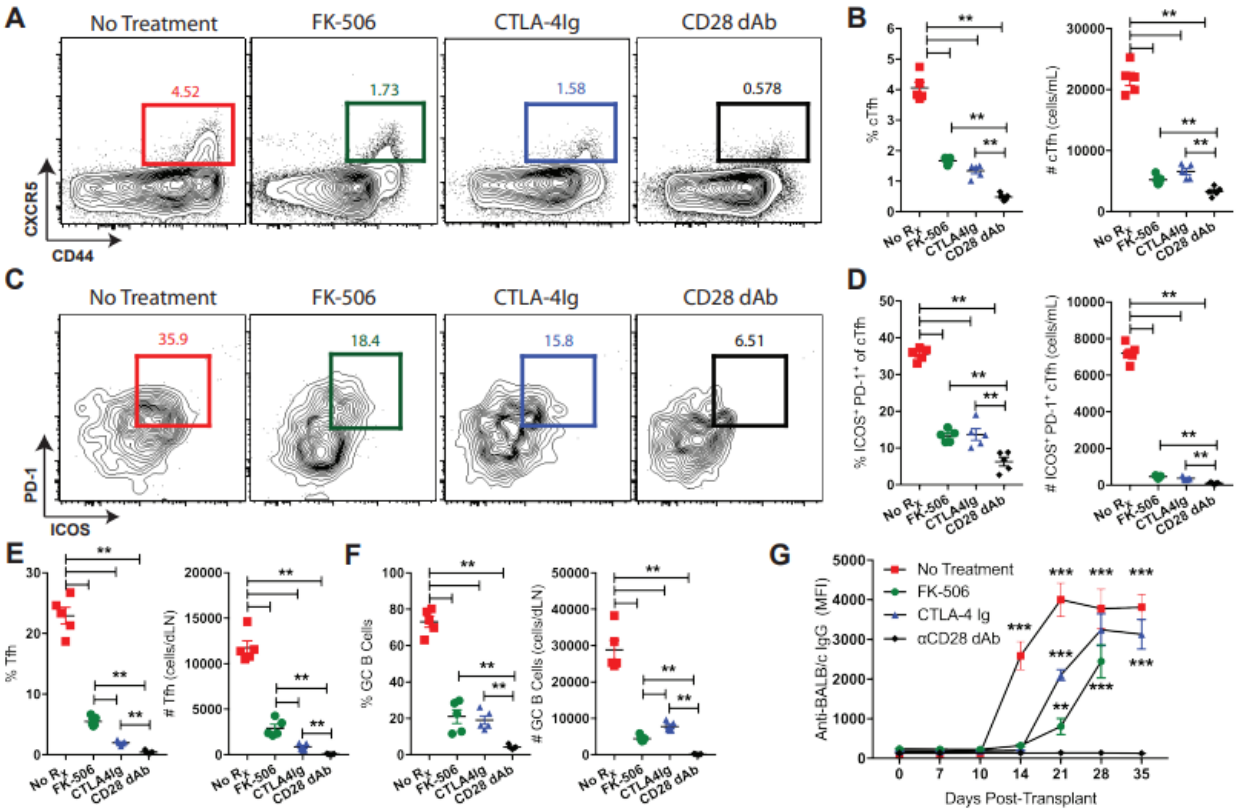


Figure 6. Circulating Tfh cells predict breakthrough alloantibodies despite immunosuppression. Naïve B6 mice were transplanted with BALB/c skin grafts and treated with tacrolimus (FK-506), CTLA-4-Ig, anti-CD28 dAb, or left untreated. BALB/c-grafted mice were sacrificed 10 days post-transplant for PBMC and graft-dLN analysis and serially bled for DSA assessment. (A) Representative flow cytometric plots (gated on $CD4^+Foxp3^-$ T cells) displaying the frequencies of $CXCR5^+$ cTfh cells. (B) Summary data of the frequencies and numbers of cTfh cells (n=5 per group). (C) Representative flow plots (gated on $CD4^+Foxp3^-CXCR5^+$ T cells) displaying the frequencies of $ICOS^+PD\text{-}1^+$ cTfh cells. (D) Summary data of the frequencies and numbers of $ICOS^+PD\text{-}1^+$ cTfh cells (n=5 per group). Summary data of the frequencies and numbers of graft-dLN (E) Tfh and (F) GC B cells (n=5 per group). (G) Summary data of anti-donor IgG formation in treated or untreated BALB/c-grafted mice over time (n=5 per group). Summary data represent mean (SE) and are

representative of three independent experiments with a total of 15 mice per group. *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

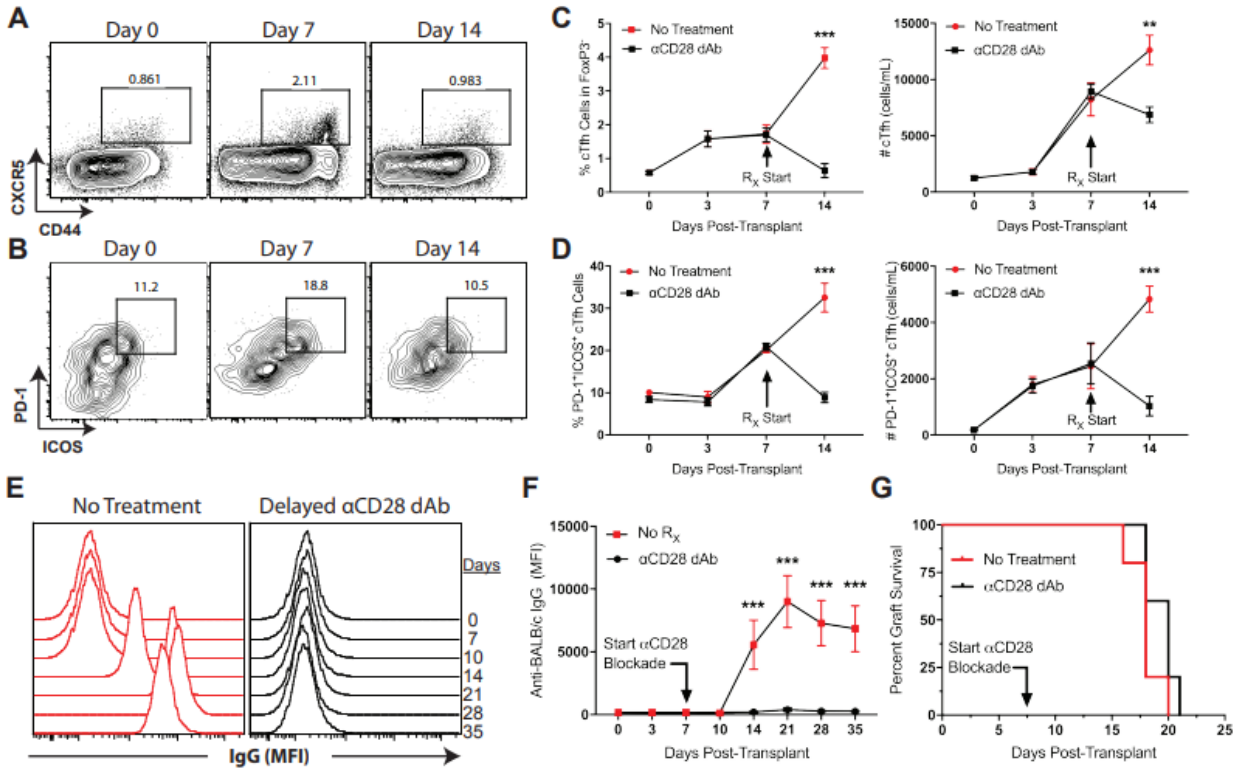


Figure 7. Delayed anti-CD28 CoB initiated after detection of ICOS$^+$PD-1$^+$ cTfh cells prevents DSA formation.

Naïve B6 mice were transplanted BALB/c skin grafts and their blood serially monitored for the development of ICOS$^+$PD-1$^+$ cTfh cells. Once detected on post-transplant day 7, immunosuppression with anti-CD28 dAb was initiated. Representative flow plots (gated on CD4$^+$Foxp3$^-$ T cells) displaying the frequencies of (A) cTfh and (B) ICOS$^+$PD-1$^+$ cTfh cells over time. Summary data of the frequencies and numbers of (C) cTfh and (D) ICOS$^+$PD-1$^+$ cTfh cells (n=5 per group). (E) Representative histograms of anti-donor IgG in untreated and delayed anti-CD28 dAb treated mice over time. (F) Summary data of anti-donor IgG over time (n=5 per group). Summary data represent mean (SE) and are representative of two independent experiments with a total of 10 mice per group. *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

References

1. Hart A, Smith JM, Skeans MA, Gustafson SK, Wilk AR, Robinson A, Wainright JL, Haynes CR, Snyder JJ, Kasiske BL, Israni AK. OPTN/SRTR 2016 Annual Data Report: Kidney. Am J Transplant. 2018;18 Suppl 1:18-113. Epub 2018/01/03. doi: 10.1111/ajt.14557. PubMed PMID: 29292608; PMCID: PMC5772947.

2. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. The natural history of chronic allograft nephropathy. N Engl J Med. 2003;349(24):2326-33. Epub 2003/12/12. doi: 10.1056/NEJMoa020009. PubMed PMID: 14668458.

3. Loupy A, Hill GS, Jordan SC. The impact of donor-specific anti-HLA antibodies on late kidney allograft failure. Nat Rev Nephrol. 2012;8(6):348-57. doi: 10.1038/nrneph.2012.81. PubMed PMID: 22508180.

4. Lefaucheur C, Loupy A, Hill GS, Andrade J, Nochy D, Antoine C, Gautreau C, Charron D, Glotz D, Suberbielle-Boissel C. Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation. J Am Soc Nephrol. 2010;21(8):1398-406. Epub 2010/07/17. doi: 10.1681/ASN.2009101065. PubMed PMID: 20634297; PMCID: PMC2938596.

5. Wiebe C, Gibson IW, Blydt-Hansen TD, Karpinski M, Ho J, Storsley LJ, Goldberg A, Birk PE, Rush DN, Nickerson PW. Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant. Am J Transplant. 2012;12(5):1157-67. Epub 2012/03/21. doi: 10.1111/j.1600-6143.2012.04013.x. PubMed PMID: 22429309.

6. Djamali A, Kaufman DB, Ellis TM, Zhong W, Matas A, Samaniego M. Diagnosis and management of antibody-mediated rejection: current status and novel approaches. Am J Transplant. 2014;14(2):255-71. doi: 10.1111/ajt.12589. PubMed PMID: 24401076; PMCID: PMC4285166.

7. Bray RA, Gebel HM. Strategies for human leukocyte antigen antibody detection. Curr Opin Organ Transplant. 2009;14(4):392-7. Epub 2009/07/18. PubMed PMID: 19610172.

8. Bray RA, Gebel HM. Monitoring after renal transplantation: recommendations and caveats. Nat Clin Pract Nephrol. 2008;4(12):658-9. Epub 2008/10/15. doi: 10.1038/ncpneph0968. PubMed PMID: 18852733.

9. Viglietti D, Loupy A, Vernerey D, Bentlejewski C, Gosset C, Aubert O, Duong van Huyen JP, Jouven X, Legendre C, Glotz D, Zeevi A, Lefaucheur C. Value of Donor-Specific Anti-HLA Antibody Monitoring and Characterization for Risk Stratification of Kidney Allograft Loss. J Am Soc Nephrol. 2017;28(2):702-15. Epub 2016/08/06. doi: 10.1681/ASN.2016030368. PubMed PMID: 27493255; PMCID: PMC5280026.

10. Crotty S. Follicular helper CD4 T cells (TFH). Annu Rev Immunol. 2011;29:621-63. doi: 10.1146/annurev-immunol-031210-101400. PubMed PMID: 21314428.

11. Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity. 2014;41(4):529-42. Epub 2014/11/05. doi: 10.1016/j.immuni.2014.10.004. PubMed PMID: 25367570; PMCID: PMC4223692.

12. Conlon TM, Saeb-Parsy K, Cole JL, Motallebzadeh R, Qureshi MS, Rehakova S, Negus MC, Callaghan CJ, Bolton EM, Bradley JA, Pettigrew GJ. Germinal Center Alloantibody Responses Are Mediated Exclusively by Indirect-Pathway CD4 T Follicular Helper Cells. Journal of Immunology. 2012;188(6):2643-52. Epub 2012/02/11. doi: 10.4049/jimmunol.1102830. PubMed PMID: WOS:000301126000020; PMCID: PMC3378630.

13. Kim EJ, Kwun J, Gibby AC, Hong JJ, Farris AB, 3rd, Iwakoshi NN, Villinger F, Kirk AD, Knechtle SJ. Costimulation blockade alters germinal center responses and prevents antibody-mediated rejection. Am J Transplant. 2014;14(1):59-69. Epub 2013/12/21. doi: 10.1111/ajt.12526. PubMed PMID: 24354871; PMCID: PMC3985346.

14. Badell IR, La Muraglia GM, 2nd, Liu D, Wagener ME, Ding G, Ford ML. Selective CD28 Blockade Results in Superior Inhibition of Donor-Specific T Follicular Helper Cell and Antibody Responses Relative

to CTLA4-Ig. Am J Transplant. 2018;18(1):89-101. Epub 2017/06/22. doi: 10.1111/ajt.14400. PubMed PMID: 28637095; PMCID: PMC5740006.

15. Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly - TFH cells in human health and disease. Nat Rev Immunol. 2013;13(6):412-26. Epub 2013/05/18. doi: 10.1038/nri3447. PubMed PMID: 23681096.

16. Ueno H. Human Circulating T Follicular Helper Cell Subsets in Health and Disease. J Clin Immunol. 2016;36 Suppl 1:34-9. Epub 2016/03/18. doi: 10.1007/s10875-016-0268-3. PubMed PMID: 26984851.

17. de Graav GN, Dieterich M, Hesselink DA, Boer K, Clahsen-van Groningen MC, Kraaijeveld R, Litjens NH, Bouamar R, Vanderlocht J, Tilanus M, Houba I, Boonstra A, Roelen DL, Claas FH, Betjes MG, Weimar W, Baan CC. Follicular T helper cells and humoral reactivity in kidney transplant patients. Clin Exp Immunol. 2015;180(2):329-40. Epub 2015/01/06. doi: 10.1111/cei.12576. PubMed PMID: 25557528; PMCID: PMC4408167.

18. Shi J, Luo F, Shi Q, Xu X, He X, Xia Y. Increased circulating follicular helper T cells with decreased programmed death-1 in chronic renal allograft rejection. BMC Nephrol. 2015;16:182. Epub 2015/11/04. doi: 10.1186/s12882-015-0172-8. PubMed PMID: 26525294; PMCID: PMC4630917.

19. Cano-Romero FL, Laguna Goya R, Utrero-Rico A, Gomez-Massa E, Arroyo-Sanchez D, Suarez-Fernandez P, Lora D, Andres A, Castro-Panete MJ, Paz-Artal E. Longitudinal profile of circulating T follicular helper lymphocytes parallels anti-HLA sensitization in renal transplant recipients. Am J Transplant. 2019;19(1):89-97. Epub 2018/06/28. doi: 10.1111/ajt.14987. PubMed PMID: 29947147.

20. Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. Cell. 1994;76(1):17-27. Epub 1994/01/14. PubMed PMID: 8287475.

21. Barnden MJ, Allison J, Heath WR, Carbone FR. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory

elements. Immunol Cell Biol. 1998;76(1):34-40. Epub 1998/04/29. doi: 10.1046/j.1440-1711.1998.00709.x. PubMed PMID: 9553774.

22. Ehst BD, Ingulli E, Jenkins MK. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. Am J Transplant. 2003;3(11):1355-62. Epub 2003/10/04. PubMed PMID: 14525595.

23. Trambley J, Bingaman AW, Lin A, Elwood ET, Waitze SY, Ha J, Durham MM, Corbascio M, Cowan SR, Pearson TC, Larsen CP. Asialo GM1(+) CD8(+) T cells play a critical role in costimulation blockade-resistant allograft rejection. J Clin Invest. 1999;104(12):1715-22. Epub 1999/12/22. doi: 10.1172/JCI8082. PubMed PMID: 10606625; PMCID: PMC409885.

24. Sage PT, Sharpe AH. In vitro assay to sensitively measure T(FR) suppressive capacity and T(FH) stimulation of B cell responses. Methods Mol Biol. 2015;1291:151-60. Epub 2015/04/04. doi: 10.1007/978-1-4939-2498-1_13. PubMed PMID: 25836309.

25. Choi YS, Gullicksrud JA, Xing S, Zeng Z, Shan Q, Li F, Love PE, Peng W, Xue HH, Crotty S. LEF-1 and TCF-1 orchestrate T(FH) differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. Nat Immunol. 2015;16(9):980-90. Epub 2015/07/28. doi: 10.1038/ni.3226. PubMed PMID: 26214741; PMCID: PMC4545301.

26. Morita R, Schmitt N, Bentebibel S-E, Ranganathan R, Bourdery L, Zurawski G, Foucat E, Dullaers M, Oh S, Sabzghabaei N, Lavecchio EM, Punaro M, Pascual V, Banchereau J, Ueno H. Human Blood CXCR5+CD4+ T Cells Are Counterparts of T Follicular Cells and Contain Specific Subsets that Differentially Support Antibody Secretion. Immunity. 2011;34(1):108-21. Epub 2011/01/11. doi: 10.1016/j.immuni.2010.12.012. PubMed PMID: 21215658; PMCID: PMC3046815.

27. He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, Sun X, Vandenberg K, Rockman S, Ding Y, Zhu L, Wei W, Wang C, Karnowski A, Belz GT, Ghali JR, Cook MC, Riminton DS, Veillette A, Schwartzberg PL, Mackay F, Brink R, Tangye SG, Vinuesa CG, Mackay CR, Li Z, Yu D. Circulating precursor CCR7(lo)PD-1(hi)

CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. Immunity. 2013;39(4):770-81. Epub 2013/10/22. doi: 10.1016/j.immuni.2013.09.007. PubMed PMID: 24138884.

28. Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, Su LF, Cubas R, Davis MM, Sette A, Haddad EK, International AVIPCPI, Poignard P, Crotty S. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. Immunity. 2013;39(4):758-69. Epub 2013/09/17. doi: 10.1016/j.immuni.2013.08.031. PubMed PMID: 24035365; PMCID: PMC3996844.

29. Bentebibel SE, Lopez S, Obermoser G, Schmitt N, Mueller C, Harrod C, Flano E, Mejias A, Albrecht RA, Blankenship D, Xu H, Pascual V, Banchereau J, Garcia-Sastre A, Palucka AK, Ramilo O, Ueno H. Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. Sci Transl Med. 2013;5(176):176ra32. Epub 2013/03/15. doi: 10.1126/scitranslmed.3005191. PubMed PMID: 23486778; PMCID: PMC3621097.

30. Schmitt N, Ueno H. Blood Tfh cells come with colors. Immunity. 2013;39(4):629-30. Epub 2013/10/22. doi: 10.1016/j.immuni.2013.09.011. PubMed PMID: 24138878; PMCID: PMC3996729.

31. Ford ML, Koehn BH, Wagener ME, Jiang W, Gangappa S, Pearson TC, Larsen CP. Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. J Exp Med. 2007;204(2):299-309. Epub 2007/01/31. doi: 10.1084/jem.20062319. PubMed PMID: 17261633; PMCID: PMC2118720.

32. Singh D, Henkel M, Sendon B, Feng J, Fabio A, Metes D, Moreland LW, McGeachy MJ. Analysis of CXCR5(+)Th17 cells in relation to disease activity and TNF inhibitor therapy in Rheumatoid Arthritis. Sci Rep. 2016;6:39474. Epub 2016/12/23. doi: 10.1038/srep39474. PubMed PMID: 28004828; PMCID: PMC5177940.

33. Badell IR, Ford ML. T follicular helper cells in the generation of alloantibody and graft rejection. Curr Opin Organ Transplant. 2016;21(1):1-6. doi: 10.1097/MOT.0000000000000260. PubMed PMID: 26727455; PMCID: PMC4702514.

34. Chenouard A, Chesneau M, Bui Nguyen L, Le Bot S, Cadoux M, Dugast E, Paul C, Malard-Castagnet S, Ville S, Guerif P, Soulillou JP, Degauque N, Danger R, Giral M, Brouard S. Renal Operational Tolerance Is Associated With a Defect of Blood Tfh Cells That Exhibit Impaired B Cell Help. Am J Transplant. 2017;17(6):1490-501. Epub 2016/11/27. doi: 10.1111/ajt.14142. PubMed PMID: 27888555.

35. Leibler C, Thiolat A, Henique C, Samson C, Pilon C, Tamagne M, Pirenne F, Vingert B, Cohen JL, Grimbert P. Control of Humoral Response in Renal Transplantation by Belatacept Depends on a Direct Effect on B Cells and Impaired T Follicular Helper-B Cell Crosstalk. J Am Soc Nephrol. 2018;29(3):1049-62. Epub 2018/01/13. doi: 10.1681/ASN.2017060679. PubMed PMID: 29321143; PMCID: PMC5827599.

36. Hale JS, Youngblood B, Latner DR, Mohammed AU, Ye L, Akondy RS, Wu T, Iyer SS, Ahmed R. Distinct memory CD4+ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection. Immunity. 2013;38(4):805-17. Epub 2013/04/16. doi: 10.1016/j.immuni.2013.02.020. PubMed PMID: 23583644; PMCID: PMC3741679.

37. Sage PT, Alvarez D, Godec J, von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. J Clin Invest. 2014;124(12):5191-204. Epub 2014/10/28. doi: 10.1172/JCI76861. PubMed PMID: 25347469; PMCID: PMC4348955.

38. Hale JS, Ahmed R. Memory T follicular helper CD4 T cells. Front Immunol. 2015;6:16. Epub 2015/02/24. doi: 10.3389/fimmu.2015.00016. PubMed PMID: 25699040; PMCID: PMC4313784.

39. Wiebe C, Gibson IW, Blydt-Hansen TD, Pochinco D, Birk PE, Ho J, Karpinski M, Goldberg A, Storsley L, Rush DN, Nickerson PW. Rates and determinants of progression to graft failure in kidney allograft recipients with de novo donor-specific antibody. Am J Transplant. 2015;15(11):2921-30. Epub 2015/06/23. doi: 10.1111/ajt.13347. PubMed PMID: 26096305.

40. Nickerson PW, Rush DN. Begin at the Beginning to Prevent the End. J Am Soc Nephrol. 2015;26(7):1483-5. Epub 2015/01/04. doi: 10.1681/ASN.2014111115. PubMed PMID: 25556171; PMCID: PMC4483596.

41. Young JS, Chen J, Miller ML, Vu V, Tian C, Moon JJ, Alegre ML, Sciammas R, Chong AS. Delayed Cytotoxic T Lymphocyte-Associated Protein 4-Immunoglobulin Treatment Reverses Ongoing Alloantibody Responses and Rescues Allografts From Acute Rejection. Am J Transplant. 2016;16(8):2312-23. Epub 2016/03/02. doi: 10.1111/ajt.13761. PubMed PMID: 26928966; PMCID: PMC4956497.

Chapter 5

Discussion

5.1. Enhancing Control of *de novo* DSA Responses Through the Application of Novel Costimulation Blockade Therapeutics

5.2. Utilizing Longitudinal cT_{FH} Frequency and Phenotype as an Interventional Biomarker for the Prevention of *de novo* DSA Following Transplantation

5.1 Enhancing Control of *de novo* DSA Responses Through the Application of Novel Costimulation Blockade Therapeutics

Solid organ transplantation is the treatment modality of choice for patients experiencing end-stage organ failure. Since the seminal discovery of cyclosporine by Jean-François Borel and its subsequent application to transplantation by Sir Roy Calne (1), advances in solid-organ transplant immunosuppression have remained rather stagnant (2). While CNI therapeutics have largely ameliorated the impact of acute rejection and greatly improved short-term outcomes, chronic rejection mediated through the development of *de novo* DSA remains a significant barrier to improving long-term success (3-5). A collaborative effort between Drs. Christian Larsen, Thomas Pearson at Emory University, Flavio Vincenti at UCSF, and scientists at Bristol-Meyers-Squibb led to the successful clinical trial "Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial" (BENEFIT), which utilized Belatacept, a second generation CTLA-4 Ig derivative CD28-directed costimulation blockade therapy, for kidney transplantation. The phase III BENEFIT study revealed that while higher incidence of acute rejection episodes occurred in Belatacept treated groups compared to CNI treated patients, significant improvements in both long-term graft survival and renal function were observed in the Belatacept treated cohorts (6-8). Post-hoc analysis of the BENEFIT trial clinical samples demonstrated a significant reduction in the generation of *de novo* DSA in Belatacept treated groups compared to those treated with CNIs (9). Although the use of costimulation blockade therapy as the standard of care for renal transplantation has not gained widespread traction (10), multi-therapeutic approaches utilizing Belatacept for maintenance

immunosuppressive therapy have thus far shown great clinical promise for improving long-term graft survival.

In recent years, the clinical burden of DSA has managed to reach the spotlight, garnering wide acceptance amongst transplant clinicians and immunologists that antibody mediated rejection portends an impasse to improved long-term graft survival. Despite the highly recognized deleterious role antibodies play in kidney transplant outcomes, large knowledge deficits exist regarding the mechanisms underlying their development and maintenance following transplantation, and robust therapeutic strategies to control DSA-mediated allograft dysfunction have yet to be developed. Currently available clinical therapies aimed at controlling established DSA responses in graft recipients are limited, ranging from immunological depletion of B cell populations to serum apheresis and IVIg therapy aimed at limiting plasma cell immunoglobulin production (11). These therapies are chiefly dilatorily and have been plagued by suboptimal efficacy, underscoring the necessity for a dogmatic shift in clinical practice away from therapies intended to ameliorate established DSA responses, with greater focus towards strategies aimed at the prevention of these processes in the transplant recipient.

As presented in the previous chapters, our approach has centered around garnering a better understanding of the immunological processes that lead to the development of *de novo* DSA following transplantation under various immunosuppressive strategies. Given the greater efficacy of T cell targeted CTLA-4 Ig based costimulation blockade therapy in controlling *de novo* DSA generation within human transplant recipients, we focused our efforts on better understanding the T cell component of antibody responses in the post-transplant phase. T_{FH}

cells are a distinct lineage of $CD4^+$ T cells that provide T cell help to B cells for germinal center (GC) formation, affinity maturation and the generation of memory B cells and long-lived plasma cells required to effectuate an adequate humoral immune response. The critical role T_{FH} cells play in the generation of antibody responses to vaccines and in autoimmune disease has been established, but little is known regarding their role in the generation of DSAs in the setting of transplantation (12). In an effort to better understand the role T_{FH} cells play in the generation of *de novo* DSA we adapted multiple murine transplant models in order to investigate antibody responses in the context of both antigen-specific immune responses and fully MHC disparate alloresponses utilizing various immunosuppressive strategies.

The work we present in Chapter Two focused on delineating the effect of CTLA-4 Ig and a novel third-generation selective CD28 costimulation blockade on T_{FH} cell and DSA responses following transplantation. Our lab had previously published observations of superior graft survival when utilizing a selective CD28 dAb that directly antagonizes CD28 receptors when compared to CTLA-4 Ig, that alternatively functions through indirect blockade of CD28 receptor ligands B7-1/2(13). We further investigated the effect of CD28 dAb in the context of antibody responses, finding that selective CD28 blockade exhibited superior suppression of DSA responses over CTLA-4 Ig in a TCR transgenic antigen-specific transplant model. Importantly, our data indicated that the suppression of DSA by selective CD28 blockade was largely attributed to improved control of both total T_{FH} and GC-specific T_{FH} cells compared to CTLA-4 Ig therapy following transplantation. Subsequent to the improved control of T_{FH} responses, we also showed that GC B cell responses were further attenuated through selective CD28 blockade. Taken together, the cellular data, along with serological analysis, revealed a substantially

impaired T_{FH}-dependent humoral response under selective CD28 blockade therapy compared to CTLA-4 Ig. Although this study did not specifically address the requirement of T_{FH} cells for the generation of *de novo* DSA, given the lack of evidence of extra-follicular antibody responses within the context of humoral alloresponses, it is likely that the greater efficacy of T_{FH} cell suppression exhibited by the selective blockade is directly responsible for the observed prevention of *de novo* DSA.

The mechanism through which CD28-directed costimulation blockade exerts global suppression of humoral immune responses has recently become a hotly contested topic within the field (14, 15). In 2017, Baan and colleagues published rather quixotic claims that belatacept does not inhibit T_{FH}-dependent B cell differentiation following kidney transplantation (15). This study aimed determine the differential efficacy of belatacept and CNI-treated renal transplant recipients through *ex vivo* PBMC assays to assess activation potential and functionality of both circulating T_{FH} (cT_{FH}) and B cells. One of the chief issues with this study revolved around the aspect that no unmodified immune responses in the absence of immunosuppression were assessed, with overstated claims made that belatacept does not inhibit T_{FH}-dependent B cell differentiation. Another major limitation to this study is the sole utilization of human subjects leading to the inability to surveil SLOs where T_{FH}-B cell interactions are actively taking place, due to the nature of complications associated with invasive procedures in immunosuppressed patients. While our group's data shows that cT_{FH} mirror ongoing GC activity in SLOs, the highly disparate phenotypes and poorly understood ontogeny of cT_{FH} *versus* SLO-derived T_{FH} cells outlined within our publication severely limit their interpretability as an unequivocal measure of immunosuppressive efficacy on the humoral alloimmune response (16). Furthermore, our

data directly contradict these claims through both *in vivo* animal models, and *ex vivo* T_{FH}: B cell co-culture assays showing that CTLA-4 Ig was capable of significantly reducing T_{FH}, GC B cell and DSA responses over unmodified instances (GM. La Muraglia II, unpublished data). Our findings have additionally been corroborated with those of other groups in various animal models, further galvanizing the notion that CTLA-4 Ig potently suppresses T_{FH} cell alloimmune responses following transplantation (17).

Based on our exciting findings that the immunosuppressive properties heralded by CD28-directed costimulation blockade therapy could be further enhanced through selective targeting of CD28 receptors, we sought to determine the mechanism by which this was afforded. Both costimulation blockade reagents are targeted to block CD28 signaling, however, they achieve this goal through two disparate immunologic strategies. CTLA-4 Ig is a fusion protein of two CTLA-4 coinhibitory receptors fused to an immunoglobulin tail, where T cell suppression is exerted through the saturation of CD28 receptor ligands CD80/86 on the surface of APCs. CTLA-4 Ig's blockade of the ligands CD80/86 prevents their ligation by CD28 on responding cognate T cells in the context of productive TCR – pMHC interactions, which subsequently induces T cell anergy (18-21). Unfortunately, this mechanism leads to the undesirable consequence of additionally impairing the CTLA-4 coinhibitory axis of T cell regulation due to the sequestration of potential binding ligands. The negative implications for this action are two-fold: the first of which regards the loss of potential CTLA-4 coinhibitory signaling in T_{FH} cells, significantly limiting their intrinsic inhibitory capacity (22, 23). This is of particular importance in the context of T cell memory, where T cell activation no longer relies on CD28 based costimulation – a paradigm that has been heavily observed during belatacept-

resistant rejection (24-26). In addition to the impairment of intrinsic T cell control, there is also the issue of attenuated extrinsic coinhibitory control through CTLA-4 within the T cell compartment. Regulatory T cell subsets essential to the extrinsic control of responding T cells constitutively maintain dense expression of CTLA-4, with a myriad of reports illustrating their dependence on CTLA-4 signaling for stimulation and execution of both cell-cell and paracrine based effector function (27-30). Importantly, these T_{REG} extrinsic inhibitory functions have been implicated as indispensable to the suppression of both humoral immune responses (22, 31, 32), and allograft rejection (33-35). Based upon the plethora of published data supporting the essential function of CTLA-4 for both intrinsic and extrinsic control of T_{FH} cell responses, preservation of CTLA-4 signaling capacity afforded through selective CD28 blockade is a palpable mechanism for the observations of improved suppression of T_{FH}-driven humoral alloimmune responses.

In Chapter Three, we sought to investigate the basic dependence of preserved CTLA-4 inhibitory potential in the enhanced control of T_{FH}-directed humoral alloantibody observed in selective CD28 blockade. We first confirmed that the administration of an agonistic anti-CTLA-4 mAb monotherapy was capable of exacerbating T_{FH} and GC B cell responses in our transplant model, demonstrating that the magnitude of the T-dependent humoral alloimmune response was significantly greater than unmodified controls. Through the application of anti-CTLA-4 in conjunction with selective CD28 blockade we were able to test the dependency of preserved CTLA-4 coinhibitory capacity on the effectiveness of selective CD28 blockade therapy. Utilizing this treatment model, we observed a significant reduction in the efficacy of immunosuppression of T_{FH}-driven humoral alloimmune responses, to a level more comparable

to CTLA-4 Ig therapy. This data therefore predicates a dominant role for CTLA-4 coinhibitory capacity in adequately controlling humoral alloimmune responses in CD28-directed costimulation blockade strategies. Previous work from our group and others supports this notion with studies citing similar findings to the dependence of CTLA-4 as the key mediator in the improvement of immunosuppression conferred through selective CD28 blockade for transplantation (13, 36-38). However, due to the rather ubiquitous expression of CTLA-4 on various immune cell subsets, global pharmacological blockade of CTLA-4 is incapable of isolating the cell lineage responsible for the improved immunosuppression observed in selective CD28 blockade.

Despite the technical limitations of interpreting our *in vivo* data, we sought to establish *ex vivo* assays capable of interrogating the role of CTLA-4 at the level T_{FH}-B cell interactions in the context of selective CD28 blockade. Through our adaption of complex T_{FH}: B cell co-culture systems pioneered by Peter Sage (39), we determined that the blockade of CTLA-4 in conjunction with CD28 dAb significantly increased; T_{FH} and B cell proliferation, B cell CSR, and IgG production in response to alloantigens compared to CD28 dAb monotherapy. These data, with the support of Dr. Sage's work in models of immunization (22), directly implicates CTLA-4 as a dominant mechanism in the enhanced inhibition of humoral alloimmune responses under selective CD28 blockade therapy. In order to further characterize the role of CTLA-4 directly on T_{FH} cells, where it is chiefly expressed within this system (GM La Muraglia II, unpublished data), we isolated CTLA-4 dependence on both T_{FH} and B cells individually through anti-CTLA-4 pre-treatment prior to the co-culture. As expected, we found that reversal of selective CD28 blockade-mediated suppression of B cell differentiation was excusive to CTLA-4 blockade on T_{FH}

cells and not B cells, despite reports of CTLA-4 expression on B cell subsets (40). Nevertheless, due to the scope of our investigations for these experiments, we cannot account for the potential of compensatory or additive suppression of B cell responses by T_{FR} or T_{REG} cells within our system.

The findings discussed so far serve to provide a framework of composed of both experimental and clinical observations through which we can further enhance the efficacy of CD28-directed costimulation blockade through the application of novel immunosuppressive therapeutics. Taken together, these data detail that preserved CTLA-4 co-inhibitory capacity potentiates the superior control of T_{FH} cell driven *de novo* DSA generation exhibited by selective CD28 blockade and is in part a T_{FH} cell intrinsic mechanism of inhibition. This topic is of particular interest due to the ensuing clinical trial for anti-CD28 dAb as a maintenance stage immunosuppressive therapy following renal transplantation here at Emory University (AB Adams, ML Ford, CP Larsen). Future studies utilizing inducible knockout systems for the conditional expression of CTLA-4 on various T cell subsets including but not limited to; T_{FH}, T_{REG}, and T_{FR} cell populations would ultimately be required utilizing both *in vivo* and *ex vivo* experimentation to unequivocally determine whether the inhibitory action is mediated through T cell intrinsic and/or extrinsic mechanisms.

5.2 Utilizing Longitudinal cT_{FH} Frequency and Phenotype as an Interventional Biomarker for the Prevention of *de novo* DSA Following Transplantation

Historically, late kidney allograft failure has been chiefly attributed to CNI nephrotoxicity and the progression of chronic allograft nephropathy (41). However, through expanded knowledge within the fields of transplantation and pathology, it is now well recognized that donor-specific HLA antibodies are an important immunologic cause of acute and chronic allograft injury that substantially curtail renal allograft survival (42). These anti-HLA directed DSAs can exist pre-transplant from various sensitizing events, or arise *de novo* following transplant and are associated with inferior transplant outcomes (5, 11, 43). Despite the major prevalence and deleterious impact of HLA antibodies in kidney transplantation, diagnostic biomarkers to reliably guide clinical management of pathologic DSA have not been developed (44).

The development and clinical application of sensitive assays for the identification of HLA antibodies has greatly enhanced pre-transplant donor-recipient matching to reduce the incidence of early antibody-mediated rejection (45). However, the utility of such data in the post-transplant setting has provided limited benefits (46, 47), due to the dearth of reproducible therapeutic options to attenuate established DSA responses in the clinic (42, 44). Therefore, there is great need for the development of alternative biomarkers that precede the generation of DSA, allowing for the detection of humoral alloimmune responses at their initial inception in transplant recipients to facilitate the initiation of therapeutic interventions prior to the establishment of robust antibody titers.

The idea of longitudinal immune monitoring of transplant recipients has been a long-time goal for the field of transplantation, in an effort to improve long-term outcomes through the provision of personalized immunosuppression (48, 49). To achieve this goal for the purposes of predictive biomarkers associated with the events preceding DSA formation, the monitoring of cT_{FH} cell activity from peripheral blood has taken the spotlight. The magnitude and phenotype of cT_{FH} cell responses have been shown to correlate with autoimmune disease, vaccine responses, and broadly neutralizing antibodies in HIV controllers (50-54), while recent clinical reports within the transplant literature have loosely described their relationship with anti-donor antibody responses (55-57). While these studies suggest an association between cT_{FH} cell activity and humoral alloimmune processes in human transplant recipients, experimental data investigating the temporal relationship between cT_{FH} cells and alloantibody formation for their evaluation as a predictive biomarker in transplantation remain of pinnacle importance.

In an effort to further understand the temporal relationship between cT_{FH} cell frequency and phenotype to GC T_{FH} responses in SLOs, we investigated the possibility of utilizing them as a non-invasive method for interventional biomarker detection prior to the establishment of DSA as discussed in Chapter Four. In this study, we provide solid experimental evidence that alloreactive, antigen-specific cT_{FH} cells are detectable in the peripheral blood and expand following transplant, correlate with ongoing GC alloreactivity in graft-dLNs, and precede the manifestation of anti-donor antibodies within the serum. Interestingly, we additionally observed progressive polarization of cT_{FH} cells towards an effector-like $ICOS^{+}PD\text{-}1^{+}$ phenotype in an allo-dependent manner post-transplant that preceded DSA formation and resolved in

parallel to the contraction of graft-dLN GC activity. While kinetic studies were carried out under conditions of unmodified rejection, we performed auxiliary experiments under various immunosuppressive protocols aimed to stratify disparate levels of GC and DSA control, which further solidify the relationship between cT_{FH} activity, GC reactivity and DSA generation following transplantation. Taken together, these data establish that cT_{FH} cell activity; directly mirrors the kinetics of graft-dLN GC responses and predicts *de novo* DSA formation, providing a non-invasive method of immune monitoring that may inform clinicians of mounting humoral immune responses within the transplant recipient population. One major caveat to our study is seated within the fact that we did not employ vascularized allografts within our longitudinal studies in large part due to the sheer number of animals required for each kinetic timepoint to accurately determine graft-dLN responses. While it is possible that the kinetics of the cT_{FH} and GC responses could be altered within the context of a vascularized graft model, no literature has reported significant modulation of immune responses between transplanted organ systems in the setting of unmodified rejection. However, future confirmatory studies utilizing heterotopic cardiac allografts for the investigation of cT_{FH} signature in relation to DSA responses could be of additional value for the breadth of experimental evidence.

Further investigation into the kinetics of the cellular components of the humoral alloimmune response in our models lead us to investigate the applicability of cT_{FH} signature as an interventional biomarker, potentially capable of providing a window for therapeutic intervention for the diminution of mounting GC responses. To this aim, we allowed unmodified allogenic grafts to proceed until robust cT_{FH} cell responses were detectable, at that time, costimulation blockade based immunosuppressive treatment was initiated, leading to the

attenuation of mounting GC responses and the prevention of *de novo* DSA generation. Interestingly, similar observations have been reported in the literature, describing the ability of costimulation blockade to reverse ongoing GC activity and prevent the generation of donor-directed antibody formation (58, 59). Our data, along with the support of other published studies within the field represent a cogent argument that humoral alloimmune responses, if detected during their infancy are receptive to interventional therapy. While our studies afford a short period of time between the detection of cT_{FH} activity and the generation of DSA, the window of opportunity for intervention is very likely to be months or years in clinical transplantation under standard immunosuppression (11, 42). A significant portion of alloantibody burden in transplantation results from the unpredictable emergence of *de novo* DSA several years after transplant (43), while antibody-mediated injury is chiefly classified as an indolent process, associated with occult histopathologic changes and cellular alloimmunity prior to the serologic detection of DSA (60). Therefore, the observed ability of cT_{FH} cell differentiation to predict delayed, breakthrough DSA despite immunosuppression strongly supports the notion that changes in cT_{FH} cells during episodes of acute allograft dysfunction or periodically over the course of a transplant could be utilized as an potent indicator of smoldering humoral alloimmunity prior to the serologic detection of alloantibodies.

The establishment of cT_{FH} cell responses as a biomarker preceding the generation of DSA represents a significant step forward in our fields efforts to prevent donor-directed antibody responses in clinical transplantation. Our data demonstrate a proof of concept that quantitative and qualitative changes in cT_{FH} cells provide a robust cellular indicator of alloantibody formation and could guide clinical management strategies to mitigate the negative

impact of DSA following transplantation. We highlight the potential for cT_{FH} cells to be utilized as a predictive biomarker for humoral alloreactivity and the generation of DSA in clinical transplantation that could trigger the initiation of therapy to prevent alloantibody-mediated allograft injury. These findings support additional investigation of cT_{FH} cells in vascularized transplant models and humans as a clinically relevant cellular biomarker for the diagnosis or prediction of anti-HLA antibody-mediated processes in transplantation.

Figures and Figure Legends

References

1. Calne RY, White DJ, Thiru S, Evans DB, McMaster P, Dunn DC, Craddock GN, Pentlow BD, Rolles K. Cyclosporin A in patients receiving renal allografts from cadaver donors. Lancet. 1978;2(8104-5):1323-7. Epub 1978/12/23. PubMed PMID: 82836.

2. Starzl TE. History of clinical transplantation. World J Surg. 2000;24(7):759-82. Epub 2000/06/01. PubMed PMID: 10833242; PMCID: PMC3091383.

3. Valenzuela NM, Reed EF. Antibodies in transplantation: the effects of HLA and non-HLA antibody binding and mechanisms of injury. Methods Mol Biol. 2013;1034:41-70. doi: 10.1007/978-1-62703-493-7_2. PubMed PMID: 23775730; PMCID: PMC3879955.

4. Everly MJ, Rebellato LM, Haisch CE, Ozawa M, Parker K, Briley KP, Catrou PG, Bolin P, Kendrick WT, Kendrick SA, Harland RC, Terasaki PI. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. Transplantation. 2013;95(3):410-7. Epub 2013/02/06. doi: 10.1097/TP.0b013e31827d62e3. PubMed PMID: 23380861.

5. Lefaucheur C, Loupy A, Hill GS, Andrade J, Nochy D, Antoine C, Gautreau C, Charron D, Glotz D, Suberbielle-Boissel C. Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation. J Am Soc Nephrol. 2010;21(8):1398-406. Epub 2010/07/17. doi: 10.1681/ASN.2009101065. PubMed PMID: 20634297; PMCID: PMC2938596.

6. Vincenti F, Blancho G, Durrbach A, Friend P, Grinyo J, Halloran PF, Klempnauer J, Lang P, Larsen CP, Muhlbacher F, Nashan B, Soulillou JP, Vanrenterghem Y, Wekerle T, Agarwal M, Gujrathi S, Shen J, Shi R, Townsend R, Charpentier B. Five-year safety and efficacy of belatacept in renal transplantation. J Am Soc Nephrol. 2010;21(9):1587-96. doi: 10.1681/ASN.2009111109. PubMed PMID: 20634298; PMCID: PMC3013525.

7. Vincenti F, Charpentier B, Vanrenterghem Y, Rostaing L, Bresnahan B, Darji P, Massari P, Mondragon-Ramirez GA, Agarwal M, Di Russo G, Lin CS, Garg P, Larsen CP. A phase III study of

belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). Am J Transplant. 2010;10(3):535-46. doi: 10.1111/j.1600-6143.2009.03005.x. PubMed PMID: 20415897.

8. Vincenti F, Rostaing L, Grinyo J, Rice K, Steinberg S, Gaite L, Moal MC, Mondragon-Ramirez GA, Kothari J, Polinsky MS, Meier-Kriesche HU, Munier S, Larsen CP. Belatacept and Long-Term Outcomes in Kidney Transplantation. N Engl J Med. 2016;374(4):333-43. doi: 10.1056/NEJMoa1506027. PubMed PMID: 26816011.

9. Bray RA, Gebel HM, Townsend R, Roberts ME, Polinsky M, Yang L, Meier-Kriesche HU, Larsen CP. Posttransplant reduction in preexisting donor-specific antibody levels after belatacept- versus cyclosporine-based immunosuppression: Post hoc analyses of BENEFIT and BENEFIT-EXT. Am J Transplant. 2018;18(7):1774-82. Epub 2018/03/25. doi: 10.1111/ajt.14738. PubMed PMID: 29573335; PMCID: PMC6055813.

10. Wojciechowski D, Vincenti F. Current status of costimulatory blockade in renal transplantation. Curr Opin Nephrol Hypertens. 2016;25(6):583-90. Epub 2016/10/18. doi: 10.1097/MNH.0000000000000268. PubMed PMID: 27517137.

11. Wiebe C, Gibson IW, Blydt-Hansen TD, Pochinco D, Birk PE, Ho J, Karpinski M, Goldberg A, Storsley L, Rush DN, Nickerson PW. Rates and determinants of progression to graft failure in kidney allograft recipients with de novo donor-specific antibody. Am J Transplant. 2015;15(11):2921-30. Epub 2015/06/23. doi: 10.1111/ajt.13347. PubMed PMID: 26096305.

12. Badell IR, Ford ML. T follicular helper cells in the generation of alloantibody and graft rejection. Curr Opin Organ Transplant. 2016;21(1):1-6. doi: 10.1097/MOT.0000000000000260. PubMed PMID: 26727455; PMCID: PMC4702514.

13. Liu D, Krummey SM, Badell IR, Wagener M, Schneeweis LA, Stetsko DK, Suchard SJ, Nadler SG, Ford ML. 2B4 (CD244) induced by selective CD28 blockade functionally regulates allograft-specific CD8+

T cell responses. J Exp Med. 2014;211(2):297-311. doi: 10.1084/jem.20130902. PubMed PMID: 24493803; PMCID: PMC3920565.

14. Schroder PM, Ezekian B, Ford M, Knechtle SJ, Kwun J. Commentary: Belatacept Does Not Inhibit Follicular T Cell-Dependent B-Cell Differentiation in Kidney Transplantation. Front Immunol. 2017;8:1615. Epub 2017/12/09. doi: 10.3389/fimmu.2017.01615. PubMed PMID: 29218048; PMCID: PMC5704107.

15. de Graav GN, Hesselink DA, Dieterich M, Kraaijeveld R, Verschoor W, Roelen DL, Litjens NHR, Chong AS, Weimar W, Baan CC. Belatacept Does Not Inhibit Follicular T Cell-Dependent B-Cell Differentiation in Kidney Transplantation. Front Immunol. 2017;8:641. Epub 2017/06/18. doi: 10.3389/fimmu.2017.00641. PubMed PMID: 28620390; PMCID: PMC5450507.

16. La Muraglia GM, II, Wagener ME, Ford ML, Badell IR. Circulating T Follicular Helper Cells Are a Biomarker of Humoral Alloreactivity and Predict Donor-Specific Antibody Formation After Transplantation. Am J Transplant. 2019. Epub 2019/06/30. doi: 10.1111/ajt.15517. PubMed PMID: 31250973.

17. Kim EJ, Kwun J, Gibby AC, Hong JJ, Farris AB, 3rd, Iwakoshi NN, Villinger F, Kirk AD, Knechtle SJ. Costimulation blockade alters germinal center responses and prevents antibody-mediated rejection. Am J Transplant. 2014;14(1):59-69. Epub 2013/12/21. doi: 10.1111/ajt.12526. PubMed PMID: 24354871; PMCID: PMC3985346.

18. Larsen CP, Pearson TC, Adams AB, Tso P, Shirasugi N, Strobert E, Anderson D, Cowan S, Price K, Naemura J, Emswiler J, Greene J, Turk LA, Bajorath J, Townsend R, Hagerty D, Linsley PS, Peach RJ. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. Am J Transplant. 2005;5(3):443-53. doi: 10.1111/j.1600-6143.2005.00749.x. PubMed PMID: 15707398.

19. Azuma H, Chandraker A, Nadeau K, Hancock WW, Carpenter CB, Tilney NL, Sayegh MH. Blockade of T-cell costimulation prevents development of experimental chronic renal allograft rejection. Proc Natl Acad Sci U S A. 1996;93(22):12439-44. Epub 1996/10/29. PubMed PMID: 8901600; PMCID: PMC38010.

20. Linsley PS, Wallace PM, Johnson J, Gibson MG, Greene JL, Ledbetter JA, Singh C, Tepper MA. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. Science. 1992;257(5071):792-5. Epub 1992/08/07. PubMed PMID: 1496399.

21. Collins AV, Brodie DW, Gilbert RJ, Iaboni A, Manso-Sancho R, Walse B, Stuart DI, van der Merwe PA, Davis SJ. The interaction properties of costimulatory molecules revisited. Immunity. 2002;17(2):201-10. Epub 2002/08/28. PubMed PMID: 12196291.

22. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. Immunity. 2014;41(6):1026-39. doi: 10.1016/j.immuni.2014.12.005. PubMed PMID: 25526313; PMCID: PMC4309019.

23. Wang CJ, Heuts F, Ovcinnikovs V, Wardzinski L, Bowers C, Schmidt EM, Kogimtzis A, Kenefeck R, Sansom DM, Walker LS. CTLA-4 controls follicular helper T-cell differentiation by regulating the strength of CD28 engagement. Proc Natl Acad Sci U S A. 2015;112(2):524-9. doi: 10.1073/pnas.1414576112. PubMed PMID: 25548162; PMCID: PMC4299196.

24. Cortes-Cerisuelo M, Laurie SJ, Mathews DV, Winterberg PD, Larsen CP, Adams AB, Ford ML. Increased Pretransplant Frequency of CD28(+) CD4(+) TEM Predicts Belatacept-Resistant Rejection in Human Renal Transplant Recipients. Am J Transplant. 2017;17(9):2350-62. Epub 2017/05/16. doi: 10.1111/ajt.14350. PubMed PMID: 28502091; PMCID: PMC5599135.

25. Mathews DV, Wakwe WC, Kim SC, Lowe MC, Breeden C, Roberts ME, Farris AB, Strobert EA, Jenkins JB, Larsen CP, Ford ML, Townsend R, Adams AB. Belatacept-Resistant Rejection Is Associated

With CD28(+) Memory CD8 T Cells. Am J Transplant. 2017;17(9):2285-99. Epub 2017/05/16. doi: 10.1111/ajt.14349. PubMed PMID: 28502128; PMCID: PMC5573634.

26. Mathews DV, Dong Y, Higginbotham LB, Kim SC, Breeden CP, Stobert EA, Jenkins J, Tso JY, Larsen CP, Adams AB. CD122 signaling in CD8+ memory T cells drives costimulation-independent rejection. J Clin Invest. 2018;128(10):4557-72. Epub 2018/09/18. doi: 10.1172/JCI95914. PubMed PMID: 30222140; PMCID: PMC6159972.

27. Zheng Y, Manzotti CN, Liu M, Burke F, Mead KI, Sansom DM. CD86 and CD80 differentially modulate the suppressive function of human regulatory T cells. J Immunol. 2004;172(5):2778-84. Epub 2004/02/24. doi: 10.4049/jimmunol.172.5.2778. PubMed PMID: 14978077.

28. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. J Exp Med. 2000;192(2):295-302. Epub 2000/07/19. doi: 10.1084/jem.192.2.295. PubMed PMID: 10899916; PMCID: PMC2193261.

29. Tang Q, Boden EK, Henriksen KJ, Bour-Jordan H, Bi M, Bluestone JA. Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function. Eur J Immunol. 2004;34(11):2996-3005. Epub 2004/10/07. doi: 10.1002/eji.200425143. PubMed PMID: 15468055.

30. Manzotti CN, Tipping H, Perry LC, Mead KI, Blair PJ, Zheng Y, Sansom DM. Inhibition of human T cell proliferation by CTLA-4 utilizes CD80 and requires CD25+ regulatory T cells. Eur J Immunol. 2002;32(10):2888-96. Epub 2002/10/02. doi: 10.1002/1521-4141(2002010)32:10<2888::AID-IMMU2888>3.0.CO;2-F. PubMed PMID: 12355442.

31. Wing JB, Ise W, Kurosaki T, Sakaguchi S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. Immunity. 2014;41(6):1013-25. doi: 10.1016/j.immuni.2014.12.006. PubMed PMID: 25526312.

32. Gamez-Diaz L, Neumann J, Jager F, Proietti M, Felber F, Soulas-Sprauel P, Perruzza L, Grassi F, Kogl T, Aichele P, Kilimann M, Grimbacher B, Jung S. Immunological phenotype of the murine Lrba knockout. Immunol Cell Biol. 2017;95(9):789-802. Epub 2017/06/28. doi: 10.1038/icb.2017.52. PubMed PMID: 28652580.

33. Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, Morris PJ, Powrie F, Wood KJ. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. J Immunol. 2001;166(6):3789-96. Epub 2001/03/10. PubMed PMID: 11238621.

34. Kingsley CI, Karim M, Bushell AR, Wood KJ. CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. J Immunol. 2002;168(3):1080-6. Epub 2002/01/22. doi: 10.4049/jimmunol.168.3.1080. PubMed PMID: 11801641.

35. Chandraker A, Huurman V, Hallett K, Yuan X, Tector AJ, Park CH, Lu E, Zavazava N, Oaks M. CTLA-4 is important in maintaining long-term survival of cardiac allografts. Transplantation. 2005;79(8):897-903. Epub 2005/04/26. doi: 10.1097/01.tp.0000158275.56248.f8. PubMed PMID: 15849541.

36. Liu D, Suchard SJ, Nadler SG, Ford ML. Inhibition of Donor-Reactive CD8+ T Cell Responses by Selective CD28 Blockade Is Independent of Reduced ICOS Expression. PLoS One. 2015;10(6):e0130490. doi: 10.1371/journal.pone.0130490. PubMed PMID: 26098894; PMCID: PMC4476729.

37. Poirier N, Azimzadeh AM, Zhang T, Dilek N, Mary C, Nguyen B, Tillou X, Wu G, Reneaudin K, Hervouet J, Martinet B, Coulon F, Allain-Launay E, Karam G, Soulillou JP, Pierson RN, 3rd, Blancho G, Vanhove B. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. Sci Transl Med. 2010;2(17):17ra0. doi: 10.1126/scitranslmed.3000116. PubMed PMID: 20371478; PMCID: PMC2860737.

38. Ville S, Poirier N, Branchereau J, Charpy V, Pengam S, Nerriere-Daguin V, Le Bas-Bernardet S, Coulon F, Mary C, Chenouard A, Hervouet J, Minault D, Nedellec S, Renaudin K, Vanhove B, Blancho G.

Anti-CD28 Antibody and Belatacept Exert Differential Effects on Mechanisms of Renal Allograft Rejection. J Am Soc Nephrol. 2016. doi: 10.1681/ASN.2015070774. PubMed PMID: 27160407.

39. Sage PT, Sharpe AH. In vitro assay to sensitively measure T(FR) suppressive capacity and T(FH) stimulation of B cell responses. Methods Mol Biol. 2015;1291:151-60. Epub 2015/04/04. doi: 10.1007/978-1-4939-2498-1_13. PubMed PMID: 25836309.

40. Quandt D, Hoff H, Rudolph M, Fillatreau S, Brunner-Weinzierl MC. A new role of CTLA-4 on B cells in thymus-dependent immune responses in vivo. J Immunol. 2007;179(11):7316-24. Epub 2007/11/21. PubMed PMID: 18025174.

41. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. The natural history of chronic allograft nephropathy. N Engl J Med. 2003;349(24):2326-33. Epub 2003/12/12. doi: 10.1056/NEJMoa020009. PubMed PMID: 14668458.

42. Loupy A, Hill GS, Jordan SC. The impact of donor-specific anti-HLA antibodies on late kidney allograft failure. Nat Rev Nephrol. 2012;8(6):348-57. doi: 10.1038/nrneph.2012.81. PubMed PMID: 22508180.

43. Wiebe C, Gibson IW, Blydt-Hansen TD, Karpinski M, Ho J, Storsley LJ, Goldberg A, Birk PE, Rush DN, Nickerson PW. Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant. Am J Transplant. 2012;12(5):1157-67. Epub 2012/03/21. doi: 10.1111/j.1600-6143.2012.04013.x. PubMed PMID: 22429309.

44. Djamali A, Kaufman DB, Ellis TM, Zhong W, Matas A, Samaniego M. Diagnosis and management of antibody-mediated rejection: current status and novel approaches. Am J Transplant. 2014;14(2):255-71. doi: 10.1111/ajt.12589. PubMed PMID: 24401076; PMCID: PMC4285166.

45. Bray RA, Gebel HM. Strategies for human leukocyte antigen antibody detection. Curr Opin Organ Transplant. 2009;14(4):392-7. Epub 2009/07/18. PubMed PMID: 19610172.

46. Bray RA, Gebel HM. Monitoring after renal transplantation: recommendations and caveats. Nat Clin Pract Nephrol. 2008;4(12):658-9. Epub 2008/10/15. doi: 10.1038/ncpneph0968. PubMed PMID: 18852733.

47. Viglietti D, Loupy A, Vernerey D, Bentlejewski C, Gosset C, Aubert O, Duong van Huyen JP, Jouven X, Legendre C, Glotz D, Zeevi A, Lefaucheur C. Value of Donor-Specific Anti-HLA Antibody Monitoring and Characterization for Risk Stratification of Kidney Allograft Loss. J Am Soc Nephrol. 2017;28(2):702-15. Epub 2016/08/06. doi: 10.1681/ASN.2016030368. PubMed PMID: 27493255; PMCID: PMC5280026.

48. Cook DJ, Bishay ES, Yamani M. The use and misuse of immunologic monitoring after transplantation: approaches that have proved useful. Curr Opin Cardiol. 2000;15(2):104-7. Epub 2000/08/30. PubMed PMID: 10963147.

49. Cravedi P, Heeger PS. Immunologic monitoring in transplantation revisited. Curr Opin Organ Transplant. 2012;17(1):26-32. Epub 2011/12/22. doi: 10.1097/MOT.0b013e32834ee402. PubMed PMID: 22186089; PMCID: PMC3285386.

50. Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, Foucat E, Dullaers M, Oh S, Sabzghabaei N, Lavecchio EM, Punaro M, Pascual V, Banchereau J, Ueno H. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. Immunity. 2011;34(1):108-21. Epub 2011/01/11. doi: 10.1016/j.immuni.2010.12.012. PubMed PMID: 21215658; PMCID: PMC3046815.

51. He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, Sun X, Vandenberg K, Rockman S, Ding Y, Zhu L, Wei W, Wang C, Karnowski A, Belz GT, Ghali JR, Cook MC, Riminton DS, Veillette A, Schwartzberg PL, Mackay F, Brink R, Tangye SG, Vinuesa CG, Mackay CR, Li Z, Yu D. Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen

reexposure. Immunity. 2013;39(4):770-81. Epub 2013/10/22. doi: 10.1016/j.immuni.2013.09.007. PubMed PMID: 24138884.

52. Singh D, Henkel M, Sendon B, Feng J, Fabio A, Metes D, Moreland LW, McGeachy MJ. Analysis of CXCR5(+)Th17 cells in relation to disease activity and TNF inhibitor therapy in Rheumatoid Arthritis. Sci Rep. 2016;6:39474. Epub 2016/12/23. doi: 10.1038/srep39474. PubMed PMID: 28004828; PMCID: PMC5177940.

53. Bentebibel SE, Lopez S, Obermoser G, Schmitt N, Mueller C, Harrod C, Flano E, Mejias A, Albrecht RA, Blankenship D, Xu H, Pascual V, Banchereau J, Garcia-Sastre A, Palucka AK, Ramilo O, Ueno H. Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. Sci Transl Med. 2013;5(176):176ra32. Epub 2013/03/15. doi: 10.1126/scitranslmed.3005191. PubMed PMID: 23486778; PMCID: PMC3621097.

54. Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, Su LF, Cubas R, Davis MM, Sette A, Haddad EK, International AVIPCPI, Poignard P, Crotty S. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. Immunity. 2013;39(4):758-69. Epub 2013/09/17. doi: 10.1016/j.immuni.2013.08.031. PubMed PMID: 24035365; PMCID: PMC3996844.

55. de Graav GN, Dieterich M, Hesselink DA, Boer K, Clahsen-van Groningen MC, Kraaijeveld R, Litjens NH, Bouamar R, Vanderlocht J, Tilanus M, Houba I, Boonstra A, Roelen DL, Claas FH, Betjes MG, Weimar W, Baan CC. Follicular T helper cells and humoral reactivity in kidney transplant patients. Clin Exp Immunol. 2015;180(2):329-40. Epub 2015/01/06. doi: 10.1111/cei.12576. PubMed PMID: 25557528; PMCID: PMC4408167.

56. Shi J, Luo F, Shi Q, Xu X, He X, Xia Y. Increased circulating follicular helper T cells with decreased programmed death-1 in chronic renal allograft rejection. BMC Nephrol. 2015;16:182. Epub 2015/11/04. doi: 10.1186/s12882-015-0172-8. PubMed PMID: 26525294; PMCID: PMC4630917.

57. Cano-Romero FL, Laguna Goya R, Utrero-Rico A, Gomez-Massa E, Arroyo-Sanchez D, Suarez-Fernandez P, Lora D, Andres A, Castro-Panete MJ, Paz-Artal E. Longitudinal profile of circulating T follicular helper lymphocytes parallels anti-HLA sensitization in renal transplant recipients. Am J Transplant. 2018. Epub 2018/06/28. doi: 10.1111/ajt.14987. PubMed PMID: 29947147.

58. Young JS, Chen J, Miller ML, Vu V, Tian C, Moon JJ, Alegre ML, Sciammas R, Chong AS. Delayed Cytotoxic T Lymphocyte-Associated Protein 4-Immunoglobulin Treatment Reverses Ongoing Alloantibody Responses and Rescues Allografts From Acute Rejection. Am J Transplant. 2016;16(8):2312-23. Epub 2016/03/02. doi: 10.1111/ajt.13761. PubMed PMID: 26928966; PMCID: PMC4956497.

59. Chen J, Yin H, Xu J, Wang Q, Edelblum KL, Sciammas R, Chong AS. Reversing endogenous alloreactive B cell GC responses with anti-CD154 or CTLA-4Ig. Am J Transplant. 2013;13(9):2280-92. doi: 10.1111/ajt.12350. PubMed PMID: 23855587; PMCID: PMC3797532.

60. Nickerson PW, Rush DN. Begin at the Beginning to Prevent the End. J Am Soc Nephrol. 2015;26(7):1483-5. Epub 2015/01/04. doi: 10.1681/ASN.2014111115. PubMed PMID: 25556171; PMCID: PMC4483596.