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B Lymphocyte Homeostasis and BLyS Directed Immunotherapy in Transplantation

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Abstract

Current strategies for immunotherapy after transplantation are primarily T-lymphocyte directed and effectively abrogate acute rejection. However, the reality of chronic allograft rejection attests to the fact that transplantation tolerance remains an elusive goal. Donor-specific antibodies are considered the primary cause of chronic rejection. When naïve, alloreactive B cells encounter alloantigen and are activated, a resilient “sensitized” state, characterized by the presence of high-affinity antibody, is established. Here, we will delineate findings that support transient B-lymphocyte depletion therapy at the time of transplantation to preempt sensitization by eliminating alloreactive specificities from the recipient B-cell pool (i.e., “repertoire remodeling”).

Recent advances in our understanding of B-lymphocyte homeostasis provide novel targets for immunomodulation in transplantation. Specifically, the TNF-related cytokine, BLyS, is the dominant survival factor for “tolerance-susceptible” Transitional and “preimmune” mature Follicular B-cells. The Transitional phenotype is the intermediate through which all newly formed B cells pass before maturing into the Follicular subset, which is responsible for mounting an alloantigen specific antibody response. Systemic BLyS levels dictate the stringency of negative selection during peripheral B cell repertoire development. Thus, targeting BLyS will likely provide an opportunity for repertoire directed therapy to eliminate alloreactive B-cell specificities in transplant recipients; a requirement for the achievement of humoral tolerance and prevention of chronic rejection.

In this review the fundamentals of pre-immune B cell selection, homeostasis and activation will be described. Also, new and current B-lymphocyte directed therapy for antibody mediated rejection and the highly sensitized state will be discussed. Overall, our objective will be to propose a rational approach for induction of B cell transplantation tolerance by remodeling the primary B cell repertoire of the allograft recipient.

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Keywords

B lymphocyte; B_{LyS}; homeostasis; selection; immunotherapy; transplantation

Introduction

Currently, the mainstay of immunotherapy for solid organ transplantation is primarily T lymphocyte-directed and focused on preventing acute rejection. As a result, allograft survival rates in non-sensitized, cross-match negative recipients of solid organ allografts are quite good. However, long-term graft survival remains unsatisfactory; a testament to the fact the true transplantation tolerance remains an unfulfilled goal. Donor-specific alloantibodies (DSA) are considered major culprits—if not *the* primary cause of chronic allograft rejection(1). Mounting clinical and basic scientific evidence provide a compelling argument that DSA contribute directly to chronic rejection via complement activation (detected by C4d deposition) and T-cell activation (2,3). However, efforts to curtail DSA producing B lymphocytes have so far been limited to select patient populations (4). Notably, patients with histological evidence of antibody-mediated graft rejection (AMR) or those sensitized after transfusion, pregnancy, or prior transplantation have received B cell depletion therapy and so-called antibody-cleansing treatments, such as plasmapheresis. Notwithstanding, It is our contention that unless B-lymphocytes are targeted at the time of transplantation (i.e., induction therapy) the emergence of DSA and chronic rejection will remain major obstacles to transplantation tolerance.

The importance of B cell mediated humoral alloimmunity in the pathogenesis of transplant rejection is undeniable (5). Terasaki et al. have documented that 23% of transplant recipients who did not have preformed HLA antibodies at the time of transplantation developed DSA within four years of transplantation (6). Importantly, this study also found that those recipients who developed DSA had significantly worse allograft survival rates compared to those who did not (58% vs. 81%, $p < 0.0001$ after deceased donor and 62% vs. 78%, $p < 0.0008$ after living donor transplantation) (6). It is essential to consider that induction of transplantation tolerance will require purging alloreactive clones from the pre-immune B-cell repertoire to minimize differentiation of DSA producing plasma cells and long-lived memory cells in the germinal center. Here, we will review the processes that govern pre-immune B-lymphocyte compartment development and its subsequent differentiation into a sensitized state. Novel approaches to induction of humoral transplantation tolerance will require elimination of alloreactive specificities from the preimmune repertoire in order to prevent maturation of DSA responses in the germinal center.

B Cell Development: Selection and Homeostasis

Selection of the recipient B lymphocyte repertoire occurs in the absence of donor alloantigens. Therefore, the participation of donor specific B-cells in the germinal center reaction and their affinity maturation to produce DSA is not surprising. The “pre-immune” B-lymphocyte repertoire originates in the bone marrow (BM) from hematopoietic stem cells. B cells are produced continuously throughout the life of the organism and pass through several selection “checkpoints” (i.e. intervals of time in the cell's ontogeny where its fate is determined) prior to entering the mature, Follicular (FO) pool. Normally, these tolerance checkpoints in B-cell compartment development ensure adequate clonal regulation of self-reactive specificities, resulting in an immunologically tolerant B-cell compartment (7–12). Immature B cells exit the BM and enter the peripheral circulation and spleen as Transitional (TR) B cells, where they undergo further differentiation before entering the mature, pre-immune B cell pool(13,14). The described mechanisms for B cell tolerance include: clonal deletion, receptor editing, clonal anergy, and competition for extrinsic factors (15). Nossal and Pike first posited B cell tolerance

via deletional mechanisms, or the “clonal abortion theory,” as the basis for immune tolerance in 1975(16).

Central B-Cell Selection and Tolerance to Self-Antigens

Central B lymphocyte development occurs in a series of steps that involve rearrangement of their immunoglobulin genes, culminating in B cell antigen receptor (BCR) expression in the BM. The first BCR-related checkpoint occurs during heavy chain rearrangement. Cells that productively rearrange an *Igμ* heavy chain pass this checkpoint, express a surface pre-BCR and join the “pre-B cell” pool where they rearrange their light chain genes and ultimately express a surface IgM at the Immature (17) B-cell stage (see Table 1). At this and all subsequent tolerance checkpoints, selection is based on BCR specificity. Substantial cell losses are incurred at each of these checkpoints, as clones bearing potentially self-reactive or “ineffective” BCR specificities are eliminated from the developing B cell repertoire (see Figure 1). It is generally accepted that IMM B cells in the BM, whose BCR binds with high affinity, either die or undergo secondary light chain rearrangements to change their specificity, a process termed “receptor editing” (18–22). Fewer than 10% of immature B cells formed in the BM actually exit to the periphery as TR B cells(23,24).

In 1989, both the Nemazee and Goodnow laboratories presented well-designed transgenic models characterizing deletional tolerance in vivo (25,26). In these systems, IMM B-cells with transgenic B cell antigen receptors are eliminated in the presence of the relevant autoantigen. These findings were confirmed in several other Ig transgenic systems specific for DNA and red blood cell antigens(27–29). Negative selection was also demonstrated in a number of in vitro mechanistic studies in which IMM B cells were shown to be susceptible to apoptosis, rather than activation, upon BCR cross-linking (30).

Peripheral B cell Selection and Tolerance to Self-Antigens

Despite the efficacy of central B-cell deletion in the BM, this process does not lead to perfect regulation of all autoreactive specificities, as relative to the periphery autoantigens are present at a lower concentration within the BM milieu. Therefore, peripheral regulatory mechanisms must be in place to ensure elimination of autoreactive specificities that are not subject to central deletion(7,9). Indeed, two thirds of the B-cells exiting the bone marrow as TR B-cells are lost as a result of peripheral B cell selection at the TR→FO checkpoint(14). Those that survive this checkpoint, enter the follicular subset in the spleen, which comprises the great majority of mature, recirculating pre-immune B cells (See Figure 1). Some IMM and TR B-cells join the marginal zone (MZ) subset in the spleen, but the criteria for differentiation into the FO versus MZ peripheral subsets remain incompletely delineated.

In contrast to the nearly “all-or-none” clonal deletion mechanism transpiring in the bone marrow, the regulation of peripheral autoreactive B-cells is thought to be more elastic (7,9). Elimination of self-reactive B cells via a process of clonal anergy has been well demonstrated in transgenic systems (27,31). In 1994 Goodnow and colleagues demonstrated that elimination of self-reactive B cells occurs as newly emerging TR B cells compete for a limited follicular resource; subsequently identified to be the cytokine BLyS (see below) (32). Thus, anergic B-cells are thought to be short-lived(33), unresponsive B-cells that differentiate from the TR B-cell subset having been excluded from the follicular pool based on their specificity-based inability to compete for the follicular niche(15,34,35). It is now a widely accepted premise that the distinction between the central and peripheral B cell tolerance checkpoints is that the former is primarily specificity based, whereas the latter is regulated by the system's homeostatic demands (36). While losses at the immature checkpoint appear to be absolute and based solely on B cell antigen receptor signal strength, stringency of negative selection at the peripheral checkpoint can be modulated based on a homeostatic feedback system, which controls the size

of the peripheral pre-immune B cell compartment. This feedback mechanism is now known to be mediated by the TNF related cytokine, BLyS, through an integrated signaling cross-talk between the BCR and BLyS-receptor starting at the TR stage and beyond (see below)(37).

Ultimately, irrespective of the efficiency of pre-immune mechanisms of central and peripheral B-cell negative selection, it remains a certainty that a fraction of autoreactive specificities escape regulation and emerge into the mature pool. Whether these specificities are subsequently activated to produce pathogenic auto-antibodies is, in part, dependent on chance encounter with the cognate autoantigen and, in part, on the availability of sufficient T-cell help to initiate a productive germinal center reaction (see below) (38–43). Indeed, in the setting of autoimmunity and transplantation, T-cell directed immunosuppression alone can suppress auto-/allo-antibody production by inhibiting the GC reaction. Nevertheless, it is a fundamental tenet of immunological tolerance that central and peripheral regulation of autoreactive specificities in the pre-immune compartment is required for the achievement of humoral tolerance. It is, thus, likely that achievement of robust transplantation tolerance will require harnessing the efficacy of pre-immune checkpoints to delete alloreactive specificities from the recipient repertoire.

BLyS Family of TNF Receptors and Ligands

The BLyS (a.k.a., BAFF) family of TNF receptors and ligands has generated considerable interest over the past decade because of its central role in the homeostatic control of primary B cells (7–9,36,44). The two BLyS receptors initially identified were TACI (transmembrane activator 1 and calcium-signaling modulator and cyclophilin ligand-interactor) and BCMA (B cell maturation antigen) (see Figure 2). These receptors also bind APRIL (another member of the TNF family of cytokines). TACI and BCMA knockouts do not demonstrate a pre-immune B cell compartment deficiency. Importantly, a third BLyS receptor was described by different groups, who coined the terms BLyS receptor 3 (BR3), or BAFF receptor (BAFF-R) (45). Mice with a spontaneous BR3 mutation leading to disrupted signaling (i.e., the A/WySnJ strain) exhibit a phenotype in which the TR B cell compartment is proportionally expanded due to a deficient primary FO compartment (46,47). Subsequent studies demonstrated that BLyS is the primary survival factor required by mature FO B cells (48–51) and that pro-survival signals are propagated primarily through BLyS-BR3 interaction (8). Of note, the BR3 deficient human homolog has been characterized in common variable immunodeficiency (CVID) patients (52). Similar to A/WySnJ mice, B cell development in these CVID patients is arrested at the transitional B cell stage causing a paucity of mature B cells and low IgG and IgM levels. Conversely, BLyS-over-expressing transgenic mice demonstrate FO compartment B cell hyperplasia and numerous autoimmune diseases (49,53), indicating the breakdown of the BLyS mediated tolerance checkpoint.

During B cell development, the immature CD23⁻ fraction of B cells demonstrates little BLyS binding, while somewhat more BLyS binding is observed in the CD23⁺ immature fraction (54). As immature cells enter the periphery their BLyS binding capacity increases, as surface expression of both BR3 and TACI increases (54). Critical interactions between B cell antigen receptor signaling and BR3 expression have been observed and dictate survival(46). B cell antigen receptor signaling provides an NF- κ B substrate, p100, for BR3 mediated processing and B cell survival beyond the TR stage (37). BLyS-BR3 binding promotes NF- κ B activation and also up-regulates a variety of anti-apoptotic proteins, including A1/Bfl-1, Bcl-xL, and Bcl-2(55,56) and activation of prosurvival pathways such as, Akt/mTOR and Pim2(57).

BCMA, TACI, and BR3 form trimeric complexes in vivo, similar to other TNF receptors(9). Of note, TACI and BCMA bind APRIL with equivalent strength and also have a 100-fold greater affinity for APRIL than BLyS (58–60). APRIL also binds heparin sulfate proteoglycans

(syndecan-1/CD138) found on the surface of plasma cells (among other cells) (61). Importantly, BR3 only binds BLyS and not APRIL (45). The soluble BLyS trimer is generally considered the only active form for primary B cell survival and is produced by several different cell types, including: neutrophils, dendritic cells, synovial fibroblasts, astrocytes, nurse-like cells, and T cells, as well as in response to some inflammatory cytokines (see Figure 2)(9). In contrast to BR3, evidence suggests oligomeric BAFF and APRIL binding to TACI may support survival of activated B cells and plasmablasts in culture (62).

The importance of BLyS levels for pre-immune B cell homeostasis and selection is now well established (9). Exogenous BLyS administration in vivo rapidly but reversibly doubles the peripheral B cell pools (50), while in vivo BLyS-neutralization using TACI-Ig or anti-BLyS mAb markedly depleted most peripheral B cell populations (63,64). When excess BLyS is present, self-reactive B cells are rescued from elimination and permitted to enter the normally “forbidden” mature B cell and marginal zone subsets(65,66). Similarly, when the TR B cell compartment is lymphopenic competition for BLyS is minimal and normal negative selection in the periphery is lost. These findings imply that BLyS level may control the stringency of negative selection at the TR→FO B-cell peripheral tolerance checkpoint (see Figure 3)(65, 67). Such is the case of the non-obese diabetic mouse model of autoimmune diabetes, in which autoreactive B cells survive and perpetuate beta cell destruction (68,69). Importantly, BLyS neutralization (i.e., using an anti-BLyS mAb) enhances the stringency of selection at the TR→FO B cell checkpoint, delaying the onset of type 1 diabetes (64). Likewise, BLyS and APRIL neutralization has shown a blunting of autoimmune activity and prevention of type 1 diabetes and the anti-phospholipid syndrome(70,71). Overall, it is becoming widely accepted that BLyS is responsible for not only regulating the steady state size of the pre-immune B-cell compartment, but importantly, the stringency of B-cell clonal elimination at the TR→FO tolerance checkpoint. As it is now evident that in vivo BLyS neutralization can ameliorate humoral autoimmunity in Lupus(72–75). It will be important to determine the tolerogenic potential of targeting BLyS to prevent the emergence of DSA following transplantation.

The Germinal Center Reaction and Homeostatic Regulation of Antigen-Experienced B cells

The germinal center is the nidus of immunological activation, where a concerted interplay between antigen driven T- and B-cell activation and cognate T-B interaction promotes an optimally activated and “affinity-matured” immune response(76). It is within the confines of the germinal center, through cognate T-B cell collaboration, that previously resting B-cells differentiate into class-switched, high affinity plasma cells (PCs)(77,78). Indeed, inhibition of T cell help alone can prevent the emergence of a high-affinity IgG response(79). The role of T cells in driving GC affinity maturation of humoral immunity is so important that irrespective of the presence or absence of antigen reactive cells in the pre-immune B-cell repertoire, provision of sufficient T cell help may ultimately promote differentiation of a high affinity IgG response(43). Under physiological conditions, the initiation of a germinal center reaction is under control of the following parameters: 1) antigen availability, 2) sufficient antigen presentation by immunogenic antigen presenting cells (e.g., dendritic cells, macrophages), 3) a threshold frequency of antigen reactive CD4 T-cells, 4) a threshold frequency of antigen reactive resting B-cells, and 5) sufficient MHC class II mediated cognate B-cell interaction with antigen specific CD4 T-helper cells. Disruption of any of these parameters can inhibit the germinal center reaction from yielding a robust humoral response.

All activated B cells participating express at least one of the BLyS family receptors (8). Although resting pre-immune B cells have surface expression of both BR3 and TACI, only BR3 is necessary for their survival (45). Any T cell dependent (TD) antigenic stimulus for B cells favors BR3 expression during germinal center reactions, while TACI expression is

favorable in the short-lived antibody-forming cells of a T cell independent (TI) response. For example, A/WySnJ mice demonstrate normal primary IgM responses for both TD and TI antigens. Nonetheless, these mutant mice demonstrate poor secondary humoral responses, low IgG levels, and compromised germinal center formation(80–82); likely because of their FO B cell lymphopenia. By influencing isotype switching—either directly or indirectly—both BLyS and APRIL are implicated in promoting an affinity-matured antibody response to inciting immunogens (8).

The role of the BLyS family members during B cell maturation into the long-lived memory B and antibody-forming cells is an area of ongoing research. Memory B cells are known to express elevated levels of TACI, at least initially and do not depend upon BLyS or APRIL to survive (the only known B2 subset to carry such a distinction) (83). High levels of surface TACI are found on short-lived antibody-forming cells(84) and high levels of BR3 are demonstrated on antigen experienced germinal center B cells(82). The factors influencing long-lived plasma cell (LLPC) survival are of great importance for influencing the outcome of transplantation patients who harbor alloantibodies (85,86). The conversion of BR3 expression to the BCMA-directed survival system suggests homeostatic compartmentalization of the LLPC pool from the preimmune pool(7). LLPCs require BLyS or APRIL for survival, as they express BCMA, and reside primarily in the bone marrow (87). Although a variety of tissues produce APRIL, its mRNA expression is quite high in human and mouse osteoclasts—suggesting the importance of this cytokine to LLPC survival (88). Importantly, the absence of either APRIL or BLyS after antigen immunization does not limit LLPC survival, but the combined absence of BLyS and APRIL significantly limits LLPC survival (83). Furthermore, LLPCs (as well as natural antibody forming cells and memory B cells) have shown continued survival after BLyS neutralization(89). Therefore, though BLyS neutralization therapy may be a potentially powerful means of modulating the stringency of clonal selection in the pre-immune repertoire, targeted elimination of the plasma cell pool is a complex feat in need of further investigation.

B Cell Immunity in Allograft Rejection

The role of B cells and their secreted antibodies during the alloimmune response has often been neglected because passive transfer of serum from sensitized hosts to naïve recipients was shown not sufficient to provoke rejection (90). Likewise, the more recent finding that B cell-deficient mice retained their predilection for allograft rejection was deemed as supportive evidence that that B cells do not make an important contribution to the pathogenesis of acute alloimmune responses (91,92). However, further analysis of B cell-deficient mice demonstrated an increased production of IL-12 by dendritic cells (DCs) and a marked polarization of the immune response toward a T helper 1 (Th1) phenotype (93). Accordingly, several discrete processes characterize the importance of B lymphocytes during an alloimmune response, such as humoral (antibody), cellular (B cell-dependent), and Ag-Ab complex mechanisms (94).

In the setting of transplant rejection, these three specific B-cell/antibody mediated mechanisms contribute to the pathogenesis of rejection. First, alloantibodies secreted by recipient B lymphocytes bind major histocompatibility antigens and vascular endothelium, activating complement and perpetuating subsequent rejection (95). The process of hyperacute rejection in response to circulating DSA is a well-described and extreme example, occurring within minutes to hours after reperfusion. Furthermore, Terasaki et al. have consistently shown the development of DSA after transplantation and before rejection, prompting the conclusion that alloantibodies can cause both acute and chronic rejection and that DSA must be removed from circulation to prevent allograft failure (1). Second, B lymphocytes act as efficient antigen-presenting cells for activation of alloreactive CD4⁺ T cells. For instance, cardiac allograft survival is markedly prolonged in mice with deficiency in B-cell mediated antigen presentation

(96,97). The importance of cognate B and T cell interactions after transplantation was further established by findings that allograft specific memory B cells and/or preformed allospecific antibodies facilitate activation and proliferation of naïve alloreactive T cells, and ultimately rejection in otherwise tolerant anti-CD154 treated recipients (98). These studies suggest that the elimination of the germinal center reactions blocks the formation of both cognate T-B cell interaction or alloantibody formation, permitting prolonged allograft survival(99). Third, alloantibodies form soluble alloantigen-alloantibody immune complexes, which can engage complement and Fcγ receptors on APCs and are processed and presented by MHC molecules. Furthermore, AlloAg-AlloAb complexes can promote APC maturation and subsequently drive alloreactive CD4+ and CD8+ T cell activation(100,101).

B Cell Directed Immunotherapy Prior to Alloantigen Sensitization

Given the prevalence of DSA and its strong association with graft rejection, it is a logical contention that the induction of robust transplantation tolerance will require abrogation of the DSA response; that is humoral transplantation tolerance(102,103). It is likely that effective abrogation of the DSA response will require B-cell directed immunotherapy prior to sensitization (i.e., induction therapy). The main objective of B-cell directed immunotherapy is to limit B cell mediated processes, such as germinal center activation and antigen presentation to alloreactive CD4 T cells. More importantly, we contend B-cell depletion at the time of transplantation provides the opportunity for clonal deletion of alloreactive specificities from the reconstituting repertoire(102). There are some anecdotal cases where B-cell tolerance is associated with long-term allograft survival and B-cell directed therapies have been used to improve clinical transplantation outcomes. For example, B cell tolerance was shown in infant ABO-incompatible heart transplant recipients (104). West et al. observed that up to eight years after ABO-incompatible infant heart transplant a persistent, selective deficiency of circulating donor-specific B cells and alloantibodies, and an absence of antibody-mediated damage (99, 104). These findings are highly suggestive that B cell tolerance can be achieved if the allograft is present in a window of B-cell development when the more malleable immature/TR B-cell compartment predominates (see Table 2). We envision recapitulating these conditions in adults using B cell directed immunotherapy prior to or at the time of transplantation. This strategy has only recently been tested experimentally and a few efforts have been made clinically (see below).

Our group has demonstrated evidence for the potential efficacy of B cell immunotherapy in a nonhuman primate model of islet transplantation(105). Induction immunotherapy with rabbit anti-thymocyte globulin and rituximab (i.e., anti-CD20) produced significant prolongation of islet allograft survival in nonhuman primates (105). Phenotypic analysis of the reconstituting B cell compartment in monkeys with long-term graft survival demonstrated a predominance of tolerance susceptible transitional B cells (105,106). In this study, hosts that rejected islet grafts had a larger proportion of mature CD27+ B cells early on after treatment (105).

In considering the role of B-cell directed therapy for transplantation, a recent randomized clinical investigation found minimal protection against acute rejection of renal allografts after B cell induction therapy with rituximab and standard maintenance immunosuppression (107). In this study however T cell induction therapy (e.g., Thymoglobulin) was not used in conjunction with rituximab induction treatment (as is the standard of care in most clinical institutions). Moreover, the timing of rejection was highly suggestive of an acute T cell mediated process—demonstrating the importance of a combined T-B cell directed approach for induction therapy in kidney transplantation. It is a generally accepted principle that acute rejection of kidney allografts is prevented most effectively under cover of T-cell directed induction therapy(108). Therefore, the results published by Clatworthy et al., are reflective of

the importance of T-cell directed induction therapy, more than proof that B-cell directed immunotherapy is ineffective or detrimental.

BLyS-Directed Immunotherapy and BLyS in Transplantation

B cell directed immunotherapy, predominantly using Rituximab, has been used in treatment of lymphoma(109) and different forms of autoimmunity(110). Interestingly, clinical studies of patients with systemic lupus erythematosus, rheumatoid arthritis, and Sjögren's syndrome have reported elevated BLyS levels and numerous autoreactive B cell clonotypes. Targeting BLyS with the fully humanized monoclonal antibody, belimumab (a.k.a., Benlysta) has shown great promise for autoimmune diseases (as have other agents targeting B cells, such as rituximab and an anti-CD22 antibody, epratuzumab) (72,73,111). Benlysta successfully passed a Phase 3 clinical trials for the treatment of lupus and awaits FDA approval(75). It is important to note that after B cell depletion, using agents such as Rituximab, an increase in BLyS levels has been observed, which is likely to allow less stringent negative selection during reconstitution (112,113); a fact that will promote the persistence of auto/allo-reactive B cell clones. Treatment of transplant recipients with rituximab should prompt consideration of elevated BLyS levels (see Figure 3)(114). For example, Sarwal et al. reported that in renal transplant recipients with "CD20+ rejection", rituximab treatment caused a significant elevation of BLyS levels at three months post-treatment. This finding was positively correlated with DSA specific for HLA-class I and negatively correlating with creatinine clearance(115).

The regulation of B-cell compartment homeostasis may be an important component of B-cell depletion therapies in the setting of transplantation. For example, Sollinger and colleagues found a higher incidence of rejection, especially antibody-mediated rejection, in patients treated with induction alemtuzumab and rituximab(116). When patients treated with alemtuzumab were followed at least 2 years post-transplant significantly elevated BLyS levels were found, while patients treated with either calcineurin inhibitors, mycophenolic acid, or steroids did not demonstrate increased BLyS levels (117,118).

Also, in the setting of bone marrow conditioning and T cell induction therapy for simultaneous kidney and bone marrow transplantation, T cell tolerance has been shown(119). However, achievement of humoral tolerance in this setting has not been uniform, as de novo donor-specific HLA antibodies were preceded by elevated BLyS levels in two of the four patients who received combined kidney and bone marrow transplantation(120). Therefore, BLyS neutralization may be a necessary component of B cell directed therapy, especially in the maintenance phase.

To date, the evidence suggests that soluble supra-physiologic BLyS may potentiate alloreactive B cell immunity and may promote allograft rejection. Also, elevated BLyS levels on the surface of CD3+ T cells has been observed in patients with evidence of allograft rejection at least 5 years after renal transplantation. Interestingly, these patients also had significantly greater levels of anti-HLA class I and II antibodies(121,122). Therefore, given the recent discoveries regarding the pivotal role of BLyS in the regulation of B-cell homeostasis and T cell tolerance, its neutralization in the induction and maintenance phase of immunotherapy for transplantation, to prevent antibody mediated rejection, must be explored carefully in the near future.

Moreover, the optimal duration of BLyS directed therapy should be determined. How long should BLyS neutralization be maintained to optimize the tolerance-promoting effects of TR B cells before full reconstitution? Of note, experimental evidence demonstrates that although TR B cells are highly sensitive to negative selection and they can still sustain responses to T-cells that have been pre-activated(123). Therefore, future studies should address when/if mature B cells should be restored to steady state levels, and hence, how long tolerance-susceptible transitional B cells should be allowed to predominate the B cell compartment.

B Cell Immunity and Directed Immunotherapy for Antibody-Mediated Rejection

The link between chronic allograft destruction and the formation of DSA has gained recent ground based on clinical and basic science observations (1,85). This recognition has prompted greater attention to the components of humoral immunity as potential therapeutic targets in transplantation. Long-lived plasma cells are terminally differentiated B cells that concentrate within the bone marrow and provide a durable source of the alloantibodies (124,125). Expression of the following transcription factors is needed of a persistent plasma cell phenotype: BLIMP1 and XBP1; conversely, the lack of expression of PAX5, BCL-6, and MTA3 also maintains the plasma cell fate(77). Interestingly, in the murine system CD93 expression is evident on LLPCs and its expression might be important for the maintenance of antibody secretion after TD immunizations (126). LLPCs are also dependent on the BCMA receptor, as withdrawal of BLyS and APRIL, terminates their survival (83,87).

Unfortunately, rituximab has demonstrated somewhat limited success for halting an ongoing humoral alloimmune response, as it does not seem capable of depleting PCs effectively (Table 3). Humanized CD20 transgenic mice were developed to analyze the in vivo factors that regulate mechanisms involved in B cell immunotherapy (127). Gong et al. found that greater than 90% of follicular B cells were depleted after rituximab, leaving only ~33% of B220⁺ splenocytes (128). This study also showed that B cell populations had a hierarchy of sensitivity to rituximab starting with the mature follicular pool, followed by the marginal zone and then the germinal center compartments. Of note, in these mice, a BLyS neutralizing BR3-Fc chimeric reagent increased the efficiency of depletion of all splenic B cell subsets when combined with rituximab (128). This study suggested that while anti-CD20 treatment depletes circulating B cells well, cells with reduced exposure to the reticular endothelial system (such as in those in the marginal zone) require other methods for efficient depletion; and that BLyS neutralization permits this to occur(128). As mentioned above, the marginal zone and germinal center pools are critical precursors to plasma cells. In the setting of antibody-mediated rejection (AMR) efficient depletion of these pools is likely important therapeutic intervention (129). Therefore, identification of targets other than CD20, such as BlyS and APRIL, is needed to efficiently target plasma cells and their precursors.

Nevertheless, patients who develop alloantibodies and AMR have been treated with B cell depletion therapy using rituximab (130) as it is the primary B cell depletion agent available (85,131). This approach has been based in clinical rationale and has been met with some limited success. Recent work has shown that approximately a third of pediatric and adult acute renal allograft rejection episodes are associated with intragraft CD20⁺ clusters (132–135). Zarkhin et al. found in patients with biopsy proven acute renal allograft rejection (with B cell infiltrates) that treatment with rituximab could improve calculated creatinine clearance relative to standard of care immunosuppression (thymoglobulin and/or pulse steroids) (136). In this study treatment caused complete peripheral and intragraft B cell depletion without change in the DSA levels (136). Although rituximab is not known to target LLPCs, it has been proposed to target activated memory cells within the graft (137). Furthermore, it has been proposed that the lack of continued stimulation of naïve B cells after B cell depletion with rituximab would permit donor-specific antibodies and plasma cells to decline over time(138). In fact in the setting of transplantation and autoimmunity, rituximab treatment has demonstrated a significant reduction of serum IgM in most studies, while IgG levels were mostly stable (131,136,139). Consequently, rituximab treatment has been combined with “antibody-cleansing” techniques, such as plasmapheresis to enhance the treatment of AMR (140). Also, the recent studies of the proteasome inhibitor, bortezomib (which depletes plasma cells and other metabolically active cells) have potentially expanded the scope of strategies to modulate AMR (141,142).

Directed Immunotherapy for Highly Sensitized Patients

Alloantigen sensitized patients carry high levels of anti-HLA antibodies and have low renal-transplantation rates. If these patients undergo transplantation their rate of allograft loss is unacceptably high (143). The development of preformed antibodies against HLA occurs as a result of prior transplantation, transfusion, or pregnancy. Approximately 30% of patients on the wait-list for renal transplantation demonstrate prior sensitization to alloantigen—and as a result B cell directed immunotherapy in these patients is urgently needed(143). Desensitization strategies have been instituted to improve the likelihood of converting highly sensitized patients with a positive crossmatch to a negative crossmatch. These protocols involve different combinations of treatment with intravenous immunoglobulin (IVIG), rituximab, and plasmapheresis with extracorporeal immunoadsorption to remove the DSAs (144,145). Some centers resort to performing an extensive search for donors who lack unacceptable antigens reactive with the recipient's alloantibodies in order to increase the likelihood of a compatible crossmatch (146).

After these measures, short-term outcomes with desensitization are favorable, while medium to long-term outcomes fall short of expectations and warrant continued follow-up investigation (146,147). Interestingly, hemophilia A patients with inhibitory alloantibodies against plasma-derived and recombinant factor VIII can achieve approximately 70% desensitization (148). In these patients, use of methotrexate and cyclophosphamide for B cell depletion, or IVIG and plasmapheresis has been described since the 1970s(149,150).

Many studies have found efficacy with rituximab administration for desensitization of highly sensitized patients (129,151–161) (162). For example, Vo et al. reported that treatment of highly sensitized patients awaiting renal transplantation with rituximab and intravenous IVIG caused a decrease in PRA from $\sim 77\pm 19\%$ to $44\pm 30\%$, permitting 16 of 20 of patients to undergo transplantation(143). The recipients demonstrated mean serum Cr levels of 1.5 ± 1.1 mg/dl one year after transplant. Nonetheless, the results of other desensitization protocols with rituximab have not been as successful likely due to its lack of specificity for LLPCs—these cells do not express CD20 (Table 3) (134). Accordingly, although rituximab effectively depletes mature peripheral B cells, it has not been shown to consistently diminish the level of preformed alloantibodies(136). Therefore, other memory B cell and plasma cell selective targets are needed to provide effective desensitization for patients awaiting organ transplantation. Critical to this effort is delineation of a more detailed basic science roadmap of plasma cell development and the parameters governing their homeostasis in vivo.

Finally, the proteasome inhibitor, bortezomib, in combination with “antibody-cleansing” techniques, has provided encouraging results, but needs further investigation as a potential alternative to current desensitization protocols (141,142,163). Certainly, plasma cells require APRIL and BLyS for their survival, among other factors, which could potentially serve as targets against the B cell alloimmune process. Of note, several autoimmunity studies describe the efficacy of atacept (formerly TACI-Ig, a fully humanized recombinant fusion protein, which binds APRIL and BLyS) for improvement in clinical symptoms and for reduction in serum IgM and rheumatoid factor levels (61,164,165). Atacept is currently in phase II/III clinical trials for multiple autoimmune disorders and B-cell malignancies in Europe(166, 167). Furthermore, in lupus and rheumatoid arthritis patients, and a small cohort of primates with circulating donor specific antibody following rejection of heterotopic heart allografts, atacept treatment was shown to markedly reduce the immunoglobulin levels(168–170). Overall, investigation into novel desensitization protocols is needed, and BLyS neutralization at the time of re-transplantation may limit further sensitization to HLA antigens after desensitization.

Small Molecule Targets for B cell Directed Therapy

Current monoclonal antibodies for B-cell directed therapy target surface B-cell molecules (such as CD20 or CD22) or B-cell survival factors (such as APRIL or BLyS). However, small molecule targets, such as microRNAs (miRNA), which play an important role in B cell development, are also an active area of ongoing investigation(171) with promising B cell phenotypes related to transplantation(172). For example, the miRNA-150 is expressed on mature B and has been shown to directly regulate c-Myb protein levels through two conserved mi-RNA binding sites on c-Myb RNA(173). Over-expression of miRNA-150 greatly impairs B cell development beyond the pro/pre-B cell transition, while conferring little effect on mature T cell, granulocyte or macrophage development(174). Furthermore, the miRNA-155 family plays an important role in adaptive immunity by regulating T helper cell differentiation, supporting germinal center formation, and controlling B cell cytokine production after antigen-induced activation(175).

Clearly, miRNA-directed therapy might have theoretical advantages over the aforementioned targets for B-cell therapy once allo-specific targets become available. However, relative to the miRNA-based approach, the current monoclonal antibody directed approaches have the advantage of overall safety and availability for clinical study. Conversely, the miRNA approach will need clinically standardized methods for delivery. Success in animal models has been found with `antagomirs' (cholesterol-conjugated single stranded RNA complimentary for miRNAs)(176) and vector-based therapy, including an adenovirus(177). Moreover, careful investigation is also needed to determine which miRNA(s) are important in chronic rejection, as only markers of acute rejection are currently described(178). Micro-RNA-based therapy for the induction B cell tolerance after transplantation holds promise for the future. Nonetheless, monoclonal antibody therapy has greater immediate translational potential.

A BLyS-Directed Strategy for Promoting Robust Humoral Transplantation Tolerance

We propose that achievement of durable B lymphocyte tolerance will require re-emergence of the preimmune B cells in the presence of alloantigen and under stringent repertoire selection. This aim would involve B-cell depletion therapy at the time of transplantation and subsequent reconstitution of the preimmune B-cell compartment under BLyS-limiting conditions. B cell depletion can be efficiently achieved with a B-cell specific mAb, such as Rituximab. To stringently purge allo/auto-reactive B cell specificities during reconstitution, systemic BLyS will be neutralized using the anti-BLyS mAb, Benlysta. In this scheme, B cell reconstitution should occur in the absence of supra-physiologic BLyS concentrations, permitting the "remodeling" of a B cell compartment devoid of allo-reactive specificities(102). The frequency of immature Transitional (TR) to mature Follicular (FO) B cells should be tracked in the peripheral blood of patients following transplantation. Once the TR to FO B cell ratio has reached a fully constituted range and the BLyS levels have been climbed back to normal range, BLyS neutralizing therapy can be safely stopped. In order to prevent acute rejection recipients will continue to undergo standard T-cell directed therapies.

Overall, this strategy would involve a finite amount of time for B cell compartment depletion before a prompt but judicious return to steady state B cell numbers. Although the ability to mount a primary immune response during BLyS neutralization is minimized(89), patients generally have safety profiles that are similar to placebo-treated controls(72). Certainly, such an approach is less immunosuppressive than some of the promising but broadly targeting therapies, such as alemtuzumab. It is important to note that BLyS neutralization does not dampen existing humoral immunity in both murine studies(89) and clinical investigations (74). The proposition that a TR predominate phenotype of the B cell compartment is associated

with transplantation tolerance (105) should be directly tested in the setting of BLyS neutralization in clinical transplantation.

Summary

The ability to induce sustained donor specific humoral tolerance is a key to achieving robust transplantation tolerance. Over the last two decades, advances in our understanding of the parameters governing B cell tolerance and homeostasis have shed light on a new class of targets currently ripe for application in the field of transplantation. In particular, the BLyS family of cytokines has been found to be critical in regulating the stringency of clonal negative selection and B-cell lifespan. It is our contention that development of alloantigen tolerance in the pre-immune B-cell repertoire can be achieved by therapeutically driving the de novo regeneration of the preimmune B-cell compartment in the presence of alloantigen. The basic science evidence is compelling that in vivo BLyS neutralization, using agents such as Benlysta, during this reconstitution period will promote effective deletion of newly emerging alloreactive specificities. On the other hand, desensitization remains a major challenge awaiting protocols based on delineation of the specific regulators of plasma cell survival. The BLyS-related cytokine, APRIL, holds some promise as a potential target. Overall, a compelling argument exists for clinical testing of B-cell directed therapy, specifically at the time of transplantation, in order to “remodel” a preimmune recipient B cell repertoire devoid of alloreactive specificities and, thereby stunted in its capacity to generate donor-specific antibodies in the germinal center.

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Figure 1. Central and Peripheral B Cell Ontogeny and Antigen-Mediated Activation

Hematopoietic stem cells produce Pro-B cells, which upon successful rearrangement of their heavy chain loci undergo a proliferative burst (yellow arrow) to generate Pre-B cells. Upon successful rearrangement of their light chain immunoglobulin loci Pre-B cells become immature (17) B cells. Receptor-specificity based clonal deletion (gray, ruffled cells) commences at the IMM B cell stage in the bone marrow. This central selection occurs in an all-or-none fashion. IMM cells that survive this central tolerance checkpoint (~10%) hematogenously emigrate from the bone marrow to the periphery where they are identifiable as newly emerging/transitional (TR) B cells. TR B cells are subject to a peripheral tolerance checkpoint regulated by the cytokine, BLyS. Following passage through this checkpoint these cells become mature follicular (MA/FO) or marginal zone (MZ) B cells (BLyS-dependent survival, gray) capable of participating T cell independent (TI, purple) or T cell dependent (TD, orange) responses. MZ B cells can also mature directly from bone marrow IMM B cells (dotted green line) and are BLyS-dependent. Short-lived plasma cells are formed following TI responses. The TD response occurs within the confines of the germinal center and is the initiating source of DSA. The germinal center reaction produces somatically hypermutated (SHM), high affinity memory B cells and plasmablasts (PBs). The resulting memory and plasma cells may reside within the periphery or in tertiary lymphoid structures. Ultimately, long-lived plasma cells find robust pro-survival conditions (yellow) within the bone marrow. Abbreviations: HSCs hematopoietic stem cells, IMM immature B cells, TR transitional B cells, MA/FO mature follicular B cells, MZ marginal zone B cells, MA/REC mature recirculating B cells, MEM memory B cells, SLPC short-lived plasma cells, PB plasmablasts, LLPC long-lived plasma cells.

Figure 2. The BLyS Family of Receptors and Ligands

The cells listed along the bottom of the figure produce soluble BLyS and/or APRIL. The B cell receptors listed along the top bind either of the trimeric peptides of BLyS, APRIL, or both. BR3 is the primary receptor for BLyS and governs peripheral B cell tolerance and mature B cell life-span.

TR → Peripheral selection → FO

Relative BLyS levels:

References:

Figure 3. The BLyS Dependent Peripheral B Cell Tolerance Checkpoint

The level of soluble BLyS (green) dictates the stringency of peripheral B cell negative selection (orange) at the TR→FO checkpoint. In the presence of high soluble BLyS levels, B cell clones normally subject to deletion (red cells) are permitted to enter the FO B cell pool. On the other hand, under BLyS limited conditions a range of TR B cell clonotypes normally capable of maturing (blue cells) do not survive. Examples of BLyS levels regulating humoral tolerance in the clinical setting are cited to the right.

Table 1

Phenotypic Markers of B Lymphocyte Development

	HSCs	Pro B	Pre B	IMM	TR	FO	MZ	GC	MEM	SLPC	PB	LLPC
PreBCR	-	-	+	-	-	-	-	-	-	-	-	-
sIgM	-	-	-	++	++	+	++	+	-	-	-	-
sIgD	-	-	-	-	-	+	+	+	-	-	-	-
sIgG	-	-	-	-	-	-	-	-	+	-	-	-
CD20	-	+	+	+	+	+	+	+	%	-	-	-
CD19	-	+	+	+	+	+	+	+	+	+	+	-
CD93	-	+	+	+	+	-	-	-	-	+	+	+
CD21/35	-	-	-	-	-	+	++	+	%	-	-	-
CD23	-	-	-	-	-	+	+	+	-	-	-	-
BR3	-	-	-	-	+	+	+	+	-	-	%	-
TACI	-	-	-	-	+	+	+	+	+	-	+	-
BCMA	-	-	-	-	-	-	-	-	-	+	%	+
CD38	-	-	-	-	-	-	-	+	-	-	-	-
CD27	-	-	-	-	-	-	-	+	+	+	+	+
CD138	-	-	-	-	-	-	-	-	-	-	+	+
IC kappa	-	-	-	-	-	-	-	%	%	++	++	++
Proliferative	-	-	++	-	-	-	-	+	-	-	-	-

Abbreviations: HSCs hematopoietic stem cells, IMM immature B cells, TR transitional B cells, FO mature follicular B cells, MZ marginal zone B cells, MEM memory B cells, PB plasmablast, SLPC short-lived plasma cells, LLPC long-lived plasma cells, BCR B cell antigen receptor, sIgM surface IgM, sIgD surface IgD, sIgG surface IgG, BR3 BLyS Receptor 3, TACI transmembrane activator 1 and calcium-signaling modulator and cyclophilin ligand-interactor, BCMA B cell maturation antigen, IC IgG intracellular IgG, - = no expression, % = intermediate expression, + = expression, ++ = high expression.

Table 2

Properties of Peripheral Preimmune B cells

	TR B cells	FO B cells
BLyS dependent survival	In later stages	yes
APRIL dependent survival	no	no
Capacity for germinal center formation	minimal	robust
Ability to activate naïve T cells	minimal	upon activation
Antigen presentation	yes	yes
Ig isotype switch	no	yes
Somatic hypermutation	no	yes
MHC II expression	yes	yes
Ig secretion	no	no

Various Immunotherapies and Their Impact on Preimmune and Antigen-Experienced B cells

Table 3

Drug	Target	Pre-Immune/Resting B Cells			Antigen-Experienced B Cells		
		TR	FO	MZ	GC	MEM	LLPCs
Rituximab	CD20	d	d	pd	<	pd	nd
Atacicept	BLyS, APRIL	XX	d	d	<	pd	d
Belimumab	BLyS	XX	d	d	<	pd	X
Epratuzumab	CD22	d	d	pd	<	pd	nd
Bortezomib	proteasome	nd	nd	nd	nd	nd	d
MPA	IMPDH	nd	nd	nd	<	nd	nd
CNI	Calcineurin	nd	nd	nd	<	nd	nd
Steroids	Multiple	nd	nd	nd	<	nd	nd

Abbreviations: Mycophenolic acid MPA, Calcineurin inhibitor CNI, Inosine monophosphate dehydrogenase IMPDH, depletion d, partial depletion pd, non-depleting nd, limits formation< evidence of depletion after 1 year x, sustains xx