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Comprehensive Review

# Lymphodepletion and Homeostatic Proliferation: Implications for Transplantation

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**Control of the alloimmune response requires elimination and/or suppression of alloreactive immune cells. Lymphodepleting induction therapies are increasingly used to accomplish this goal, both as part of tolerance induction protocols or to reduce the requirements for maintenance immunosuppression in the peritransplant setting. However, it is well recognized that lymphopenia induces compensatory proliferation of immune cells, generally termed "homeostatic proliferation," which favors the emergence of memory T cells. Paradoxically therefore, the result may be a situation that favors graft rejection and/or makes tolerance difficult to achieve or sustain. Yet all depletion is not alike, particularly with respect to the timing of reconstitution and the types of cells that repopulate the host. Thus, to design more effective induction strategies it is important to understand the homeostatic mechanisms, which exist to maintain a balanced repertoire of naïve and memory T and B cells in the periphery and how they respond to lymphodepletion. Here we will review the biology of homeostatic proliferation stimulated by lymphopenia, the effects of specific depleting agents on reconstitution of the T- and B-cell immune repertoire, drawing from both from animal models and human experience, and potential strategies to enhance allodepletion while minimizing the adverse effects of homeostatic proliferation.**

**Key words: T cell, memory, tolerance**

**Abbreviations APC, antigen presenting cell; APRIL, a proliferation inducing ligand; ATG, antithymocyte globulin; BAFF, B-cell activating factor, TNF family; BCMA, B-cell maturation; BCR, B-cell receptor; BLyS, B lymphocyte stimulator; BR3, B-cell–activating factor receptor 3; CNI, calcineurin inhibitor; CTL, cytotoxic lymphocyte; DSA, donor specific antibody; FO, follicular; FoxP3, forkhead box P3; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; HP, homeostatic proliferation; IFN gamma, interferon gamma; IL-7R, interleukin 7 receptor; ITP, idiopathic thrombocytopenic purpura; MHC, major histocompatibility complex; MZ, marginal zone; RAG-/-, recombinase activating gene deficient; rATG, rabbit antithymocyte globulin; SCID, severe combined immunodeficiency; SLO, secondary lymphoid organ; TACI, transmembrane activator and calcium-modulating cyclophilin ligand interactor; TCR, T-cell receptor; TNFR, tumor necrosis factor receptor; TREC, T-cell receptor excision circles.**

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## Introduction to Lymphoid Homeostasis

Throughout adult life, the size and composition of the peripheral lymphocyte pool is tightly regulated and, in the absence of disease, is maintained at relatively constant levels (1,2). A few general principles govern the physiologic response to perturbation of this balance. First, insults that induce depletion of the peripheral compartment such as viral infection, or drugs used in peritransplant induction therapy or cancer chemotherapy, stimulate replenishment of that compartment until "fullness" is reachieved. Second, mechanisms conversely exist to ensure that "fullness" is not exceeded. For instance the rapid peripheral expansion of CD8+ T cells in response to viral antigens is followed by massive effector cell death once the immune response is curtailed (3), resulting in restoration of T-cell numbers. Third, different lymphocyte subsets occupy different "niches" as determined by their use of distinct growth and survival factors and in some cases their occupancy of different anatomical sites. For example, the numbers of T cells are not increased in B-cell–deficient mice and vice versa (2,4,5). In this article, we will review the basic biology of the response to lymphocyte depletion, with a particular emphasis on how this impacts strategies to prevent rejection and induce tolerance in the transplant setting.

## T-Cell Reconstitution After Lymphodepletion

T-cell reconstitution after lymphodepletion depends on two sources: (1) thymopoiesis which results in the generation of new naïve T cells; and (2) spontaneous expansion of the

existing peripheral T-cell pool, often termed "homeostatic proliferation (HP)". The relative contribution of each pathway to replenishment of the peripheral T-cell pool varies with age, thymic function, availability of homeostatic cytokines and antigenic stimulation and time from lymphodepletion. Moreover, the balance between these two sources of T cells during immune reconstitution may influence allograft responses.

### *HP comes in two flavors*

HP is typically defined as the "spontaneous" proliferation of peripheral T cells under lymphopenic conditions. We put the term "spontaneous" in quotes, because as outlined below, there are indeed stimuli and signals required for proliferation. The extent to which HP occurs is proportional to the degree and duration of lymphopenia (6,7). The mechanisms that drive HP have been studied using various mouse models of lymphopenia. These show that there are two main types of HP: a slow (~one cell division per 24–36 h), nonexogenous antigen driven process which occurs in response to a "sensing of empty space" and a fast (~one division per 6–8 h), antigen-specific process, undergone by a smaller subset of T cells that is likely driven by presentation of antigens from commensal bacteria (gut flora; 8–10). In mouse models, naïve T cells undergo only slow HP in animals which have been made acutely lymphopenic by irradiation or drugs, whereas the additional component of fast HP (also known as "spontaneous" HP) also is observed in mice lacking T cells because of genetic defects (8–11). Whether it is abnormalities associated with congenital T-cell deficiency or the "chronicity" of depletion *per se* which results in the occurrence of rapid homeostatic expansion is not known, nor have these findings in mice been experimentally confirmed in lymphopenic patients.

### *Signals required for HP*

Both naïve and memory T cells undergo HP in response to lymphopenia, with partially overlapping signal requirements (Figure 1 and Table 1).

**Naïve T cells—slow HP:** The two best characterized signals required for slow HP of naïve T cells are (1) direct contact with major histocompatibility complex (MHC) II and MHC I for CD4 and CD8 T cells, respectively (10,12–16) and (2) the cytokine IL-7 (17–20). Slow HP of naïve T cells occurs in the absence of any exogenous antigen; instead it is supported by self-peptide:MHC complexes on antigen presenting cells (APCs; 14,15 21–23). Under lymphoreplete conditions, these low-affinity interactions between T-cell receptors (TCRs) and self-peptide:MHC complexes in the periphery impart tonic "survival signals" to naïve T cells but do not induce division (12–14). However, as discussed further below, under conditions of lymphopenia these same signals stimulate T-cell division. Although the majority of naïve CD4 and CD8 T cells undergo HP (24), the extent is determined by the strength of interaction between the TCR and self-peptide:MHC complexes (25,26). Thus HP preferentially expands T cells with a greater specificity and a stronger avidity for self. This may, in part, be implicated in the development of autoimmunity induced by lymphopenia, as has been described with use of alemtuzumab and other depletional agents (27,28). Importantly, slow HP can proceed independently of conventional costimulatory signals such as CD28, CD40 and LFA-1 and thus may be resistant to therapeutic costimulatory blockade (6,10,29).

**Role of IL-7 and other cytokines in slow HP:** Competition for IL-7, a key cytokine required for naïve T-cell survival, is one of the primary drivers of slow HP. IL-7 is produced at relatively constant levels primarily by stromal cells in the bone marrow and thymus and by the fibroblastic reticular cells of the secondary lymphoid organs (SLOs; 17–19,30). It is also produced by dendritic cells and macrophages albeit in far smaller amounts (31–33). Under normal physiological conditions, IL-7 concentrations are limiting and circulating levels are insufficient to support cell division in the face of tonic TCR–MHC interactions. However during severe lymphopenia the effective amount of IL-7 per cell is increased (34–37). In mouse models, this increase in IL-7 has been attributed to diminished use because of reduced T-cell mass, i.e. the number of T cells competing for the same amount of IL-7 is drastically lower (33). In a further refinement of this concept, a recent longitudinal study of HIV patients receiving highly active antiretroviral therapy (HAART) demonstrated that fluctuations in IL-7 levels correlated more closely with IL-7 receptor expression on reconstituting T cells rather than absolute T-cell numbers *per se* (38). However, older studies of HIV infected humans have proposed that IL-7 production is increased in T-cell–depleted tissues (35). Obviously, these two options are not mutually exclusive, and in either case, normal IL-7 levels are restored when CD4 T-cell numbers recover (37). These increased levels of IL-7 drive immune reconstitution by enhancing T-cell proliferation in response to low-affinity antigens: the stronger the TCR affinity the less IL-7 is required (25,26). These observations support a model of slow HP in which there is a threshold strength of TCR affinity for self-peptide:MHC ligands required for cell division to occur. The increased availability of IL-7 during lymphopenia lowers this threshold by augmenting TCR signals in response to binding of self-peptide:MHC complexes. As T-cell numbers increase, the availability of IL-7 decreases. In addition, increased levels of IL-7 cause downregulation of the IL-7 receptor alpha chain which is expressed on most resting T cells, leading to decreased responsiveness to IL-7 and eventual termination of the homeostatic response (39). This model favors IL-7 levels as the main signal which is "sensed" by naïve T cells when "space" is created during lymphopenia, making IL-7 levels a "rheostat" that modulates the "gain" on TCR signals for naïve T cells (10,37,40).

Other cytokines have been suggested to play a role in slow HP of naïve T cells; this has been studied best in CD8 T cells. For instance, several cytokines including IL-2, IL-4, IL-6 and IL-15 modulate IL-7 receptor alpha chain expression


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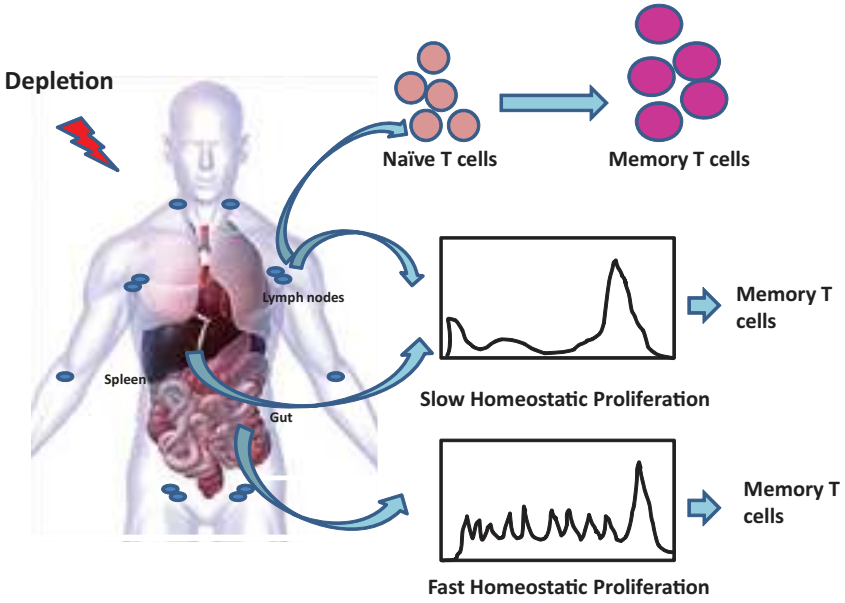


**Figure 1: Types and consequences of homeostatic proliferation after lymphodepletion.** Slow homeostatic proliferation of naïve T cells occurs in the secondary lymphoid organs, requires IL-7 and MHC contact and is resistant to costimulatory blockade. Fast homeostatic proliferation of naïve T cells is IL-7 and MHC-independent, requires costimulatory signals and may be driven by gut bacteria. Whether fast and slow homeostatic proliferation occur in humans remains unconfirmed. Homeostatic proliferation of naïve T cells results in their conversion to memory T cells. Memory T cells themselves also undergo homeostatic proliferation in response to lymphopenia.

on CD8 T cells thus rendering them more sensitive to the proliferative effects of IL-7 when that cytokine is limiting (39). *In vitro*, both IL-6 and IL-21 can synergize with IL-7 to stimulate TCR-independent proliferation of naïve CD8 T cells (41,42). The role of these cytokines and mechanisms for physiologic *in vivo* lymphocyte repopulation remains to be determined.

**Trafficking through SLOs:** Slow HP of naïve T cells in mature animals is spatially confined to the T-cell zones of SLOs, specifically the periarteriolar lymphocyte sheaths of the spleen and the paracortical regions of the lymph nodes (43,40). The CCR7 ligands CCL19 and CCL21 are responsible for directing naïve T cells into the lymph nodes, and migration through these organs is required for HP (30,44,45). CCL19 may also serve as a survival factor for naïve T cells independent of its role as a chemokine, as evidenced by the inability of CCL21 to compensate for CCL19 deficiency despite facilitating entry into the lymph node (30). Lymphoid organs provide a rich network of extracellular matrix and stromal cells where naïve T cells can encounter APCs. The fibroblastic reticular cells, which produce IL-7 also reside in the SLOs. In lymph nodes, IL-7 does not diffuse freely through the interstitium but is immobilized in the stroma attached to extracellular matrix components (46). This may achieve the effect of providing locally high concentrations of IL-7 to cells trafficking through the lymph nodes and also defines a spatially constrained niche for naïve cells competing for IL-7 under lymphopenic conditions.

**Naïve T cells—fast HP:** Fast HP, sometimes termed "spontaneous proliferation" (7–9,11) has been described in chronically lymphopenic hosts such as RAG–/–, SCID mice or other strains completely lacking T cells because of one or more genetic defects. In contrast to slow HP, which is IL-7 dependent and driven by self-peptide:MHC complexes, fast proliferation occurs in response to exposure to foreign antigens and is IL-7 independent. In contrast to slow HP which is unaffected by costimulatory blockade, fast HP requires costimulatory signals and results in swift upregulation of memory surface markers and T effector function such as increased IFN gamma and IL-2 production, consistent with a typical antigen specific immune response (7–9,47,48). Notably, fast HP is greatly reduced when recipient mice are housed in germ-free conditions leading to the conclusion that it is likely directed against commensal gut flora (8). Given the fact that this type of proliferation has all the hallmarks of an antigen specific immune response, albeit in a severely lymphopenic animal, it may be a misnomer to classify it as a "homeostatic" response *per se*.

**HP of memory T cells:** T memory cells also undergo HP during lymphopenia, but with certain key differences. First, memory T cells expand more quickly. In a study of partially lymphodepleted mice where proliferation rates of congenically marked and adoptively transferred trace cell populations were studied, it was observed that memory T cells underwent much more rapid and extensive homeostatic expansion than did naïve T cells (49). Another difference is the signals required. In contrast to naïve T cells, HP of memory T cells can occur without homing to SLOs and generally does not require contact with MHC molecules (50–52). CD4 and CD8 T memory cells have distinct but overlapping requirements. HP of CD8 memory T cells is driven by the cytokine IL-15; however both IL-7 and IL-15 are required for their long-term survival (20,53). The picture for CD4 memory T cells is more complicated. IL-7 alone is required for HP under lymphopenic conditions but both IL-7 and IL-15 are required for the low degree of "basal"

**Table 1:** Requirements for homeostatic proliferation in response to lymphopenia

| Cell type | Slow HP | Fast HP |
|---|---|---|
| Naïve CD4 T | IL-7 dependent | IL-7 independent |
| | Self-peptide driven | ? foreign antigen driven |
| | MHC II dependent | MHC II independent |
| | Costimulation independent | Costimulation dependent |
| | Homing to SLOs required | SLO independent |
| Naïve CD8 T | IL-7 dependent | IL-7 independent |
| | Self-peptide driven | ? foreign antigen driven |
| | MHC I dependent | MHC I independent |
| | Costimulation independent | Costimulation dependent |
| | Homing to SLOs required | SLO independent |
| Memory CD4 T | Unclear if occurs in response to lymphopenia | IL-7 dependent |
| | | Mostly MHCII independent* |
| | | Costimulation independent |
| | | SLO independent |
| Memory CD8 T | Unclear if occurs in response to lymphopenia | IL-15 or high IL-7 dependent |
| | | MHC I independent |
| | | Costimulation independent |
| | | SLO independent |
| T regulatory | Proliferate in response to lymphopenia with kinetics intermediate between slow and fast HP | |
| | Unclear cytokine, MHC and antigen requirements | |
| B2 B | BLyS dependent | |
| | IL-7 independent | |
| | Proliferation rate increased with high BLyS levels | |
| | Unclear antigen requirements | |
| | Unclear requirements for T-cell help | |

*A small MHCII-dependent subset of rapidly proliferating CD4 memory T cells has been described (see Ref. 55).

HP that occurs under lymphoreplete conditions (54–56). Although HP of CD4 memory T cells can proceed independently of MHC II molecules, a small, rapidly dividing subset of CD4 memory T cells does require contact with MHC II molecules for continued division (52,55). Further work is required to determine whether this subset of CD4 T cells is responding to foreign antigens such as commensal bacteria versus self-antigens.

**Kinetics of recovery: CD4 versus CD8 T cells:** In both mice and humans, during lymphopenia naive CD8 T cells undergo HP at faster rates than naïve CD4 T cells. This results in more rapid reconstitution of the CD8 compartment whereas CD4 T-cell reconstitution lags. This finding has been observed in numerous studies of human lymphopenia induced by chemotherapy, sepsis and transplant induction therapy as well as in various mouse models of lymphopenia (33,57–62). One possible explanation for this observation is the differential capacity of IL-7 to promote HP of naïve CD4 versus CD8 T cells. In cancer patients treated with recombinant IL-7 CD8, T cells were preferentially expanded over CD4 T cells (63). In patients with high serum levels of IL-7 because of lymphopenia, CD4 T-cell counts were inversely proportional to the concentration of IL-7, leading to unexpected hypothesis that high levels of IL-7 may inhibit proliferation of CD4 T cells (35,36). Consistent with this, one recent study in mice found that high levels of IL-7 produced by lymphoid stromal cells downregulate expression of MHC II on dendritic cells that are required for HP of CD4 T cells. These investigators reasoned that this provides a negative feedback loop to avoid uncontrolled CD4 T-cell proliferation in the setting of lymphopenia (33). Further work is required to determine whether the same mechanisms are at work in humans. However, these findings have clinical relevance because they suggest an explanation for the observation that CD4 T-cell recovery is prolonged in patients recovering from depletional induction therapies such as alemtuzumab or antithymocyte globulin (ATG; 60,64,65).

### *Consequences of HP*

An important consequence of HP of naïve T cells is their conversion to cells with a functional memory phenotype such as rapid induction of cytotoxic lymphocyte activity (CD8 T cells) or IFN gamma secretion (CD4 T cells) in response to TCR engagement, and relative resistance to costimulatory blockade (7,6,49,66–71). This conversion has been described in depletional models as well as in neonatal mice in which the first wave of naïve T cells to populate the SLOs undergoes HP and acquires memory markers (71). Sener et al. have demonstrated that the expansion of memory T cells in mice treated with ATG derives primarily from homeostatically proliferating naïve T cells (68). As these memory T cells do not pass through an effector intermediate and in some experimental systems exhibit subtle differences in T-cell activation markers they have been deemed "pseudo" memory T cells (50,69,72,73). However, this may be only a semantic issue, as a growing body


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of evidence supports the fact that these "memory-like" cells can function as *bona fide* memory T cells, for example in their ability to generate protective responses to pathogens such as *Listeria monocytogenes* comparable to conventional CD8 T cells (74), and that the memory phenotype is stable over time (67,68,75,76). Collectively, these findings indicate that HP is insufficient to reexpand the naïve T-cell pool in the face of T-cell depletion. Without the generation of naïve T cells via thymopoiesis, there is a net loss of naïve T cells in the peripheral pool compared to predepletion levels and skewing toward a memory phenotype with all its attendant consequences (68,75,77).

### *Regulation of HP*

As described above, the two most important factors regulating HP are competition for homeostatic cytokines such as IL-7 and access to MHC:self-peptide complexes. Relevant to this, the effects of cellular competition on HP have been studied experimentally using adoptive transfer experiments in various mouse models of lymphopenia. These experiments illustrate the fluidity of competitive niches under conditions of lymphopenia. For example under lymphopenic conditions memory CD4 T cells inhibit HP of naïve CD4 T cells and prevent their conversion to a memory phenotype; this inhibition was not observed under lymphoreplete conditions during which HP of naïve and memory CD4 T cells seemed to be independently regulated (73). Others have shown that transfer of large numbers of CD8 T cells can inhibit HP of CD4 as well as CD8 T cells suggesting that there is overlap in the regulation of CD4 and CD8 compartments (15,43). These investigators also demonstrate that HP of naïve T cells can be suppressed by the addition of more naïve T cells. These cells must be capable of entering the T-cell zones of the SLOs where HP takes place, suggesting competition for a resource spatially limited to these organs such as IL-7. Inhibition of HP by these so-called "bystander" cells does not involve competition for MHC/peptide complexes. In fact, T cells with a low affinity for a given peptide can effectively compete with T cells with a high affinity for the same peptide (43).

### *T regulatory cells and HP*

The question of whether T regulatory cells undergo HP at the same rate as nonregulatory cells is subject of ongoing investigation and some controversy. Studies in mice have yielded conflicting results with one study demonstrating reduced proliferation rates compared to memory cells (49), one study showing full restoration (78) and one study showing a 28-fold expansion (67). These seemingly contradictory results are likely because of differences in experimental systems. The ability of homeostatically expanded T regulatory cells to contain alloreactivity also differs depending on the experimental model. In mice treated with depleting antibodies, small numbers of exogenously added T regulatory cells were able to prevent graft rejection (49). However, in RAG–/– mice reconstituted with donor splenocytes, rapid allograft rejection proceeded despite massive homeostatic expansion of T regulatory cells and large numbers of FoxP3+ cells infiltrating the kidney allografts (67).

A related question is whether T regulatory cells are preferentially expanded after depletion with specific drugs. Several investigators have reported the selective expansion of T regulatory cells after treatment with ATG (64,79–81). Gurkan et al. demonstrate that these expanded Foxp3+ T regulatory cells were primarily of thymic origin in pediatric but not adult kidney recipients (64). Expansion of Foxp3+$T_{regs}$ after alemtuzumab treatment has also been described by some investigators (65,82) but not others (83). An alemtuzumab expanded CD28-CD8+ T cell with regulatory capacity has also been characterized in humans (60). The clinical consequences of this expansion likely depend on the balance between T regulatory cells, naïve cells and memory cells in the reconstituting T-cell pool. The high rate of early acute rejection in renal transplant recipients who received alemtuzumab without maintenance therapy argues that expansion of T regulatory cells, to the degree that it occurs, is not by itself sufficient to control alloreactive T cells (72,84).

A distinct issue is the effect of T regulatory cells on the process of HP itself. In some mouse models, T regulatory cells have been shown to suppress HP. Shen et al. demonstrated that T regulatory cells could efficiently prevent HP of T cells with low TCR avidity but less effectively for cells with high TCR avidity (85). Winstead et al. showed that T regulatory cells effectively inhibited fast "spontaneous" HP but failed to inhibit slow HP of naïve T cells (86). These investigators also reported that absence of T regulatory cells during homeostatic reexpansion of the T-cell pool resulted in a constricted T-cell repertoire and impaired ability to mount an antigen specific response to certain pathogens (87). This argues for a role for T regulatory cells in preventing uncontrolled expansion of rapidly proliferating dominant clones at the expense of repertoire diversity.

### *Does HP predispose toward alloreactivity?*

Mouse models have shown that HP can function as a barrier to transplant tolerance by generating allospecific T memory cells resistant to costimulatory blockade. Wu et al. showed in a cardiac allograft model using depleting antibodies that residual T cells undergoing HP are resistant to costimulatory blockade, and remain so even after proliferation has ceased (6). As noted above, work using the same experimental model showed that that memory T cells undergo accelerated HP compared to naïve and T regulatory cells after depletion, leading to skewing of the lymphocyte pool toward a memory phenotype. These investigators concluded that HP favors the expansion of tolerance-resistant memory T cells both by more efficient proliferation of T memory cells and conversion of naïve T cells to memory T cells (49). They hypothesize that reconstitution of memory cells in the presence of an allograft may exaggerate the development of allospecific

responses, a mechanism that would be operative in autoimmune settings as well. A recent report from Nadazdin et al. using a nonhuman primate kidney allograft model and a lymphodepleting induction regimen showed that a high pretransplant frequency of donor reactive T memory cells was the best predictor of allograft rejection (88). These data suggest that HP *per se* may not always predispose to alloreactivity. Rather HP may contribute to alloreactivity only when preexisting alloreactive T cells are expanded and/or converted to a memory phenotype.

#### *The role of the thymus after lymphodepletion: balancing thymopoiesis and HP*

It has long been recognized that thymic function declines with age and that thymic involution begins after the first year of life (89). Thymic function can be estimated by determining levels of TCR excision circle (TREC) bearing cells in the blood as markers of cells that have recently left the thymus, so-called recent thymic emigrants (90). Data from stem cell transplant recipients, HIV patients and healthy controls demonstrate an inverse correlation between TREC levels and age (90–94). However, TREC levels are readily detectable in adults through age 60 and measurable up to age 70, indicating that some thymic function persists through adulthood (89,91,92). In patients with diminished thymic function, HP is the main contributor to expansion of the peripheral pool in the immediate aftermath of lymphodepletion whereas the impact of thymopoiesis may be slower (62). Impaired thymic output may extend the duration of lymphopenia leading to a longer period of HP, which could further skew the T-cell pool toward a memory phenotype as well as restrict repertoire diversity (77,93,95).

Most of the human data on the role of thymopoiesis after lymphodepletion derives from cancer patients who have received thymotoxic myeloablative conditioning regimens. In these patients thymopoiesis is slow and reconstitution of the T-cell repertoire may take 1–2 years (58,62). In one study of breast cancer patients who received autologous stem cell transplants, patients between 30 and 40 years of age had a high probability of restoration of peripheral naïve CD4 T-cell numbers because of thymopoiesis; this declined significantly after age 40 and was less than 10% for those over age 50 (93). However, studies of HIV patients treated with HAART illustrate that young- to middle-aged adults who have not received thymotoxic therapies retain appreciable thymic reserve which can rebound after injury (89,90). Similar findings have been observed in the transplant setting. A recent study of 58 adult and 17 pediatric kidney transplant recipients treated with rabbit antithymocyte globulin induction therapy suggests that thymopoeisis is functional in adult transplant patients, and that recent thymic emigrants contribute significantly to early reconstitution of the CD4 and CD8 pool in adults (64). These data suggest that it may be useful to consider preservation of thymic function in the design of transplant induction regimens.

It should also be appreciated that there is some degree of cross-regulation between HP and thymopoiesis, as naïve T cells and recent thymic emigrants compete for resources in the periphery. Transferred naïve T cells undergo increased HP in thymectomized lymphopenic mice (68,71). Under lymphopenic conditions, recent thymic emigrants are preferentially incorporated into the peripheral pool. However, under lymphoreplete conditions, preexisiting naive T cells retain a survival advantage as recent thymic emigrants express lower levels of the IL-7R, and IL-7 is limiting when the immune compartment is intact (96).

## B-Cell Reconstitution After Lymphodepletion

Similar to T cells, reconstitution of B cells after lymphodepletion relies on *de novo* lymphopoiesis (the recruitment of B-cell precursors from the bone marrow) as well as on HP in the periphery. Note: this section pertains only to the effect of lymphodepletion on B2 B cells. The effect of lymphodepletion on the slow rate of self-renewal of peritoneal and pleural B1 cells has not been well characterized.

#### *B-cell subsets during development*

During development, B cells transit through a highly regulated series of intermediate steps resulting in phenotypically and anatomically distinct B-cell subsets. In the bone marrow, most high-affinity autoreactive clones are eliminated (97). Surviving cells then exit the marrow and migrate to the spleen for further rounds of selection before differentiating into mature primary (naïve) B cells: either follicular (FO) or marginal zone (MZ) B cells (98,99). The adult mouse bone marrow generates about 15 million immature B cells daily, only 10% of which ever leave the marrow and fewer than 5% of which are represented in the mature naïve pool, reflective of the high efficiency of selection mechanisms (100). Thus, under lymphoreplete conditions, most immature B cells die. Survival is dictated by B-cell receptor avidity and specificity and competition for homeostatic cytokines as described below.

#### *The central role of BLyS*

The cytokine BLyS (also known as B-cell activating factor) is the main homeostatic cytokine for both naïve and memory B cells, responsible for controlling their survival and differentiation (97–99,101,102). In addition, BLyS promotes T-independent B-cell antibody responses and survival of plasmablasts and plasma cells (103). BLyS is a member of a family of tumor necrosis factor receptor (TNFR) ligands and interacts with at least three known receptors: BR3 to which it binds most avidly, TACI and BCMA. APRIL is a second TNFR ligand in the same family which binds TACI and BCMA with100-fold higher affinity than BLyS but does not bind BR3 (97,99,102). Homeostatic niches are defined by different expression patterns of BLyS family receptors and ligands (98). Earlier in development expression


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of BR3 predominates on immature, mature naïve and recently activated B cells, favoring binding of BlyS. Successful competition for BLyS as determined by BR3 receptor density determines the lifespan of FO and MZ B cells and dictates whether transitional B cell will complete differentiation (98,104). Later, in B-cell development, expression of BCMA and TACI promote survival of long-lived plasma cells through their interaction with APRIL (97–99).

Importantly, BLyS functions as the main sensor of B-cell "space" under conditions of lymphopenia, analogous to the role of IL-7 for naïve T cells (Figure 2; 98). This has been demonstrated most clearly at the transitional B-cell stage. Serum BLyS levels increase after B-cell depletion probably because of decreased receptor mediated clearance. Survival of transitional B cells depends on intraclonal competition for BLyS (105,106). Thus, under lymphoreplete conditions where BLyS is limiting, immature B cells with lower avidity autoreactive specificities are eliminated at the transitional B-cell stage. However, under lymphopenic conditions when BLyS is in excess, selective stringency is relaxed and all clones including those with autoreactive specificities may be allowed to mature (101,106). This presents an apparent paradox with regard to how B-cell depletional therapy with drugs such as rituximab can be effective in autoimmunity (discussed below).

Consistent with the above, elevated BLyS levels after B-cell depletion have been correlated with antibody mediated autoimmunity in a variety of diseases including systemic lupus erythematosus (SLE; 107,108), Sjogrens (109,110), Wegener's Granulomatosis (111) and ITP (112). A cohort of multiple sclerosis patients treated with alemtuzumab, an antibody that depletes both B and T cells, was found to have sustained elevations of BLyS levels despite relatively rapid recovery of peripheral B cells (113). Although this study did not correlate BLyS levels with clinical relapse one series of alemtuzumab treated MS patients had a 20% incidence of autoimmune thyroiditis and a 3% incidence of autoimmune thrombocytopenia (27). The effect of elevated BLyS levels on alloimmunity are less clear. Rituximab has been used with generally favorable results for treatment of sensitized transplant recipients (114–117). However, various investigators have observed an increased rate of antibody mediated rejection in renal transplant recipients treated with alemtuzumab (82,118–121). Bloom et al. found that BLyS levels were elevated in renal transplant recipients treated with alemtuzumab; patients who experienced rejection had increased levels of BLyS at the time of calcineurin inhibitor withdrawal and positive DSA at the time of rejection (119). Optimally, the use of B-cell depletional therapies might be paired with measures to blunt or prevent BLyS-driven permissive expansion of autoreactive and alloreactive B cells, especially in sensitized recipients. However, the effects of depletion and of BLyS on regulatory B cells are not well understood and may need to be considered when manipulating BLyS in this setting.

### *HP of B cells: unresolved issues*

HP of B cells has been reported in different mouse models of lymphopenia (122–126). However recent data characterizing the requirements and consequences of B-cell HP and the B-cell subsets involved are sparse. Older studies demonstrate that unpurified, small resting splenocytes or lymph node cells enriched for B cells proliferate when transferred into genetically immunodeficient mice (122,125). More recent experiments identify a late transitional CD21+ B-cell population, which preferentially undergoes HP (124). Proliferation has been shown to be BLyS dependent (122,124) and independent of IL-7 (122). However, there is a lack of consensus on a number of issues, including whether B-cell HP is antigen-dependent (124) or antigen-independent (123), does (Rawlings, personal communication) or does not (123) require T cells, and induces an activated phenotype (125) or leaves B cells in a naïve state (122,126).

### *B-cell depletion: How does it work?*

A growing body of evidence suggests that the therapeutic effect of B-cell depletion derives from its long-term effect on B-cell reconstitution in addition to more immediate deletion of pathogenic B cells. In data derived from the autoimmune and cancer literature, B-cell depletion was associated with prolonged expansion of CD24hiCD38hi transitional B cells and delayed reemergence of B memory cells compared to healthy controls, consistent with an immature phenotype (107,127–130). In some cases, memory B cells did not reappear for several years (127). In SLE, rheumatoid arthritis (RA) and lymphoma patients treated with rituximab expansion of transitional B cells and decreased memory B cells correlated with clinical remission and/or absence of autoantibodies (127,128,130). Conversely, autoimmune disease relapse was associated with early recovery of B memory cells. Emergence of transitional B cells may also predict favorable outcomes in transplantation. A recent study of spontaneously tolerant kidney recipients found that tolerance was associated with a B-cell gene signature and higher percentages of peripheral transitional B cells (131). In a nonhuman primate model of islet cell transplant using induction with rituximab and ATG followed by maintenance with sirolimus monotherapy, a high ratio of transitional B cells to memory B cells was associated with allograft acceptance in the absence of immunosuppression (132). At present, it is unclear whether these benefits are mediated directly by the transitional B cells themselves or simply represent the relative absence of B memory effector cells.

## Clinical implications

**The paradoxical effects of B-cell depletion: implications for tolerance:** As noted above, depletion can lead to permissively high BLyS levels associated with lymphopenia, which may promote the emergence of new clones with

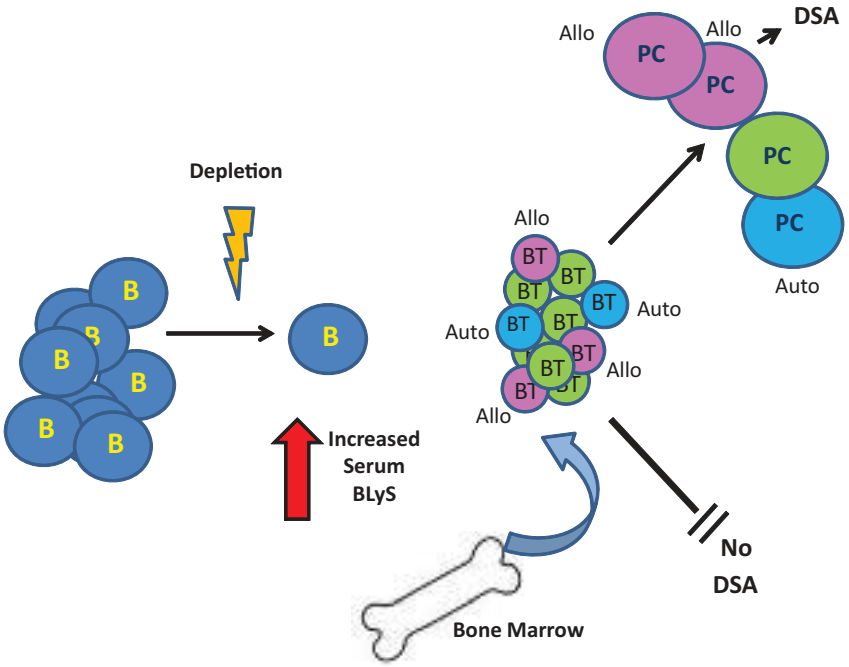


**Figure 2: B-cell depletion results in elevated BLyS levels and expansion of transitional B cells.** When BLyS is in excess, selective stringency is relaxed allowing B-cell precursors with autoreactive and possibly alloreactive specificities to emerge. Maturation of alloreactive transitional B cells may result in antibody-producing plasma cells. However, paradoxically, depletion is also associated with expansion and delayed maturation of the transitional B-cell compartment. As long as these cells remain immature they do not produce antibodies. The factors which regulate transitional B-cell maturation after B-cell depletion, require further characterization. Key: B, B cell; BT, transitional B cell; Allo, alloreactive; Auto, autoreactive; PC, plasma cell; DSA, donor-specific antibody.

autoreactive and alloreactive specificities. Thus, at first glance it seems paradoxical that depletion can be effective therapy for auto- and alloimmunity. However, the explanation is likely that the primary cells which emerge postrituximab therapy are transitional B cells, which, although possibly autoreactive, fail to mature into antibody-secreting cells (127). Nonetheless, accepting that high BLyS levels may be undesirable, the combination of rituximab followed by belimumab, a BLyS inhibitor, may prove a useful approach to reduce alloantibody production in sensitized patients (133). However, these benefits should be weighed against the risk of prolonged B-cell depletion. Furthermore, the lack of dependence of plasma cells and some B memory cells on BLyS may limit the utility of this approach (134,135).

**Lessons learned from alemtuzumab and ATG: the persistence of memory:** Memory T cells are expanded after lymphocyte depletion with alemtuzumab or ATG, in some cases far exceeding pretransplant levels (60,64,65,136). Alemtuzumab has been shown to preferentially expand effector memory T cells in both nonhuman primates and renal transplant recipients (60,83) whereas induction with ATG expands both effector memory and central memory T subsets (64). In addition, Pearl et al. have shown that CD4+ effector memory T cells are relatively resistant to depletion by ATG or alemtuzumab and that episodes of rejection were preceded by a significant increase in CD4+ T effector memory cells in lymphopenic patients treated with alemtuzumab (72). Thus, after treatment with lymphodepleting antibodies multiple avenues can lead to memory T-cell expansion: (1) incomplete depletion, (2) HP and (3) homeostatic conversion of naïve T cells to memory T cells. It would seem that all roads lead to Rome.

### *Allospecific depletional strategies*

Specific depletion of alloreactive clones presents an attractive strategy because HP could be avoided. As yet, however, there are not effective proven means to accomplish this goal of "clonal deletion". Strategies such as anti-CD25 immunotoxins or pruning CFSE-labeled alloreactive cells have not proven practical and donor specific transfusion paired with cytotoxic agents have yielded mixed results (137–141). Other approaches still in developmental stages include the use of a mutant IL-15 molecule (nonstimulatory, but complement fixing) to delete effector cells (142,143). Importantly, a key problem, which hinders the development of these approaches is the absence of quantitative assays such as MHC tetramers to test whether or not alloreactive cells have been deleted.

## Conclusion

Lymphodepleting agents are becoming a common component of transplant induction therapy and are used to promote allograft acceptance. However, paradoxically these agents may predispose to rejection by promoting the development and expansion of alloreactive B and T memory cells. Furthermore preexisting memory T cells may be relatively resistant to lymphodepletion. Thymopoiesis can counter this trend by contributing naïve T cells to the peripheral T-cell compartment and increasing T-cell repertoire diversity. New induction strategies should focus on minimizing the effects of HP, and developing means to


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specifically detect and deplete alloreactive T cells. Consideration should be given to the effects of induction strategies on thymic function and perhaps prolonged lymphodepletion should be reserved for those patients with robust thymic reserve.

## Disclosure

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. LAT and family members have a financial interest in Novartis.

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