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Recollective Homeostasis and the Immune Consequences of Peritransplant Depletional Induction Therapy

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Summary

Ones cellular immune repertoire is composed of lymphocytes in multiple stages of maturation–the dynamic product of their responses to antigenic challenges and the homeostatic contractions necessary to accommodate immune expansions within physiologic norms. Given that alloreactivity is predominantly a cross-reactive phenomenon that is stochastically distributed throughout the overall T cell repertoire, ones allospecific repertoire is similarly made up of cells in a variety of differentiation states. As such, the continuous expansion and elimination of activated memory populations, producing a "recollective homeostasis" of sorts, has the potential over time to alter the maturation state and effector composition of both ones protective and alloreactive T cell repertoire. Importantly, a T cell's maturation state significantly influences its response to numerous immunomodulatory therapies used in organ transplantation, including depletional antibody induction. In this review, we discuss clinically utilized depletional induction strategies, how their use alters a transplant recipient's cellular immune repertoire, and how a recipient's repertoire influences the clinical effects of induction therapy.

Keywords

homeostasis; induction; memory T cell; tolerance; transplantation

Introduction

Ones immune repertoire is dynamic–the ever-changing product of expansions responding to antigenic challenges and compensatory contractions required to maintain an immune system within the confines of human physiological norms. As such, each new immunological experience creates a burst of protective memory while requiring, particularly in adults, a reactive elimination of previously established memory to make space for the most proximal immune response (1). Whether this contraction is stochastic or programmed is unclear; however, it is increasingly evident that human immune responses to any given pathogen have the potential to alter the existing immunity of prior responses. We exist as a dynamic product of our memories formed and forgotten, in a constant state of "recollective homeostasis".

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As with homeostatic condensation in response to lymphoproliferation, lymphopenia, whether as an intended consequence of therapy or as a result of viral infection, evokes similarly compensatory effects, particularly expansion borne through homeostatic activation and proliferation that present dynamic opportunities for changes in the immune repertoire (2). This too is likely to be an evolutionarily conserved phenomenon with broad and persistent immunological effects. Evolutionarily relevant examples are seen in viral infections, such as infections with the human immunodeficiency virus (HIV) and other viruses that induce varying degrees of lymphopenia (3). While resolution of the infection, either through immunity or successful antiviral therapy, prompts homeostatic immune reconstitution, the cellular activation responsible for this process carries with it the risk of maladaptive inflammation and autoimmunity, a phenomenon also seen following recovery from lymphopenia after bone marrow transplantation (4, 5). In recent years, the reciprocal responses to T cell activation and elimination have been increasingly well characterized. Commensurate with this understanding has been a marked rise in the use of therapeutic T cell depletion in organ transplantation as part of multimodal immunosuppressive regimens to prevent allograft rejection (Table 1).

Immunosuppressive regimens can generally be classified as induction, maintenance, and/or rescue therapies and are applied to varying degrees based on the organ transplanted (6). Induction immunosuppression consists of potent strategies to prophylactically control the intense immune response to alloantigen immediately following organ implantation. Induction agents and strategies are so potent as to be prohibitively toxic for long-term use. Once the initial assault of the peritransplant immune response subsides, the need for induction therapy lessens and can be replaced by maintenance immunosuppression. Maintenance regimens are highly variable from organ to organ and transplant center to transplant center, but all seek to balance the benefits and risks of immunosuppression. During acute rejection episodes, maintenance regimens are augmented by rescue therapies that include agents similar to induction in their intensity, efficacy, and toxicity. In clinical practice, there are multiple immunosuppressive medications that, depending on dose, route, and schedule, can be used as induction, maintenance, or rescue.

The use of induction therapy has steadily increased, with 86% of all renal transplants and greater than 50% of all heart, lung, and pancreas transplants receiving some form of induction immunosuppression in 2010 (7, 8). Comparatively, in 1994, only 25% of renal transplant recipients underwent induction (9). While several induction regimens are used frequently in human transplantation, and others have been trialed, the majority of kidney transplant recipients in 2010 underwent induction with depletional therapy–therapies that have an intended effect of marked but transient lymphocyte elimination (Table 1). Extensive studies in rodent and nonhuman primate (NHP) models have begun to elucidate the lasting immune consequences of induction immunosuppression and while this is clearly the clinical trend, the long-term aggregate effects are as yet unclear.

In this review, we will discuss how induction therapy mollifies the alloreactive immune response and ultimately shapes the post-induction immune landscape. We concede that there are multiple methods for induction, including treatment with non-depletional antibodies (agents whose effect does not lead to a gross effect on lymphocyte number), and alternative

depletional therapies such as total lymphoid irradiation. However, for the purposes of this review we will focus on the depletional induction regimens that dominate the current clinical realm: polyclonal rabbit anti-thymocyte globulin and monoclonal antibody therapy with alemtuzumab. We also avoid discussing the influence of maintenance drugs used at the time of induction, focusing instead on the specific effect of depletion, though we recognize that the maintenance regimen chosen most certainly influences the character of repopulation. Work over the last decade has demonstrated that lymphopenia-inducing immunosuppressive regimens have profound and persistent effects on the immune repertoire, the consequences of which are only now becoming apparent.

The Biological Rationale for Depletional Induction

Numerous factors conspire at the time of transplantation to favor an aggressive alloimmune response, and thus compel a potent immunosuppressive barrier to avoid early rejection. Upon allograft revascularization, ischemia-reperfusion injury evokes endothelial damage leading to adhesion and costimulation molecule expression, chemotactic chemokine liberation and activating cytokine production. All of these processes promote lymphocyte and antigen presenting cell (APC) accumulation, foster alloimmune presentation, and increase the likelihood that a productive effector response will be generated within the allograft. Local complement cascade initiation, an innate response to parenchymal and endothelial injury, makes numerous complement components available that similarly lower the threshold for T cell activation (10, 11). All of these processes are effective in impelling immunity, and their effect is amplified on cells with prior antigen experience (9, 11–16). Although memory T cells are most responsive to these innate aspects of engraftment, naïve T cell activation also is increased though the mobilization of donor-derived passenger APCs to the recipient lymphoid organs (17) where they activate recipient alloreactive T cells. These remotely activated cells are attracted back to grafts by chemokines expressed by the injured graft parenchyma and endothelium (12). Importantly, due to the mechanisms of thymic repertoire determination, the alloresponsive precursor frequency involved in this multipronged activation is several logs-fold higher than the precursor frequency to a pathogen (18–22), and accordingly make the magnitude of any potential alloimmune response non-physiologically large (23). Thus, the initial response to an allograft is large, polyclonal, multimodal, both local and systemic, and highly redundant.

Although drugs specific to all of the aforementioned early processes exist, including chemokine and chemokine receptor blockade, chemotactic signal inhibitors, costimulation pathway blockade, and conventional immunosuppressants, no agent is sufficiently broad, or tolerated in sufficiently high doses, to cover all aspects of immunity simultaneously. In addition, the relative contribution of each of these processes varies considerably based on the clinical circumstances (e.g. MHC mismatch, ischemic time, organ type, etc.), such that the relative choice of these varied agents is highly individual. As a result, relatively non-specific elimination of T cells until such time as the stimulating effects of engraftment have waned has become increasingly adopted. T cell elimination essentially removes the effector arm of cellular immunity as well as the required help for *de novo* alloantibody formation. In general, depletional induction is a functional “all of the above” choice, that addresses to

some degree most pathways driving rejection until the transplanted organ has become a less immunostimulatory environment.

Although depletional induction is perhaps an unsatisfyingly blunt tool from a mechanistic standpoint, its ease of use, breadth of applicability, and efficacy in reducing early rejection episodes has propelled it into common practice. Compared to historical standards of calcineurin inhibitors, antimetabolites, or steroids, use of depletional induction agents in renal transplant results in fewer rejection episodes and permits some degree of maintenance therapy minimization (24–27). Induction in simultaneous kidney-pancreas transplant offers moderate improvement in rejection rates (28, 29). Use of induction therapy in small bowel transplantation has risen dramatically over the last two decades (Table 1), indicative of the perceived benefit induction has on graft rejection and survival (30, 31), while liver allograft rejection is not improved by any induction regimen. Although induction efficacy in heart transplantation remains unclear (32), approximately 50% of recipients received induction of some type, reflective of a heart allograft's high-risk status and the lack of a means for detecting rejection without biopsy.

Depletional Induction Agents

OKT3

The first monoclonal agent employed for any clinical purpose in humans was the murine antihuman CD3-specific antibody OKT3 (33). Binding of OKT3 to the T cell receptor (TCR) causes TCR internalization and subsequent cell activation and death. Some depletion occurs as a result of opsonization and antibody-dependent cell-mediated cytotoxicity since complement is not strongly activated (34, 35), but at the doses tolerated clinically, the depletion in largely peripheral and short lived. Early trials of OKT3 in kidney, liver, and heart transplantation demonstrated that it was an efficacious induction agent when combined with maintenance immunosuppression (36–38), but OKT3 did not provide adequate immune impairment to function as a sole agent due to the development of anti-OKT3 antibodies that limited its efficacy over time (39). Furthermore, the cytokine release syndrome that accompanies lymphocyte depletion with OKT3 causes fever, rigors, hypotension and pulmonary edema. The general intolerability of the therapy and its unacceptably high rate of post-transplant lymphoproliferative disorder (PTLD) led to its withdrawal from the market. It is mentioned out of historical deference, but will not be considered further in this review.

Polyclonal Antibody Preparations

Heterologous antibody preparations are relatively easy to develop compared to monoclonal therapies and were available in some form in the 1960s. Given this, polyclonal antilymphocyte antibody therapy has been used in human transplantation over the last half-century, with horse antithymocyte globulin first being used clinically by Starzl in 1966 (35, 40, 41). As expected, owing to their broad specificity, polyclonal agents have been shown to have a wide range of immune properties.

There are three polyclonal preparations currently in clinical use for induction therapy: ATG-R (Thymoglobulin, Sanofi-Genzyme, Cambridge, MA) and ATG-F (Fresenius Biotech, Waltham, MA), two rabbit derived agents, and one horse derived preparation (ATGAM,

Pfizer, New York, NY). ATG-R is most commonly used and most studied, although all three have been rigorously tested as induction therapy added to maintenance immunosuppression regimens in renal, heart, and liver transplantation (9). All have been shown to be effective adjuvants to maintenance immunosuppressive regimens but none can be used as a single agent. Although the specific make up of each of these agents is distinct, general impressions tend to assume that effects attributed to one of these agents are active with the others. Most clinical organ transplant data relevant to this review come from formal studies with ATG-R.

Corticosteroid minimization and withdrawal is possible with ATG-R induction (42, 43), and ATG-R can facilitate calcineurin inhibitor withdrawal in long-term renal transplant maintenance immunosuppression (44). When given prior to reperfusion, ATG-R has been shown to provide good graft survival with maintenance monotherapy, irrespective of organ type (45). In a randomized trial comparing ATG-R to the non-lymphodepleting IL-2 receptor antagonist monoclonal antibody, basiliximab, ATG-R showed superior reduction in the incidence of acute cellular rejection (46). This finding is likely reflective of the fact that profound lymphodepletion at the time of transplant effectively blunts the intrinsic alloimmune response generated as a result of heterologous immunity. The widespread use of polyclonal induction agents in multiple organ transplants is a clear indication of their overall positive tolerability.

Given the polyclonal nature of these antibody preparations, the mechanisms of action are diverse (47). Within the first several hours of ATG administration, profound lymphodepletion of the peripheral blood is evident. Indeed, an initial depletion of monocytes is also seen that sometimes exceeds that of lymphocytes. With repeated dosing, lymph node and splenic compartments are depleted of T cells in a dose-dependent manner (48). These effects are sustained for months, with some patients having indefinite inversion of their CD4 to CD8 ratio. Although the primary immunosuppressive properties of all ATGs appear to stem from T cell depletion, antibodies against NK cell, B cell, and plasma cell antigens as well as adhesion and chemokine molecules can be found in these preparations (34, 47, 48). The mechanism of lymphocyte depletion in clinical practice is unclear. At high *in vitro* doses, ATG-R activates the complement cascade and induces T cell lysis, but these concentrations are difficult to achieve *in vivo* (48). Apoptosis of activated T cells via Fas/FasL-dependent (49) and Fas/FasL-independent (50) mechanisms occurs at low ATG concentrations, but T cell death via this mechanism only occurs several days after initial treatment, indicating that this may not be the primary mechanism of lymphopenia in a clinical setting (51). Finally, *in vitro* data shows that rabbit IgG can bind to human Fc receptors, suggesting that ATG-R may induce antibody-dependent cell cytotoxicity on T lymphocytes (49). It is likely that each of these putative mechanisms for T cell depletion occurs in clinical use, however the timing likely differs, and regardless, the effects of ATG-R persists for months after initial dosing (52). While the effects are manifold, at least in animal models and *in vitro*, the most apparent mechanism of immunosuppression is lymphodepletion. Polyclonal ATGs have immediate and profound effects on the immune repertoire, and the resulting impact on the immune system is discussed below.

Alemtuzumab (Campath-1H)

Campath antibodies are complement-fixing anti-CD52 antibodies, directed to the membrane glycoprotein CD52, which is expressed by all T and B lymphocytes and a majority of monocytes, macrophages, eosinophils, NK cells, and dendritic cells (53). The first iterations, Campath-1G and Campath-1M (rat anti-human IgG and IgM monoclonal antibodies, respectively) depleted lymphocytes *in vitro* and were efficient for use in bone marrow depletion for cellular transplantation (54, 55). These first isotypes were, however, very immunogenic, making clinical implementation difficult. Subsequently, Campath-1H (alemtuzumab), a humanized rat IgG1 anti-CD52 monoclonal antibody, was developed and proved to be more suitable for *in vivo* use (56). Indeed, alemtuzumab was the first humanized monoclonal antibody of any specificity (57). As expected, administration of alemtuzumab results in rapid lymphocyte depletion in the peripheral and central lymphoid compartments (58). Lymphocyte depletion with alemtuzumab in human studies is much more rapid (within hours), potent (requiring <1mg/kg as opposed to > 5mg/kg) and profound (95–99% depletion including lymph nodes and spleen) than with polyclonal agents. Rebound is slow, taking 6–18 months, but the eventual repertoire is less likely to be permanently altered compared to ATGs (58–61).

Alemtuzumab efficiently cross-links complement, and CD52 persists on the cell surface despite alemtuzumab binding; this lack of receptor internalization allows for prolonged antibody availability for complement fixation, and it is generally thought that this is a significant mechanism fostering alemtuzumab's effective lymphodepletion (35, 62–64). Some groups have shown that at least *in vitro*, alemtuzumab may induce lymphocyte apoptosis via caspase-dependent and independent pathways (65, 66). Each of these likely plays a role in lymphocyte depletion, but neither has been proven as the dominant mechanism in clinical use. While the primary immunosuppressive effect of alemtuzumab is prompt depletion of lymphocytes, further evidence suggests that, like ATG, alemtuzumab administration may have broader consequences. Alemtuzumab has been shown to deplete monocyte-derived dendritic cells in the presence of complement, as this population of APCs expresses high levels of CD52, but depletion is limited to circulating peripheral blood DCs, protecting tissue-resident dendritic cells (67, 68). Alemtuzumab has been shown to induce apoptosis and depletion of B cells, although to a lesser extent than T cells (6, 57, 58). Natural Killer (NK) cells express CD52 and have been shown, at least *in vitro*, to be susceptible to alemtuzumab depletion (69, 70). Alemtuzumab has also been shown to induce degranulation and apoptosis of NK cells via a FasL-dependent mechanism, implicating alemtuzumab as a significant source of impairing antiviral and antibacterial immunity (70). Thus, treatment with anti-CD52 antibodies likely impairs multiple arms of the alloimmune response.

As alemtuzumab gains clinical popularity, other mechanistic insights have been uncovered. When used as monotherapy, alemtuzumab prevents acute rejection, but many renal transplant patients eventually show evidence of chronic allograft vasculopathy mediated by alloantibody (71). This finding is likely attributable to the increase in B cell activating factor, BAFF, a member of the TNF-ligand family, found in recipients treated with

alemtuzumab, particularly in the setting of prior allosensitization, or inadequate maintenance immunosuppression (72).

Experimental use of alemtuzumab in NHPs has been limited, however, since CD52 is expressed on erythrocytes of most Old World monkeys resulting in fatal hemolytic anemia with drug administration (73). Use in selected macaques has been reported, but the dosing kinetics and depletional effects in this setting differ markedly from that seen in humans (74). Despite this, alemtuzumab has made the successful leap into clinical practice. Originally approved by the FDA for treatment of refractory CLL and other hematological malignancies, alemtuzumab has now been used off-label for induction therapy in solid organ transplantation for years. Calne and colleagues first used the drug for renal transplantation induction with cyclosporine monotherapy maintenance inducing a “near” tolerance state (75, 76). Subsequent uncontrolled studies demonstrated good results with alemtuzumab induction and minimal or single-agent maintenance regimens, often steroid-free (61, 77), and comparison of alemtuzumab to historical controls with ATG induction showed equivalent rates of patient and graft survival with a trend toward increased late rejection episodes in the Campath groups (78, 79). Thus far, two randomized, prospective trials in human renal transplantation have shown that groups using alemtuzumab induction are able to minimize maintenance drug dosing and use steroid-free regimens with equivalent rates of rejection, compared to ATG-R induction (27, 80). In low-risk renal transplant patients, alemtuzumab was superior to ATG with respect to biopsy-proven rejection episodes in the first year post-transplant; in high-risk patients the two approaches were equivalent (81). So, although alemtuzumab is not FDA-approved, it has clinical efficacy and is commonly used as an off-label solid organ transplant induction regimen.

The Lymphocyte Repertoire at Baseline

An appreciation of the effects of depletional induction requires an understanding of the general make-up of ones unmanipulated lymphocyte repertoire. First, the allospecific repertoire represents a non-physiologically high portion of the total repertoire relative to cells specific for any single pathogen. The frequency of antigen-specific naïve T cell precursors to any nominal antigen is estimated to be on the order of, at most, 100/million cells, and the number of precursors directly impacts the magnitude of the response to a given antigen (19). Antigen exposure leads to rapid proliferation and expansion of this precursor pool, provided the proper activation threshold is met. The alloreactive T cell precursor pool has been shown to be log-fold higher than for any nominal antigen, on the order of 1–10% of T cells, a feature related to the requirement for MHC-binding during of thymic selection (18, 20–22). Expectedly, in renal transplantation, the pretransplant frequency of donor-reactive lymphocytes correlates to post-transplant rejection episodes (23). This preexisting pool of potentially allo-MHC reactive lymphocytes is the paramount driving force behind prophylactic depletional induction immunosuppression.

In addition to the number of responsive T cells influencing the vigor of an alloimmune response, the activation state of the responding cells is important to consider. Following antigen exposure, antigen-specific T cells expand and differentiate into effector T cells. The majority of the effector pool then involutes, leaving a small population of long-lived

memory cells, which can be characterized by cell-surface marker expression. Memory T cells are able to mount an immune response much quicker upon antigen stimulation compared to naïve T cells, and they possess antigen-independent survival advantages that result in their relative longevity (82, 83). While naïve T cells are restricted to the lymphoid tissues so that they may encounter antigen presented on APCs with proper costimulation, memory T cells can circulate in the periphery, allowing them to mount an immune response within non-lymphoid tissue (84).

Memory T cells can be characterized based on their expression of numerous surface markers, but a common classification is based on work by Lanzavecchia (85) which segregates cells differing in migratory and functional characteristics. “Central” memory (T_{CM}) T cells are $CCR7^{+}CD62L^{hi}$, whereas “effector” memory (T_{EM}) T cells are $CCR7^{-}CD62L^{lo}$. $CCR7^{-}$ cells can be further characterized with regard to their CD45 isoform expression with robust armed effectors expressing CD45RO, and terminally differentiated cells (see below) expressing CD45RA. $CCR7^{+}$ cells that express CD45RA are classified as naïve or non-antigen experienced cells, with T_{CM} expressing CD45RO. T_{CM} produce little to no effector cytokines, but like naïve cells, have substantial proliferative capacity; they circulate in secondary lymphoid tissues and produce IL-2, likely serving as a repository for the memory pool to replenish as needed. T_{EM} have little proliferative capacity, but can produce perforin, IFN-γ, and IL-4 and circulate in nonlymphoid tissues to mediate effector functions of rapid antigen elimination (85–87). This basic characterization of memory T cell subsets, has recently been expanded dramatically, with research revealing a gradient of diverse T cell subsets that have numerous blends of characteristics of memory and effector T cells (88).

Recent studies suggest that humans also have significant compartmentalization of the T cell repertoire (89). CD45RO-expressing memory T cells predominate in human organs and lymphoid tissues, whereas there is a relative mixture of terminally differentiated effector $CD45RA^{+}$ T cells and memory T cells in peripheral blood. Expectedly, expression of the human lymphoid homing receptor CCR7 on tissue-resident or circulating CD4 and CD8 T cells is lineage- and tissue-specific, and the patterns of expression are conserved among individuals. As in murine work, the T cell lineage-specific functional capability differs in human tissues, with the majority of T cells being quiescent memory phenotype and capable of IL-2 production and a limited number of effector T cells able to produce IFN-γ and having an effector phenotype. Suffice it to say that within the repertoire of any human, there exists substantial variation in the maturation state of the lymphocytes, some of it highly relevant to therapeutic immunosuppression (as discussed below) and thus critically important to the capacity of the individual to mount immune responses, including alloimmune responses, in the setting of clinical transplantation.

Memory T cells respond to antigen stimulation more quickly than naïve T cells (peak response in hours) and the number of effectors generated is higher than with naïve T cells (82, 90). Furthermore, memory T cells have a lower threshold of activation as compared to naïve T cells and do not require traditional costimulation when encountering antigen in order to produce effector cytokines (91, 92). Studies in mice also suggest that memory T cells retain some functional plasticity in that their ultimate effector functions are determined by

the environment of the recall stimulus (93). They have also been shown to mediate allograft rejection in the absence of secondary lymphoid tissues, and thus their effector functions can be unleashed solely via peripheral mechanisms (84). Thus, given their propensity for rapid and robust response, memory T cells can substantially augment the magnitude of an immune response.

While it is understandable that alloantigen-specific memory T cells can arise if an individual receives a blood transfusion, has been pregnant, or previously had another organ transplant, it is less intuitive how alloreactive memory cells would be present in patients who have never been exposed to alloantigen (23). This memory results from lymphocytes that are specific for viral or bacterial antigen and cross-react with alloantigen, a phenomenon termed heterologous immunity. Murine studies and clinical investigation have shown that heterologous immunity plays an important role in solid organ allograft recognition (94–96). Furthermore, cross-reactivity is common in some experimental models, with nearly half of virus-specific T cell clones having some alloreactivity (97). Thus, just as ones basal repertoire consists of an admix of naïve and memory cells, so too does each repertoire contain a variable number of alloresponsive cells in various stages of maturation. The presence of sensitized alloreactive T cells in rodent models of transplantation overcomes tolerance induced by powerful costimulation blockade (98), and there is growing acceptance that one reason that tolerance-inducing immunosuppressive strategies that work well in naïve rodents often do not translate to clinical practice, is that through heterologous immunity, no human is truly naïve.

Elegant work by Adams and coworkers demonstrated that virally induced CD8 T_{CM} are a specific barrier to transplant tolerance (99). In a dose-dependent manner, these cross-reactive memory T cells can generate an alloreactive immune response thwarting establishment of mixed chimerism, and in addition to sheer numbers of T_{CM} present, multiplicity of viral infections generates a hierarchical resistance to tolerance induction. These data suggest that, as a result of heterologous immunity, a virus-experienced recipient faces formidable challenges to graft acceptance. Human transplant recipients are, by nature, sick and generally heavily antigen-experienced. Acute infections (100), chronic infections necessitating transplant (e.g., Hepatitis C) (94), intermittent bacteremia or viremia following interventions like hemodialysis can all generate potentially cross-reactive memory (101). This concept is relevant because as we will discuss, memory T cells seem to be particularly resistant to depletion with commonly employed lymphodepletional induction agents, and this differential susceptibility has potential to be relevant during post depletional repopulation.

Effects of Therapeutic Lymphodepletion

The predominant induction regimens in renal transplantation, ATG-R and alemtuzumab, produce a profound and rapid state of lymphopenia. The concept here is to prophylactically reduce the frequency of donor-reactive T cells in order to prevent acute rejection. However, as many groups have shown, induction therapy alone is not successful in securing allograft acceptance; paradoxically, without maintenance immunosuppression, allograft rejection proceeds with relatively normal kinetics even when the peripheral lymphocyte repertoire is

apparently completely devoid of T cells (75). Indeed, the post-depletional repertoire appears more alloreactive on a cell for cell basis than the unmanipulated repertoire. One explanation for this could be a selective persistence of cross-reactive, viral-specific memory T cells.

ATG-R and alemtuzumab rapidly deplete essentially all T cell subsets in NHP and human models, but evaluation of the post depletional repertoire has revealed some degree of selective resistance amongst the various cellular phenotypes (48, 58, 102–107). In human studies of renal transplant recipients, aggressive lymphodepletion uncovers a relatively resistant population of T_{EM} and $CD45RA^+$ terminal effector cells that predominate within the first month post-depletion (102, 105, 107). Both CD4 and $CD8^+$ T cells can be seen and the dominance varies by report. This may be related to the concomitant maintenance regimen employed or differing pre-transplant antigen exposure or thymic capacity. Regardless, cells with a surface phenotype suggesting prior antigen experience heavily dominate the initial post depletional landscape. The mechanisms conferring resistance to therapeutic depletion have not been elucidated. Memory cells are known to have reduced surface expression of some molecules targeted by ATGs. However, their expression of CD52 appears to be the same as naïve cells making the resistant state likely to be more complex. Memory cells are known to have altered survival gene expression, and this and other cell intrinsic mechanisms are probably at play in selecting cells that survive depletional induction.

The persistence of this memory population has been shown, in NHPs, to allow the recipient to retain fungal and viral recall responses and alloreactivity in the absence of thymopoiesis (103). This certainly contributes to the striking immune competence of patients with exceptionally low T cell counts post induction. While opportunistic infections are seen with higher incidence post depletion, they are not as prevalent as would be expected based on the paltry peripheral T cell counts. T_{EM} cells present post-depletion are immunocompetent and importantly are resistant to steroids and sirolimus, producing recall cytokines and proliferating in the presence of these drugs. They are, however, exquisitely sensitive to calcineurin inhibitors *in vitro* (102), a feature likely related to the reliance on TCR signaling for memory T cell activation. This gives mechanistic insight into clinical observation that calcineurin inhibitor maintenance therapy is particularly efficacious following depletional induction. As T_{EM} cells have limited proliferative capacity, it is unclear whether these cells participate in repopulation, or if the dominant repopulation is driven by thymic output of naïve cells. As repopulation can occur without the thymus, either may be possible, and is likely variable based on the capacity of the individual recipient. This is addressed further below.

Importantly, the post-depletion pool of memory T cells possesses a constrained repertoire, making its capabilities stochastically spotty. In the first few months following depletion, analysis of TCR diversity reveals oligoclonal spectratypes with limited diversity, but this repertoire diversifies over time, indicating an important role for homeostatic repopulation (102). Taken together, these findings provide insight into why profound lymphodepletion is actually reasonably well tolerated in the clinical setting: lymphopenia relatively preserves quiescent memory T cells, which can rapidly respond to foreign antigen, thus preventing overwhelming infection. The caveat, however, is that there is a significant subset of these

memory T cells available to cross-react with alloantigen and ultimately threaten graft survival. Heterologous immunity therefore produces a population of immunosuppression-resistant donor-reactive effectors that can compete with the naïve population during post-depletion expansion.

Postdepletional Homeostatic Proliferation and Expansion

The size and diversity of the lymphocyte pool is of crucial importance in maintaining an adequate adaptive immune response. There is limited space in the host to house all the requisite lymphocytes needed to produce a robust response to a diverse array of foreign antigens. Clonal expansion of antigen-specific T cells and subsequent contraction into a long-lived memory population is an evolutionary solution to the constraint of space (1). Attrition of the memory pool conserves diversity while further saving physical space needed to house immune effectors. This delicate homeostasis requires various survival factors, cytokines, chemokines, and continuous engagement of self-peptide/MHC-complexes (108–111). In the setting of lymphopenia, expansion of the remaining peripheral lymphocytes and *de novo* lymphopoiesis combine to repopulate the lymphocyte pool, termed homeostatic proliferation. The clonality of the existing T cell pool influences the resulting diversity of this homeostasis. Monoclonal or oligoclonal T cells cannot effectively inhibit homeostatic proliferation of naïve T cells whereas a diverse memory T cell population can limit homeostatic expansion (112), thus preserving diversity within constraints of limited size.

The implications of homeostatic repopulation can be profound in the clinical setting. In certain scenarios, reconstitution of the immune system after a period of relative lymphopenia can result in hyperinflammatory phenotypes of overt autoimmune diseases. For example, following initiation of anti-retroviral therapy in HIV patients, case reports of Graves' disease have surfaced as the immune system is repopulated (113). Similarly, a significant portion of patients treated with alemtuzumab for multiple sclerosis generated elevated levels of IL-21, correlating to development of autoimmune thyroiditis (114). IL-21 is produced by follicular T helper cells, which seem to be resistant to depletional therapy and provide cognate help to B cells for alloantibody production (115). Autoimmune thyroiditis has also been reported in a renal transplant patient following aggressive lymphodepletion with alemtuzumab and minimal maintenance immunosuppression (116). These findings suggest that either development of the T cell repertoire after lymphodepletion can be skewed towards an autoimmune phenotype, or that T cells that are resistant to depletion can escape regulatory mechanisms necessary to prevent autoimmunity. While these are relatively isolated and specific reports, the number of solid organ transplant recipients who are subjected to lymphodepletional induction therapy is substantial. Furthermore, in high-risk recipients, profound depletion is the norm. The relative paucity of autoimmune eruption in transplant patients may relate to the ubiquitous effects of maintenance immunosuppression keeping residual autoreactive memory clones in check. Regardless, there is a risk-reward profile for depletional induction that drastically changes the resulting immune landscape.

As discussed above, human and animal studies have corroborated that memory T cells predominate early after use of depletion induction. Farber and colleagues have reported that mouse memory CD4 T cells are susceptible to depletion. Indeed, all reports of depletional

effects have suggested this to be true, with differences in the remaining repertoire related to relative and not absolute susceptibilities. However, this study went on to specifically examine the nature of the cells contributing to repopulation. They demonstrated that the post-depletion repertoire is a result of naïve T cell expansion, at least in this murine model (117). There are several important caveats here though: first, this was not a transplant model and the post-depletion cohort developed in the absence of allogeneic tissue; second, the chimeric memory population was relatively constrained in diversity as compared to a sensitized human host, and other work has shown that naïve T cell repopulation is limited by the clonality of the remaining memory pool (112). Interestingly, the thymic state of the host (thymectomized versus euthymic) had no impact on the ability of memory and naïve T cells to proliferate post-depletion, but no determination of TCR repertoire diversity was made (117). These data imply that young (euthymic) and old (relatively athymic) transplant recipients have equivalent potential to generate a diverse post-depletional T cell pool, although this is at odds with the known waning of TCR diversity with age (118). Clinically, most patients regain a similarly normal absolute number of lymphocytes post depletion, but the types of cells that make up the repertoire are highly and individually varied.

To date, evidence of homeostatic proliferation in human T cell populations following lymphodepletion has largely been observational, but recent work with a humanized murine model suggests that human T cells behave similarly to murine lymphocytes (119). When naïve CD4 and CD8 T cells are introduced into a lymphopenic humanized SCID mouse, rapidly proliferating cells acquire a memory phenotype and the ability to produce IFN-g, whereas slow proliferating T cells retain naïve functional and phenotypic characteristics. As predicted from murine studies, homeostatic proliferation of human T cells in this humanized mouse model requires human APCs, and the degree of chimerism influences the success of repopulation. While this study does not specifically address the role for immune reconstitution following depletional induction chemotherapy, the model will likely prove useful in future studies on human T cells.

In human transplantation, this seeming polarity to the post-depletion immune response is likely more blended. Clearly, memory T cells are a predominate subset of the postdepletion lymphocyte population. Likely, there are pools of antigen-experienced T cells that resist depletion and rapidly respond to the lymphopenic vacuum through homeostatic proliferation. Simultaneously, naïve T cells that survived depletion undergo proliferation and likely differentiation into memory T cells based on their antigen specificity. Finally, lymphopoiesis and maturation of marrow-derived emigrants seek to replenish the overall T cell compartment. Indeed, the state of lymphodepletion provides, in mice, specific stimulus for thymopoeisis and fosters the activation and proliferation of recent thymic emigrants until a physiologic lymphocyte number is achieved (120). A similar mechanism could be anticipated in humans, particularly in light of studies demonstrating anatomical recrudescence of the thymus in cancer patients repopulating after chemotherapeutic depletion (121–123). Each of these mechanisms, however, occurs in the face of varying levels of acute inflammation from reperfusion injury and constraining influences of maintenance immunosuppressants.

Costimulation blockade is currently an active area of research for development of effective maintenance immunosuppression therapies in the hopes of inducing prolonged alloantigen tolerance. Following lymphodepletion and subsequent homeostatic expansion, Wu, et al, showed in a murine model that the resulting T cell population is highly resistant to costimulation blockade (124). In this study, post-depletion proliferation occurred regardless of the thymic status, suggesting that the homeostatic proliferation of memory T cells would be relevant to pediatric (euthymic) and elderly (relatively athymic) patients. Adoptive transfer of this expanded population also confers resistance to tolerance therapy, consistent with the predominance of memory T cells in this population. Interestingly, subsequent work by the same group showed that regulatory T cells were capable of limiting tolerance resistant memory T cell proliferation following lymphodepletion (125). The resistance to tolerance induction was overcome by transferring naïve regulatory T cells at the time of transplant and by administering non-depleting CD4 and CD8 antibodies to block homeostatic proliferation. Finally, recent data show that the post-depletion immune response is so robust that it is capable of effecting rejection of established, tolerized allografts, suggesting that memory T cells undergoing homeostatic proliferation do not require endothelial damage by reperfusion injury which often marks the graft as foreign (126). Taken together, these findings imply that there is a delicate balance between memory, regulation (discussed below), and naïve T cells, and that broad-spectrum lymphodepletion alters this balance in a way that is highly individual and difficult to anticipate for a given patient.

Early clinical studies have suggested that terminally differentiated effectors that have lost the critical costimulatory receptor CD28 dominate post-depletional recovery (105, 127). While intuitively, this could suggest a fundamental indifference to CD28-B7 pathway directed costimulatory blockade, preliminary in vitro assessment by Trzonkowski, et al, using human cells has shown that these cells are largely inert and may in fact serve to blunt the allospecific response. However, others, including Lo, et al, have shown that $CD28^{-}$ T cells account for the bulk of in vitro responsive T cells responding despite the B7 specific agent belatacept (128). Additional work is needed to fully understand what role $CD28^{-}$ T cells have in clinical allograft rejection.

Since memory T cells appear to present such a barrier to tolerance following depletional induction therapy, and could, in some situations, be resistant or indifferent to traditional B7-CD28 costimulation blockade, some groups have identified costimulation pathways that are unique to memory T cells. Blockade of the ICOS-ICOSL pathway, which is crucial for activation of sensitized/memory CD8 T cells, results in prolongation of allograft survival in rodent models (129, 130). Similarly, impairment in the OX40-OX40L pathway on memory CD4 T cells prolonged murine cardiac allograft survival (82, 131). Despite these promising early results in animal models, memory-specific costimulation blockade has yet to make the leap to clinical trials.

Given that the costimulation pathways are significantly redundant, it is unlikely that single-pathway blocking agents will have marked impact on clinical graft survival. But, if depletion is so efficacious for other T cell subsets, why not deplete memory T cells directly? Alefacept, an anti-LFA3Ig molecule causes rapid, selective depletion of memory T cells and

facilitates costimulation blockade tolerizing regimens in NHPs *in vivo* (132) and in humans *in vitro* (128). However, a recent randomized trial comparing alefacept to placebo as an adjunct to maintenance immunosuppression failed to show any improvement in the incidence of acute rejection and the alefacept group had a higher rate of malignancy (133). It is unclear what long-term effects wholesale memory T cell depletion will have on the immune system, but responsiveness to infection and malignancy will likely be drastically impaired. Indeed, recent studies in kidney and islet transplant models suggest that the benefits to memory depletion in terms of eliminating costimulation resistant clones are not sufficient to overcome their risks with regard to impaired protective immunity (134, 135).

Perhaps more significant than the variable effect lymphodepletional induction therapy has on differing T cell subsets, the resulting diversity of T cell repertoire is markedly limited. Studies in rodents demonstrate that the T cell repertoire, based on TCR diversity screening, is restricted after thymectomy (136), and human studies have shown that while they retain reactivity to viral pathogens, renal transplant recipients who received depletional induction therapy had an oligoclonal T cell repertoire post-depletion (102, 137). Thus, while depletion and subsequent repopulation of the immune system results in alteration of the balance of T cell subsets and an overall restricted TCR diversity, clinical evidence suggests that when alloantigen is present during repopulation the resulting T cell clones tend to be unresponsive either due to exhaustion or senescence.

Effect of Aging and Thymic Status on Post-Depletion Repopulation

Clinical transplantation does not occur in a vacuum. Donor and recipient factors play roles in graft and patient outcomes. While immunosuppressive regimens are generally tailored to the organ and recipient circumstances, the resultant effects likely depend on the immune status of the recipient at the time of transplantation. Animal and human studies are starting to uncover the impact that aging and subsequent thymic status has on the post-depletion repertoire (118). Although the thymus involutes with age and does retain some capacity for regeneration and recovery during a lymphopenic state, the extent and speed of recovery never reaches its original potential (138). Furthermore, as people age, their overall repertoire becomes more memory-like, to include substantial loss of CD28 expressing lymphocytes (139). Thus, therapeutic manipulation of the immune repertoire is likely to yield substantially different effects between a child and an older adult.

Reconstitution of the immune system following lymphoablative therapy requires active thymopoiesis. Without the thymus, post-depletion T cell expansion is limited, and the repertoire is less diverse, indicating that the thymus is required for generation of diversity (122, 140). Thus, in an aged solid organ transplant recipient treated with depletional induction therapy, the post-depletion immune landscape is markedly different than that of a younger recipient. As previously discussed, the memory T cell repertoire is specifically resistant to depletion by conventional induction agents in clinical use, and their expansion post-depletion tends to be oligoclonal unless there is significant naïve T cell diversity to compete. So, in older recipients with limited or delayed thymopoietic potential, the post-induction immune system is likely to be predominantly oligoclonal and potentially highly donor-reactive. In younger transplant recipients, the post-depletion T cell repertoire would

be expected to be more diverse with robust thymic output, however, the memory population is smaller with limited antigen experience, likely predisposing to viral and bacterial infections.

Postdepletional Regulation

Increasing attention is being directed toward the effects of depletional induction agents on regulatory T cell populations. Both direct and indirect effects are becoming evident. Polyclonal ATGs appear to influence regulatory T cell function directly, presumably through binding of antibodies with as of yet undefined specificities found on regulatory cells promoting immunosuppressive features (141–143). Within 24h of treatment *in vitro*, ATG-R converts $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T cells, which display enhanced expression of FoxP3 and are capable of antigen-specific suppression of the alloimmune response (142). Interestingly, ATGAM depleted $FoxP3^+$ regulatory T cells *in vitro* while ATG-R promoted regulatory T cell expansion (141). It is unclear as to whether or not direct induction of regulatory T cells occurs in clinical therapy with either preparation.

Induction therapy with ATG-R skews the balance of regulatory and non-regulatory T cells, both through some degree of selective resistance (144), and selective thymopoiesis of thymic derived (CD31 positive) Tregs, particularly in children where thymic activity is robust (107). As compared to adult recipients, T cells with a Treg phenotype post-depletion in pediatric patients were predominantly a result of thymic emigration while adult recipients had a blend of recent thymic emigrants and peripheral expansion. These findings are consistent with the selectivity described above for T_{EM} populations that spares previously activated cells. While the maintenance regimen can be anticipated to influence these residual regulatory populations, no systematic evaluation of this has been performed in humans.

Alemtuzumab therapy also has been shown to foster an increase in regulatory T cells following lymphocyte depletion, theoretically promoting immunoregulation of the alloimmune response (106, 145). *In vitro*, alemtuzumab treatment results in a dose-dependent preferential increase in regulatory T cells that are capable of regulating an alloimmune response in an antigen-specific fashion, and addition of the common maintenance immunosuppressant sirolimus synergized this response (146). Long-term, however, depletion induction with alemtuzumab seems to result in a relative decrease in the $FoxP3^+$ regulatory T cell population in humans, with a concomitant rise in T_{EM} and $T_{EM}RA$ memory T cells (147). This finding suggests that while Treg cells may be resistant to depletion initially, they may not posses a similar capacity for longevity or expansion seen with memory T cells. Moreover, in patients experiencing episodes of acute cellular rejection, the immune repertoire is further skewed to favor a predominance of T_{EM} cells (147). Theoretically, the preponderance of regulatory T cells post-depletion should boost allograft tolerance, but it is unclear whether the post-depletion increase in regulatory T cells is a result of their relative resistance to depletion, a specific increase in *de novo* $FoxP3^+$ T cells, or a result of different maintenance immunosuppression regimens (64), and clinical studies have yet to clearly demonstrate that modulation of the regulatory T cell repertoire is a primary mechanism of immunosuppression with depletion induction.

Interestingly, while Treg persistence is postulated to be beneficial in transplantation, its ability to regulate the immune system is generally assumed to be detrimental to the antitumor immune response. Several rodent models of malignancy and clinical antitumor therapies rely on a robust effector T cell response, which is blunted by regulatory T cells that predominate in the lymphopenic state. Selective depletion of Treg with the anti-IL2R alpha monoclonal antibody, daclizumab, following lymphopenia resulted in robust effector responses to tumor antigen and maintenance of antiviral immunity (148). Thus, these data imply that use of daclizumab induction in the setting of solid organ transplantation may be unwise, since it produces a lymphopenic state without the benefit of preserved alloregulation.

Clearly, the role of immune regulation and the fate of regulatory T cells following depletion induction therapy in clinical transplantation are uncertain. Perhaps the relative ratio of Treg to T_{EM} in the recipient periphery may simply be a useful marker of acute rejection. Although promotion of an overall regulatory state is presumably beneficial to graft tolerance, no clinical regimen has reliably and clearly generated this environment. Further investigation is necessary to determine the impact current induction regimens have on the postdepletion regulatory response.

Conclusions

Adaptive immunity is now recognized as a continuous cycle of expansions and homeostatic contractions that respectively foster anamnestic elimination of antigen, and require that memories once held, eventually fade without periodic reexposure. Through heterologous immunity, a bystander effect is concomitant ebbs and flows of the alloantigen specific repertoire. Although prophylactic depletional induction therapies have been demonstrated to be efficacious and appropriately gained favor in most transplant settings, their influence on established memory is incomplete, and is influenced by numerous variables, the most unpredictable of which is a patient’s prior immunological experience. Thus, while T cell depletion can be reliably expected to reduce the allospecific precursor frequency and generally blunt the early alloimmune effector response, its ultimate effect will remain dependent on the combined product of the residual immune repertoire, the dynamics of lymphocyte repopulation, and regulatory controls shaping the resulting repertoire. All of these factors will be influenced by the maintenance regimens layered on induction regimens. While the initial impact of depletion appears to be relatively consistent, the late effects are less well defined requiring that modern attention to the use of these agents be shifted toward the best use of adjuvant therapies used in the depletional aftermath. Indeed, the most critical period for attention to maintenance therapies is likely the period between 6 and 18 months post transplant, when homeostatic repopulation and regulation is most dynamic. This later time zone has not been the traditional focus of immunosuppressive trials, which have instead set maintenance therapies at the time of transplant and if anything gradually reduced them with time. Indeed, many conversion trials propose changing immunosuppression precisely in this most unstable of times. We would favor an approach whereby the duration and scope of postdepletional homeostasis could be monitored, and intentionally shaped with agents that allowed for salutary regulatory effects to remain in play, and controlled residual allospecific memory. Based on the available data in NHPs, we anticipate that wholesale elimination of

memory will be poorly tolerated, and instead support the use of antigen specific modes of memory elimination. Given the terminal differentiation state of most depletion resistant cell types, mechanisms of activation induced cell death that are triggered by TCR engagement combined with deprivation of costimulation and/or cytokine signals seem mechanistically attractive. Early experience with this approach appears promising (134, 135, 149).

Depletional induction therapy has proven benefits, but its best use remains to be established. Rather than considering it as a means of eliminating the risk of rejection, it is perhaps best deployed with the intent on shaping the framework within which new and existing immune experiences are balanced (Figure 1). In doing so, clinicians might mold a recipient's recollective homeostasis to retain desired memories while eliminating those best forgotten. Perhaps the secret to success is selective memory.

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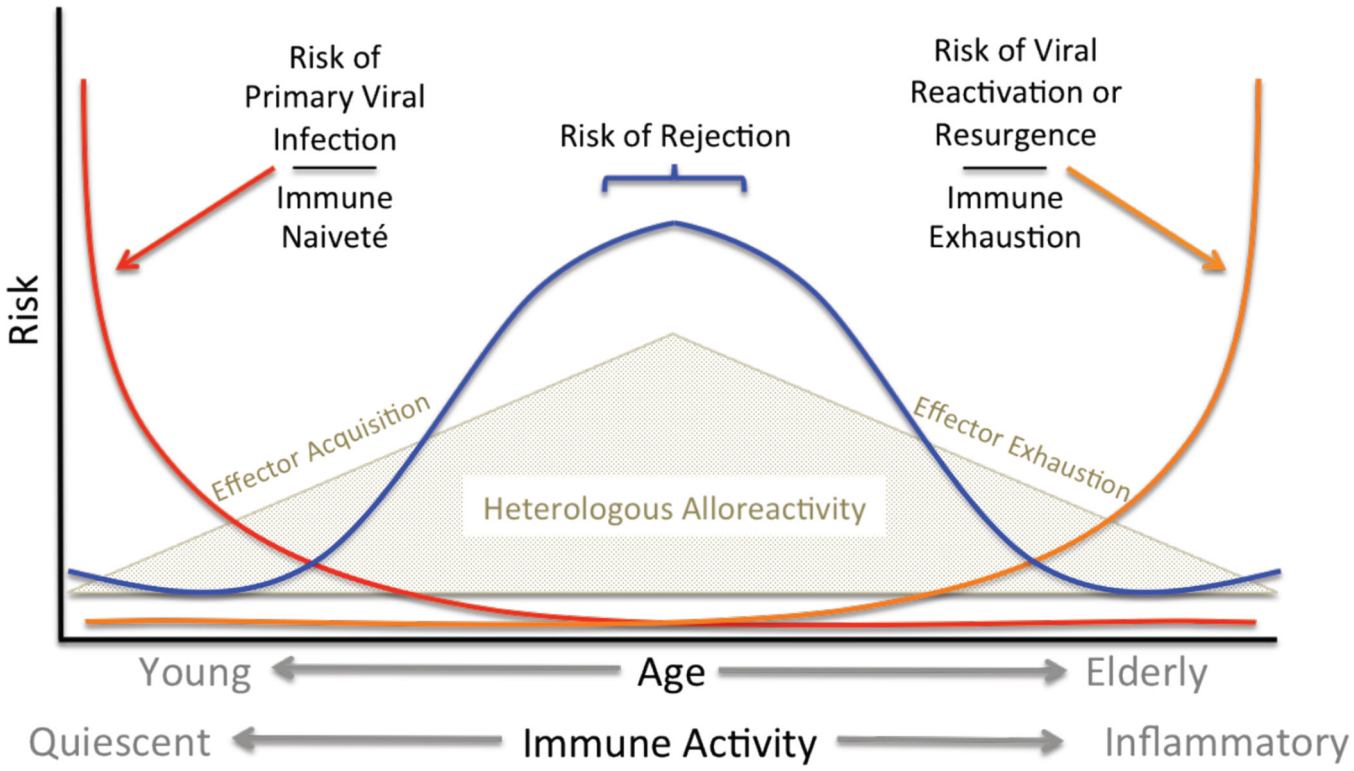

Figure 1.

Factors influencing by the dynamic expansion and contraction of ones protective and alloresponsive immune repertoire as they relate to the risk of infection and transplant rejection. On the y axis is relative risk and on the x axis is a spectrum of age (as a rough surrogate for immune experience) or immune activity, such as that associated with graft implantation and reperfusion or homeostatic activation post depletion. In patients with limited experience or during quiescent states, immune naiveté dominates making the risk for primary infection high and for allograft rejection low. On the opposite end of the spectrum, extensive immune experience makes the risk of primary infection low, but through exhaustion, the risk of viral reactivation high and at the same time allograft rejection low. In intermediate conditions, accumulations of immune experience without substantial requirements for exhaustion or homeostatic contraction limit the risk of infection or reactivation but bolsters the potential for heterologous immunity and its consequent risk of rejection.

Table 1

Trends in induction therapy from 1994 to 2011 by organ and agent in the United States[a]

Organ	Any Induction			OKT3			ATGAM			ATG-R			Alemtuzumab			Basiliximab			Daclizumab		
Year	**1994**	**2003**	**2011**	**1994**	**2003**	**2011**	**1994**	**2003**	**2011**	**1994**	**2003**	**2011**	**1994**	**2003**	**2011**	**1994**	**2003**	**2011**	**1994**	**2003**	**2011**
Kidney	25	70	86	24	<1	1	<1	1	1	6	34	50	0	4	13	0	22	26	0	13	<1
Pancreas	30	79	90	30	0	10	0	2	0	0	35	71	0	35	7	0	8	15	0	10	0
KP	48	79	90	48	<1	2	2	2	2	0	49	63	0	7	15	<1	16	13	<1	8	0
Liver	13	20	31	9	<1	2	5	<1	<1	0	6	10	0	1	<1	0	7	19	0	6	0
Intestine	13	74	80	17	2	25	0	2	2	0	46	53	0	9	10	0	2	19	0	16	0
Heart	36	48	50	16	5	<1	20	8	5	0	13	18	0	0	2	0	9	27	0	15	<1
Lung	25	44	49	6	1	1	19	5	4	0	3	3	0	1	6	0	19	36	0	16	<1

[a]Numbers represent percent of total transplants performed in the year noted receiving the noted induction agents (7–9).