Mechanisms of Tolerance Induction by Hematopoietic Chimerism: The Immune Perspective

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Key Words. Transplant tolerance • Hematopoietic cell transplants • Hematopoietic chimerism • Central tolerance • Peripheral tolerance • Regulatory T cells

SUMMARY

Hematopoietic chimerism is one of the effective approaches to induce tolerance to donor-derived tissue and organ grafts without administration of life-long immunosuppressive therapy. Although experimental efforts to develop such regimens have been ongoing for decades, substantial cumulative toxicity of combined hematopoietic and tissue transplants precludes wide clinical implementation. Tolerance is an active immunological process that includes both peripheral and central mechanisms of mutual education of coresident donor and host immune systems. The major stages include sequential suppression of early alloreactivity, establishment of hematopoietic chimerism and suppressor cells that sustain the state of tolerance, with significant mechanistic and temporal overlap along the tolerization process. Efforts to devise less toxic transplant strategies by reduction of preparatory conditioning focus on modulation rather than deletion of residual host immunity and early reinstitution of regulatory subsets at the central and peripheral levels.

SIGNIFICANCE STATEMENT

Reconstitution of vital tissues and organs by means of transplantation relies on severe detrimental side effects of protracted immunosuppressive therapy. Emerging regimens of induction of hematopoietic chimerism by means of hematopoietic stem and progenitor transplantation are markedly advantageous, yet optimization of the procedures is ongoing.

INTRODUCTION

Induction of transplant tolerance has been the focus of intense investigation along evolution of techniques for surgical implantation of healthy organs, and tissue regeneration from stem cells to substitute a defective parenchyma. The main hurdles limiting tissue/organ transplants are acute and chronic rejection, typically treated by immunosuppressive agents that cause end-organ failure (including the graft), pose risk of infections, and increase the incidence of malignancies. One of the emerging successful techniques to induce robust and unbreakable tolerance is hematopoietic chimerism through transplantation of hematopoietic stem and progenitor cells (HSPC) from the same donor, which alleviates long-term administration of immunosuppressive agents. The rationale is based on resetting of a chimeric immune system that is permissive to indefinite survival of mismatched grafts with specific configuration of the donor hematopoietic system while resuming full immunocompetence of the recipient to respond to unrelated antigens. However, the complex immunology of bone marrow transplants (BMT) includes reciprocal reactions generated by confrontation of coresident disparate immune systems: host versus graft reaction (HVG) leads to acute and chronic rejection, and graft versus host disease (GVHD). The current gold standard to alleviate these immunogenic reactions is post-transplant administration of immunosuppressive agents, in addition to pretransplant conditioning for BMT. Here, we attempt to provide insights into the process of induction of tolerance by hematopoietic chimerism and describe the evolution of hypothetic thinking along emergence of experimental information.

The term tolerance is synonymous to allowance and acceptance, however, a clear distinction has to be made from the immunological point of view. Acceptance is easier to achieve and is characteristic of situations associated with acute or sustained immune nonresponsiveness of the host, commonly achieved by immunosuppression. Acceptance may be also mediated by transient states of immune nonresponsiveness, which are reversible and easily terminated, resulting in delayed acute rejection. Unfortunately, this is essentially the outcome reported by most experimental studies that use the term tolerance without challenging host immune system [1, 2]. In variance,
true transplant tolerance (referred here as tolerance) is an active immunological process of mutual education of two immune systems to accept donor antigenic makeup, which requires either modulation of central selection or institution of indefinite peripheral suppression.

**EVOLUTION OF A CONCEPT**

Building on seminal contributions to identification of the activity of the immune system in late 19th century and early 20th century [3–5], tolerance was elaborated in respect to fetal cosanguinity in bovine twins (Fig. 1) [6]. Seminal information on the immune nature of rejection and tolerance has evolved from careful observation of very simple experiments over six decades ago [7]. These early murine studies defined the tempo of immune reactions (starting within ~3 days and peaking at 7 to 12 days in mice), hyperacute rejection caused by antigen-selective immunization, induction of tolerance by preemptive exposure of the fetus to the foreign antigen, the alloantigen-specific nature of tolerization, and adoptive tolerance transfer [7], later termed infectious tolerance [8]. Recognition of the capacity of the adult immune system to acquire tolerance to mismatched antigens has evolved along the emerging hypothesis of self-discrimination [9–11], which attributes a dominant role to cellular immunity over antibody-mediated humoral pathways in rejection and tolerance [12]. Aberrant recognition of self in the thymus leads to eruption of autoimmune disorders and involves an “information code” that participates in the process of clonal selection underlying immunogenic reactions against nonself and pathogens. The engagement mode was later characterized as “germline-encoded” affinity of the T-cell receptor (TCR) for a distinct major histocompatibility complex (MHC) molecule, which defines a “general” orientation to the antigenic makeup of an individual [13].

**GRAFT REJECTION**

Differences in Sensitization to Tissue/Organ and Hematopoietic Cell Grafts

Induction of tolerance to hematopoietic cell and tissue/organ grafts across antigenic barriers is in essence synonymous, with several distinct features. First, infusion of a hematopoietic graft exposes the host to robust systemic sensitization, particularly at sites of cell trapping by filtration in the liver and lungs, and directed homing to the bone marrow. Thus, the mode, site, and intensity of host sensitization are different and often more powerful in hematopoietic cell transplants, although antigen-presenting cells (APC) in some donor tissues such as skin are potent stimulants. Second, a particular prerequisite of conditioning for BMT is to free bone marrow space for seeding and engraftment of donor progenitors. Third, while major histocompatibility antigens set the context of self versus nonself recognition, the immune reaction targets primarily minor antigens: both tissue and immunohematopoietic cells express distinct tissue-associated antigens while only the latter display minor histocompatibility antigens (miHA). Fourth, unlike most parenchymal tissues, hematopoietic grafts include cellular elements, primarily T cells, capable to counteract residual host alloreactivity and also hold the capacity to generate vicious GvH reactions. Fifth, most conditioning regimens suppress the hematopoietic and immune systems and induce collateral

Figure 1. Milestones in development of approaches to induction of tolerance by hematopoietic chimerism. Efforts to decipher the nature of the immune system extend over more than a century, with gradual transition from cellular to molecular research and characterization. This knowledge has been adopted to develop strategies to induction of transplant tolerance by hematopoietic chimerism for more than six decades. The conceptual transitions from aggressive conditioning and full chimerism to reduced intensity conditioning and mixed or transient donor chimerism follow the evolution of experimental approaches to immunosuppression. The general trend is reduction of preparatory conditioning to a yet undefined minimum that prevents acute rejection and is permissive to hematopoietic engraftment. Current efforts are directed to develop approaches to immunomodulation and diversion of the function of immune cells without depletion. Abbreviations: ATG, anti-thymocyte globulin; TBI, total body irradiation, TLI, total lymphoid irradiation.
tissue injury, in particular to relatively fast cycling tissues such as the gut, that contributes to immune sensitization also through release of danger signals [14].

**Cellular Effectors of Rejection**

Immune responses to mismatched grafts are shaped by recognition, uptake and processing of alloantigens by professional APC to host T cells in two ways: direct presentation of intact donor MHC and peptide complexes (pMHC) or indirect presentation of donor peptides associated with recipient MHC molecules in a self-restricted manner [15]. Professional APC such as dendritic cells (DC) of crucial importance to evolution of alloreactions and rejection, with redundant activities of donor (direct pathway) and host (indirect pathway) APC in cross priming of residual host immune cells against donor alloantigens [16] and reciprocally, both modes are redundant triggers of GVHD (Fig. 2). In the process of antigen presentation, professional APC determine T-cell function and sensitivity to activation-induced cell death (AICD), therefore affecting the pace of graft rejection or acceptance [17].

Alloresponses are restricted to a finite number of CD4 and CD8 T-cell clones endowed with compatible TCR rearrangements, selected by antigen recognition from a wider repertoire of potentially responsive T cells [18]. Sensitization occurs only in T cells capable to recognize distinct allodogeneic pMHC complexes, and alloreactivity evolves primarily by clonal expansion of numerous T cells with high avidity to a single peptide [19]. The mode of T-cell stimulation critically depends on TCR interactions (signal 1) and costimulation (signal 2), as cytokines (signal 3) are often redundant and their inherent absence does not prevent rejection [20].

**Which Antigens Are Targeted in the Process of Graft Rejection and Tolerization?**

Immune reactivity (HVG and GvH) is generally more severe as a function of increasing MHC disparity between the donor and the host, with haploidentical and xenogeneic transplants being more prone to rejection than less disparate pairs (Fig. 2). It is questioned what are the specific antigenic targets attacked in the process of rejection: tissue, major or minor MHC antigens? One proposition suggests that transplant tolerance is specific to donor class I and II MHC [19, 21], endorsed by the capacity of hematopoietic chimerism to induce tolerance to a variety of donor-matched tissue and organ grafts [22]. The prevalent explanation of alloreactivity suggests that T cells responses to peptide-MHC complexes are less peptide specific than T-cell recognition of foreign MHC (also termed “degenerate” response) [23]. Another proposition states that tissue-specific antigens are of prime importance to elicit immune reactivity as well as tolerance. Negative selection focuses T-cell responses to foreign peptides bound to self rather than foreign MHC alleles because the “germline-encoded TCR” displays affinity to common MHC sequences [24]. Minor MHC antigens expressed by all immune-hematopoietic cells can elicit vigorous immune reactions and may serve as the true antigenic targets [25, 26]. The same apparent cumulative contribution of tissue, minor and major MHC participates in reciprocal sensitization of mature donor T cells that mediate GVHD, though the mechanisms of HVG and GvH reactions are not synonymous [27].

**TOLERANCE BY HEMATOPOIETIC CHIMERISM**

Tolerance of tissue/organs grafts is an active immune process that can be induced by preceding or cotransplantation of hematopoietic progenitors from the same donor. The common denominator of the various modes of tolerization by hematopoietic chimerism is selective nonresponsiveness to the donor while retaining intact immune responses to unrelated antigens (third party) and infections. The types and mechanisms of immune nonresponsiveness depend on the intensity and nature of preparative conditioning, the levels of donor chimerism, and the quality of tissue/organ grafts.

**Simultaneous Hematopoietic and Tissue Transplantation**

Proof of concept for the tolerizing activity of HSPC transplantation has evolved from clinical situations where a second transplant was performed as a lifesaving procedure. For example, secondary heart transplants have been performed to treat end-organ failure caused by BMT and GVHD and conversely, HSPC transplants have been performed to correct hematopoietic deficiency after heart grafting [28]. In selected cases, additional benefit of potent graft versus tumor (GvT) reactions has been achieved by simultaneous kidney and bone marrow transplantation in multiple myeloma patients suffering of end-stage renal failure [29]. Induction of tolerance by hematopoietic chimerism alleviates the adverse effects of immunosuppressive therapy and reduces the threat of break of
tolerance while restoring immunocompetent responses to pathogens. Chimerism essentially sustains tolerance while obviating administration of post-transplant immunosuppressive therapy, often termed operational clinical tolerance in tissue/organ transplants [30], which is most frequent attained by gradual weaning of immunosuppressive therapy in cases that have not displayed significant acute rejection [31]. However, cumulative morbidity and mortality of simultaneous transplants of HSPC and donor-matched tissues/organs is a major limiting factor, and has been achieved so far on a limited basis in clinical islet, kidney, liver, lung, and heart transplants from cadaveric donors [32–35]. Although gradual transition to live donors would allow sequential induction of hematopoietic chimerism followed by kidney and liver grafting, the condition of the patients may require simultaneous transplants.

Transition from Myeloablative to Non-Myeloablative Conditioning

Transplantation of any graft requires preparative conditioning, commonly attained by modulation of T-cell responses or suppression of host immunity by lymphopherection, however, transient immunosuppression per se only slows the tempo but does not prevent graft rejection, and evolution of donor hematopoietic chimerism is essential. The same types of immunosuppressive agents used to induce immune nonresponsiveness to tissue allografts are essentially employed for preparatory conditioning for BMT (Fig. 3), in conjunction with a cytoreductive element that frees space for donor HSPC engraftment such as irradiation [36]. Earliest transplant studies showed that robust tolerance is attained when the host immune system is wiped out by high-dose total body irradiation (TBI) and is substituted by full donor chimerism, resulting in recognition of the donor as self [37, 38]. In fact, most experimental and clinical information available to us originates from myeloablative hematopoietic cell transplants that substitute host immunohematopoietic system. Thereafter, substitution of TBI with selective total lymphoid irradiation (TLI) and fractionation into multiple low TLI doses has reduced the morbidity of this procedure [39].

Transition from Non-Myeloablative to Minimal Lymphoreductive Conditioning

Mixed chimerism involves reciprocal acceptance and coexistence of two disparate immune systems through a process of mutual education, which can be attained by coinfusion of host and donor bone marrow cells [40]. This approach to tolerance evidently requires stable engraftment of donor hematopoietic progenitors, but mixed chimerism often becomes with time polarized to dominant donor or host phenotypes (Fig. 3) [39].

Transition from minimal lymphoablative conditioning to minimal lymphoreductive conditioning is rather associated with reduction of the intensity of conditioning, with successful implementation of nonmyeloablative regimens that alleviate the threat of eminent death in case of hematopoietic failure. The general approach to induction of tolerance by hematopoietic chimerism has focused on the least toxic conditions permissive to donor progenitor engraftment using various nonchimeric conditioning regimens [22, 41, 42]. Thereafter, two conceptual modifications proved effective: reducing TBI to sublethal doses by combination with T-cell depleting antibodies [43] and focused high-dose irradiation of the thymus [44]. A myriad of subsequent regimens combined low-dose TBI or TLI with high-dose thymic irradiation and depleting monoclonal antibodies against CD2, CD3 [45], CD5, CD4, CD8 and combinations [44, 46, 47], ATG [48], anti-lymphocyte serum (ALS) [49], inhibition of CD40 and activating immunoglobulin of cytotoxic T lymphocyte antigen-4 (CTLA-Ig) [50, 51]. Thymic irradiation is effectively substituted by increased doses of monoclonal antibodies [42] and irradiation may be obviated by diversion of T-cell recovery [52, 53].

Figure 3. Immune profiles of the various conditioning strategies. Myeloablation eradicates host immunity and activity of hematopoietic progenitors, awarding an advantage to creation of full donor chimerism. The nature of conditioning affects primarily early reconstitution, which is polarized to either dominant donor or host stable multilineage chimerism at later periods. Reduced intensity conditioning includes lower doses of preparatory agents, selective lymphoaclation by immunosuppressive therapy, selective lymphoreduction and cytoredemption (aiming to free space in the bone marrow), and modulation of immune responses without cell depletion. Notably, residual host hematopoietic progenitors exposed to conditioning agents engraw slower that exposed donor progenitors.

Mechanisms of Tolerization by Hematopoietic Chimerism

Robust transplant tolerance in mixed chimeras is based on evolution of stable multilineage reconstitution with immune progeny
mutually nonresponsive to both donor and host antigens (Fig. 4). The intrinsic mechanisms responsible for institution of reciprocal donor-host acceptance are not fully understood, and experimentation of diverse transplant regimens underlines dominant involvement of distinct cellular and molecular mechanisms including central and peripheral deletion as well as sustained suppression. We believe that various treatments do not activate fundamentally different modes of immune nonresponsiveness but rather accentuate various stages of the tolerizing process to achieve the necessary threshold for acceptance of donor-matched grafts.

**Induction of Nonresponsiveness in the Early Post-Transplant Period**

In variance from myeloablation or aggressive lymphodepletion that abrogate the capacity of the immunosuppressed recipient to recognize and reject the graft, non-myeloablative and minimal lymphoreductive conditioning are defined by preserved host proficiency of generate HVG rejections, imposing obligatory containment of the initial immune reaction under various tolerizing regimens. Therefore, the first and earliest event required to secure graft acceptance involves peripheral negative regulation of residual host cells that acquire alloreactivity at the time of transplantation. It is yet unclear whether depletion of alloreactive host immunocytes is mandatory or functional inactivation is sufficient to induce transplant tolerance. The requirement for physical elimination is apparent from resistance to induction of transplant tolerance in recipients deficient in intrinsic and receptor-associated apoptosis [61], persistence of the pathogenic potential under conditions of anergy [62], and other states of transient nonresponsiveness that are insecure and easily reversed under clinical conditions of transplantation [63].

**Anergy and Consequent T-Cell Death**

It is possible that states of anergy have significant contribution to initial graft acceptance prior to deletion of alloreactive host T cells and long before establishment of hematopoietic chimerism [64, 65]. Anergy consists of an “abortive T-cell response that maintains T cells in an inactive but functionally competent state” [66] attained by inhibition of costimulatory signals such as CD28 and CD40 or CTLA-4 stimulation [50, 51]. Early anergy is best emphasized by approaches using costimulatory blockade [67], which is indeed associated with apoptosis of potentially reactive anergic cells through mechanisms independent of the canonical receptors that mediate AICD [68]. An essential contribution of deletional mechanisms accompanying functional nonresponsiveness to the process of tolerance induction [69, 70] is based on susceptibility of anergic cells to physical elimination by “passive death” due to cytokine withdrawal [61, 71] and activation of mitochondria-associated apoptotic pathways [72].

**Counteracting Rejection by Active Deletion of Alloreactive T Cells**

Initial acceptance of grafts, hematopoietic progenitor engraftment, and institution of stable multilineage chimerism in the presence of residual host immunity critically depend on activity of donor T cells [73], which exert both supportive immunogenic and nonimmunogenic activities [74] as well as potentially lethal GVHD [75]. Efforts to dissociate between T-cell subsets with graft supportive functions from GVHD effectors according to phenotype have been largely inconclusive [76] and attempts are being pursued to dissociate these activities by T-cell function rather than phenotype [27].

The straightforward and apparently most important activity of donor T cells involves direct deletion of residual host T cells that
acquire alloreactivity \([77, 78]\) using canonical mechanisms of apoptosis such as Fas-ligand (FasL), tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)), TNF-related apoptosis-inducing ligand (TRAIL), and perforin/granzyme \([79–81]\). The deletional mechanism is the major ingredient of the veto effect shown to protect from HVG rejection by counterattack of residual host immunity, attributed to mature donor CD8 T cells \([80, 82–84]\) through FasL-mediated AICD \([74]\). Similar activity is displayed by megadoses of hematopoietic progenitors able to counteract rejection across antigenic barriers through apoptotic signaling mediated by TNF\(\alpha\) \([85]\). This cytolytic mechanism may be simulated and reinforced by ectopic expression of apoptotic ligands to defend allogeneic hematopoietic cell grafts \([74]\), which can be applied because hematopoietic progenitors are inherently insensitive to apoptotic signaling \([86]\).

Wide individual variability imposes critical difficulties on the timing of elimination of donor T cells engineered to express a suicide gene after transplantation \([87]\). Depletion or inhibition of activated T cells at the time of peak mutual donor-host sensitization has a distinct advantage of joint abrogation of HVG and GvH reactions \([46, 88]\), particularly in the case of cadaveric donors that do not allow sufficient recipient preconditioning. This principle has been applied by early post-transplant administration of immunosuppressive agents \([46]\), TLI and T-cell antibodies \([89]\), negative costimulation and Rapamycin \([90]\). Ongoing efforts of GVHD prophylaxis are expected to advance the safety of hematopoietic transplants because treatment of established disease not only interferes with immuno-hematopoietic reconstitution but also blunts the active process of tolerization \([27, 91]\).

Evolution of Hematopoietic Chimerism

Requirement of Durable Rather than High Levels of Chimerism. Beyond initial abrogation of host alloresponses, tolerance is consolidated by evolution of nonresponsive progeny through hematopoietic chimerism, however; the meaning of peripheral chimerism is a matter of controversy \([42]\). The general rule states that tolerance does not depend on the level of donor chimerism but on stability and durability of multilineage reconstitution \([22, 92]\). The time frame of hematopoietic progenitor engraftment depends on the source and quality of the graft, with sequential evolution of the mononuclear and lymphoid lineages. Sequential engraftment of committed, noncommitted progenitors and later on of stem cells, along recovery of residual host HSPC yields progeny tolerant to both host and donor genotypes, which creates the state of mutual tolerance characteristic of mixed chimerism (Fig. 5). Thereafter, polarized chimerism evolves with near-absolute dominance of either host or donor peripheral immuno-hematopoietic progeny in most experimental and human cases, while tolerance generally persists throughout the entire spectrum of levels of chimerism.

Is There a Threshold Level of Hematopoietic Chimerism Required for Tolerance?. It is then questioned what is the degree of mixed hematopoietic chimerism required for acceptance of tissue/organ grafts from the same donor. There is no apparent threshold for induction of transplant tolerance and at times, hematopoietic chimerism fades away while tolerance to the donor is preserved. Persistent circulation of few donor hematopoietic cells is in fact evidence of selective immune nonresponsiveness, and systemic distribution of donor cells further contributes to institution and conservation of the state of tolerance. Although peripheral mixed chimerism is in fact a biomarker of tolerance under borderline transplant conditions (determined by low intensity conditioning and size/quality of the hematopoietic cell graft), detection of peripheral microchimerism neither correlates nor specifies a state of tolerance \([93, 94]\).

Transient Chimerism Contributes to Tolerance. Persistent acceptance of tissue/organ grafts under decaying levels of chimerism is not surprising because continued presentation of donor antigens by the tissue graft preserves tolerance \([47, 64, 70, 95, 96]\). Establishment of stable or transient hematopoietic chimerism often results in protracted survival of kidney grafts after discontinuation of post-transplant immunosuppressive therapy \([33, 35, 97]\). Interestingly, effective suppression of myeloma despite decaying levels of donor chimerism \([29]\) emphasizes mechanistic dissociation between kidney acceptance and protracted GvT, and indicates that detectable chimerism at a certain time point is not a prerequisite or indicative of sustained tolerance. The evolving scenario suggests that a certain level of donor hematopoietic chimerism is required to induce but not to sustain transplant tolerance, the latter being sustained by peripheral regulatory mechanisms \([98]\). The duration and quality of transient chimerism induced by various conditioning strategies for distinct tissue grafts \([34]\) remains to be determined \([42]\).

Sustaining Tolerance: The Cellular Perspective

Recovery of Donor and Host Immune Cells Is Essential. The tolerization process critically depends on post-transplant evolution
of donor and host immune cells, both being nonresponsive to alloantgens of the mismatched partner and therefore having neither GvH nor HVG activities, respectively. Development of functional donor T cells from engrafting progenitors is obligatory to create tolerance by durable chimerism [99, 100] including MHC class II interactions of CD4 T cells [101] and the same mechanism reciprocally applies to recovery of tolerant host lymphoid progeny [102]. Evolution of tolerant lymphoid progeny is not an unique event but includes several waves of immunohematopoietic progeny, which is sequentially produced by committed and uncommitted progenitors and delayed definitive reconstitution from hematopoietic stem cells (Fig. 5).

Establishment of Central Tolerance: Modulation of Thymic Function. The observation that focused thymic irradiation substitutes and reduces the morbidity associated with high-dose TBI led to the concept that the role of preparative conditioning is to reset thymic function [44]. Tolerance by mixed chimerism is considered as a pure central clonal deletion mechanism mediated by both donor and host APC of bone marrow origin [21] operating in the thymus to select clones reactive against reciprocally mismatched antigens [22, 42]. The evolving argument attributes a major role to continuous elaboration of APC to ensure negative selection of newly developed thymocytes, whereas peripheral chimerism and persistence of alloantgens are dispensable [103, 104]. A central role of the thymus in generation of alloreactive T-cell clones is emphasized by reversal of tolerance induced by costimulatory blockade following depletion of donor cells in the presence of a functional thymus, whereas tolerance persists if thymectomy precedes depletion [103, 105].

Modulation of thymic function in the context of tolerance induction has been also explored using direct interventions: induction of thymic chimerism by direct inoculation of tissue alloantgens attempts to bypass the process of cell egress from the hematopoietic graft, systemic circulation, and colonization of the thymus. For example, nonresponsiveness induced by intrathymic antigen inoculation [106] leading to acceptance of islet grafts [107] has been attributed to clonal deletion mediated by direct alloantigen recognition by host APC [108], which is sustained by peripheral suppressor cells [99]. The limitations of this approach in simulating central unresponsiveness have been soon recognized because transplantation of thymic fragments containing epithelium-expressing alloantgens does not absolutely prevent rejection [109] despite induction of suppressor cells [110]. Inasmuch as the thymus holds the capacity to control the reactivity of newly developed thymocytes by positive and negative selection [103, 105], direct inoculation into thymus is a rather unreliable mode of tolerization to alloantgens [107, 111].

Establishment of Peripheral Tolerance: Antigen-Presenting Cells. Consistent with the requirement for a competent immune system to induce tolerance, DC often play significant roles in peripheral tolerization [112]. For example, apoptotic cell uptake and presentation of tissue-restricted antigens by immature DC residing in regional lymphoid tissues promotes peripheral cross-tolerance [113], through diversion of CD4 and CD8 T cells from evolution into IFNγ-producing cytotoxic cells [114]. The state of DC maturity and the nature of antigen presentation is in fact determined by exogenous signals evolving in part from the injured tissue, with more potent DC maturation following encounter of necrotic rather than apoptotic cells [115].

Establishment of Peripheral Tolerance: Effector Cells. Additional pathways of peripheral education have to be recognized because central modulation of thymic function is largely insufficient to explain some approaches to tolerization by non-myeloablative conditioning. For example, dispensable modulation of the thymus in tolerization by fractionated TLI [39], extrathymic anergy [68, 103] and deletion of mature alloreactive host cells [51, 65] underline the significance of peripheral mechanisms, which may sometimes be sufficient for acceptance of donor tissue/organ grafts [116]. Peripheral tolerance is mediated by T-cell inactivation through clonal deletion [64, 77] mediated by extrinsic receptors [117, 118] and mitochondria-associated apoptotic pathways [72], functional unresponsiveness [69, 70, 119] and active suppression by regulatory T cells (Treg) [120].

T cells are tolerized in the periphery by diverse mechanisms and display distinct characteristics in terms of epigenetic imprinting, transcriptional regulation and microRNA profiles [121], as well as individual factors that tune CD8 T-cell responses by attenuation of TCR signaling [122]. Blockade both of TCR (signal 1) or costimulation (signal 2) induces transplant tolerance in presensitized rats through distinct mechanisms: the first abolishes both Th1 and Th2 cytokine phenotypes whereas the latter spares the Th2 profiles [123]. It is considered that TCR signaling is disengaged from cell cycle reentry in tolerant T cells, preventing exit from the quiescent state, cycling and clonal expansion triggered by cognate antigen stimulation characteristic of naive and effector/memory T cells [121]. Quite paradoxical tolerizing phenomena have been attributed to costimulatory receptors and activating cytokines such as IFNγ and IL-2 (signal 3), which trigger negative feedback mechanisms and limit alloimmune responses [20]. Interestingly, IFNγ may facilitate long-term allograft survival by limiting expansion of activated T cells under conditions of costimulatory inhibition [124] and IL-2 both determines the susceptibility of activated T cells to apoptosis and plays pivotal roles in Treg development and homeostasis [125, 126].

Establishment of Peripheral Tolerance: Suppressor Subsets. Discrepant results have been reported concerning the role of Treg in induction and maintenance of tolerance and the capacity to create infectious tolerance [116]. Most regimens critically depend on evolution of regulatory T cells of either donor [127] or host origin [128] to sustain the state of tolerance. The source of suppressor cells is either re instituted thymic function as a source of naturally occurring Treg (nTreg) or peripheral interconversions of naive T cells and Treg precursors [129]. Irrespective of their origin, the prime site of activity of peripheral suppressor cells is at the level of the tissue/organ graft [130], with apparent superior efficacy of donor antigen-specific host Treg [131]. For example, acceptance of tissue grafts critically depends on graft-infiltrating suppressor cells under conditions of costimulatory blockade and modulation of T-cell reconstitution with Rapamycin [132] or grafting of immune privileged tissues [133], and high Treg frequencies are usually characteristic of simultaneous non-myeloablative HSCT and renal transplants without sustained post-transplant immunosuppression [42, 134]. The power of suppressor cells to impose tolerance, often termed dominant tolerance, is best emphasized by their capacity of adoptive transfer of the tolerant state, often termed infectious tolerance or linked suppression [135].

Some preparatory regimens are less dependent on sustained Treg activity to maintain tolerance, however, suppressor cells are required in initial stages of graft acceptance. For example, Treg...
抑制早期CD8 T细胞应答[102]下非同源性和嵌合性共刺激阻断[136]和一致性的消除CD8 T细胞消除了需要抑制Treg介导的抑制[137]。进化和功能的Treg是相对可替代的在保持耐受性条件下抑制的准备性条件由共刺激阻断[138]和TLI与单克隆抗体[139]。其次，消除CD4 T细胞（包括nTreg）数月后移植后并不停止抑制[140]和相互性，转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为主要的来源的胸腺自衡的效应器和抑制性力量：移植相关的免疫反应的缩小反映自衡的Treg在诱导和或维持耐受性中[110, 150]。Treg的贡献向局部耐受性抑制是托着一种的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。在本质上，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。在胸腺内，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸膜的次级供体组织的来源。Treg贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。在本质上，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。Treg的贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸膜的次级供体组织的来源。Treg贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。Treg的贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸膜的次级供体组织的来源。Treg贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。Treg的贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸膜的次级供体组织的来源。Treg贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。Treg的贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸膜的次级供体组织的来源。Treg贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。Treg的贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。Treg的贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸膜的次级供体组织的来源。Treg贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。Treg的贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸膜的次级供体组织的来源。Treg贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。Treg的贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸膜的次级供体组织的来源。Treg贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。Treg的贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸膜的次级供体组织的来源。Treg贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。Treg的贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸膜的次级供体组织的来源。Treg贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸腺的次级供体组织的来源。Treg的贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。Treg的差异性角色在诱导和或维护移植耐受性在受者和供者组织的双方参与中是主要的源头。因此，Treg向局部耐受性抑制是细胞之间的网络，作为胸腺的原发地点和胸膜的次级供体组织的来源。Treg贡献向局部耐受性抑制是胸腺的原发地点，和或转移的混合脾细胞准备从嵌合性小鼠到免疫缺陷性受者不改变耐受性向供者供体[105]。
lymphoid tissues [163]. These events take place during the period of rebound homeostatic expansion following lymphoreduction, which predisposes to sensitization of effector cells and evolution of effector/memory T cells [164]. This mechanism is not dominant in early rejection because most T cells expressing the effector/memory hallmark CD44 proliferate at fast rates and undergo apoptosis [165].

Breaking Tolerance

It is difficult to determine which factors might break tolerance when the multiple mechanisms of induction are not fully characterized. A clear distinction should be made between failure to induce tolerance, break in true tolerance and resumed allograft acceptance under conditions of relative unresponsiveness. In this context, tolerance has to be defined as acquired central or peripheral inherent nonresponsiveness to the mismatched donor antigens. Therefore, to determine a break in tolerance it is first required to prove that indefinite acceptance of grafts has been achieved in the absence of residual alloreactive potential [2].

Situations such as rejection following Treg depletion remain unsolved: does it represent a break in sustained peripheral tolerance due to withdrawal of suppressor mechanisms? We prefer to include scenarios that attribute leading roles in the tolerization process to mutual education and peripheral negative regulation, thus including suppressor subsets required to sustain tolerance prior to the recovery of thymic function. Therefore, breaks in tolerance may be triggered by various factors under different immune configurations at distinct time points.

Multiple Breaks in Different Modes of Tolerization. Remarkably, breaks in tolerance are specific to the mode of preparatory conditioning under various experimental regimens. For example, protracted GvT and sustained kidney acceptance only in the presence of durable donor chimerism following conditioning with monoclonal antibodies [29, 166]. Break in tolerance was attained both by infusion of naive host T cells and depletion of donor cells following conditioning with depleting CD4 and CD8 antibodies in conjunction with low dose TBI and thymic irradiation [104]. Consistent with the requirement of a functional thymus to break tolerance by antigen withdrawal [47], donor cell neutralization, and thymectomy break tolerance under costimulatory blockade [42, 105, 138]. In contrast, selective skin rejection following Treg depletion and loss of chimerism following T-cell infusion emphasize distinct activities of tolerogenic mechanisms [136].

A Complex Algorithm of Tolerance by Hematopoietic Chimerism

A simplistic view infers that hematopoietic engraftment and evolution of chimerism induces unbreakable nonresponsiveness to the donor, which can be harnessed for protracted survival of additional tissues. Data gathered here emphasize the diversity of the major mechanisms involved in induction and maintenance of transplant tolerance in reference to the conditions used to establish hematopoietic chimerism. Pure thymic or peripheral tolerance are dominant in different modes of tolerization and are common components of a complex network of immune interactions that mediates acceptance of tissue/organ grafts under most conditions of mixed chimerism. We propose that the three main consequent mechanisms required for induction of tolerance are dissociated in time: (a) control early peripheral alloreactivity, (b) engraftment of donor hematopoietic progenitors, and (c) withstand the state of tolerance. Each individual mechanism depends on numerous procedural variables that interact with and affects the transition to the other stages.

The nature and intensity of preparatory conditioning and quality of the hematopoietic cell graft have determinant impact on the mechanism and tempo of tolerance induction. Transition to less lymphoreductive and more lymphomodulatory regimens imposes peripheral suppression of early alloimmune responses for variable periods of time, which is best achieved by donor T cells and immunomodulatory agents that inhibit or delete the APC mediators and/or residual host T-cell effectors of rejection. Donor T cells have a dominant veto effect counteracting HVG, but T-cell replete hematopoietic grafts are associated with significant morbidity and mortality caused by GVHD.

Tolerance is an active immune process that may be induced by transient and low-levels of donor hematopoietic chimerism, recovery of regulatory clones, and thereafter resumed thymic function (wherever the functional thymus resides in the adult). It is unlikely that immunosuppressive therapy per se terminates autoimmune reactions through resetting immune homeostasis at the thymic level [167] and consequently, acceptance of allogeneic tissue is superposed on nonresponsiveness to graft antigens that belong to the self-repertoire. Complexity of the tolerizing algorithm further expands in view of the differential modes of tolerization emphasized by split tolerance and induction of nonchimeric graft acceptance under selected experimental conditions. Our detection methodology is quite limited, such as decaying donor hematopoiesis may reflect transient chimerism, undetectable donor progeny despite a state of dominant tolerance to the donor or independence of tolerance from persistent chimerism [29, 116, 155, 157].

The pace and quality of engraftment define the conditions for substitution of the immune system with host and donor progeny unresponsive to both sets of alloantigens. Discrepant data regarding the role and significance of suppressor subsets in induction and maintenance of tolerance, ranging from apparent independence to adoptive tolerance transfer. A critical role is attributed to suppressor cells under preparatory conditions that spare a fraction of Treg, such as focused irradiation and costimulatory blockade, revealing that active peripheral suppression is a potentially effective ingredient of initial suppression of HVG alloresponses. Sequential recovery of Treg by peripheral interconversions precedes in time the delayed tempo to reinstatement of thymic function including output of suppressor cells, which consolidates the state of tolerance and makes a major contribution to its maintenance.

The proposed model of induction and maintenance of transplant tolerance includes three sequential mechanisms with significant temporal overlap, stressing the importance of the continuum of the tolerization process rather than deterministic activity of singular events. The relative significance of the sequence of repressed alloreactivity, establishment of chimerism, and sustained tolerance is quite variable under different transplant regimens and may dynamically shift in reference to events taking place in the post-transplant period such as incidental infections and end-organ injury. In our view, understanding the process of tolerization and definition of the mechanisms of each individual regimen will improve our
capacity to apply hematopoietic cells for induction of indefinite tolerance to tissue/organ grafts.

ACKNOWLEDGMENTS

Selective referencing of a large body of research and interpretation is evidently subjective and therefore I apologize for possible omission of substantial contributions to the field of tolerance. The overall effort was to credit the earliest publications on each topic for the conceptual and experimental contribution.

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AUTHOR CONTRIBUTIONS

E.S.Y., H.S., and N.A.: manuscript writing, final approval of the manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

ESY and HS have significant equity interest in Apoimmune (Louisville, KY, USA) and NA has equity in Cellect Biomed (Kefar Saba, Israel).


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