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PERSPECTIVES

Mechanisms of Tolerance Induction by Hematopoietic Chimerism: The Immune Perspective

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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. STEM CELLS TRANSLATIONAL MEDICINE *2017;6:700–712*

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

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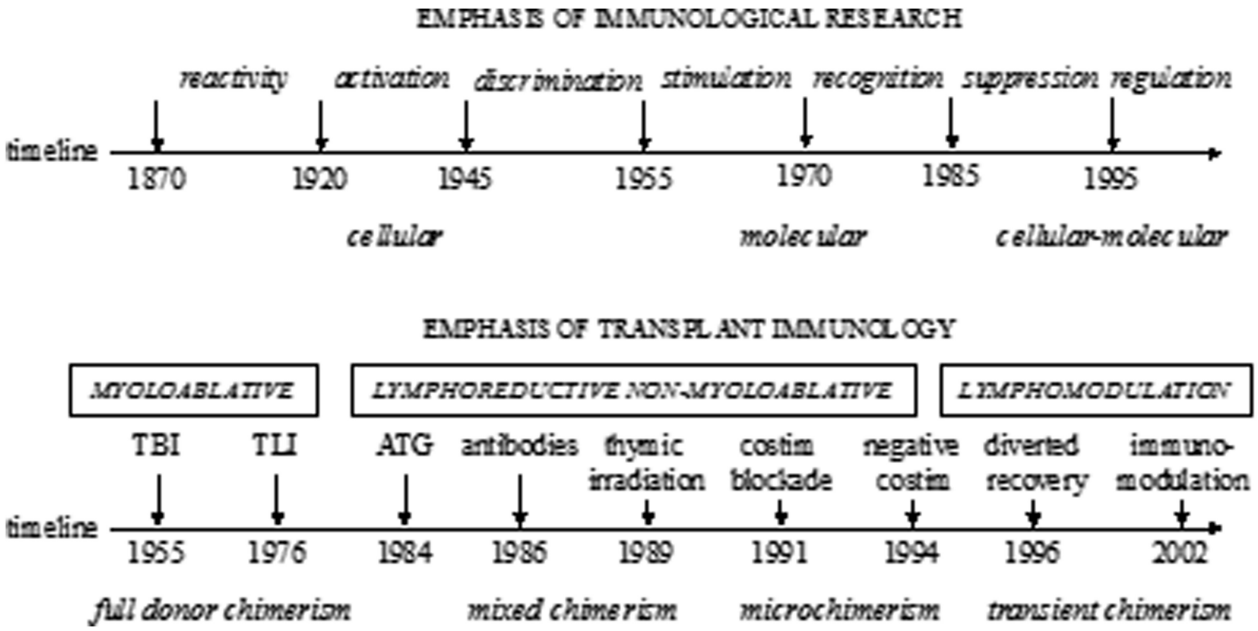

Figure 1. Milestones in development of approaches to induction of tolerance by hematopoietic chimerism. Efforts to decipher the nature of the immune system extend over more than a century, with gradual transition from cellular to molecular research and characterization. This knowledge has been adopted 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.

(referred here as tolerance) is an active immunological process of mutual education of two immune systems to accept donor antigenic makeups, 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 tissue injury, in particular to relatively fast cycling tissues such as the

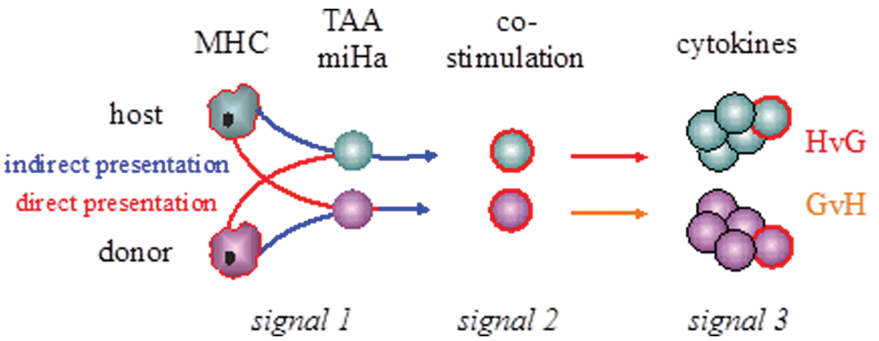

Figure 2. Inductive interactions in immune activation. In first stage, indirect antigen-presentation in the context of MHC compatibility and direct presentation in the context of incompatible MHC induce T-cell receptor-dependent T-cell stimulation (signal 1). The same interactions serve for delivery of costimulatory signals (signal 2) and T-cell activity is further activated by cytokines and environmental factors (signal 3). Uncontrolled reactivity results in adverse immune reactivity: of residual host immune cells as mediators of acute HVG rejection and of donor T cells as mediators of graft versus host disease. Abbreviations: HVG, host versus graft; GvH, graft versus host; MHC, major histocompatibility complex; TAA, tissue-associated antigens; miHA, minor histocompatibility complex antigens.

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) are 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 allogeneic 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

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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 lymphoreduction, 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 immunosuppressive therapy to hematopoietic chimerism is rather associated with reduction of the intensity of conditioning, with successful implementation of non-myeloablative 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

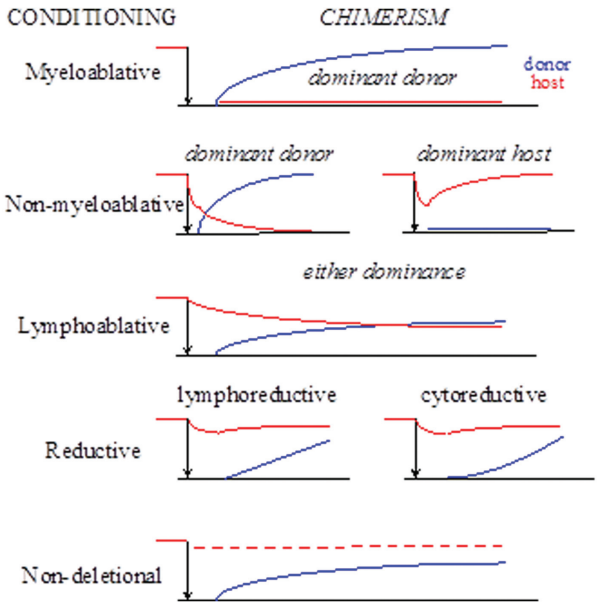

Figure 3. Immune profiles of the various conditioning strategies. Myaloablation eradicates host immunity and activity of hematopoietic progenitors, awarding an advantage to creation of full donor chimerism. The nature of conditioning affects primarily early reconstitution, which is polarized to either dominant donor or host stable multilineage chimerism at later periods. Reduced intensity conditioning includes lower doses of preparatory agents, selective lymphoablation by immunosuppressive therapy, selective lymphoreduction and cytoreduction (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 engraft slower that exposed donor progenitors.

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].

Transition from Minimal Lymphoreductive to Nonreductive Conditioning

The quest to induce hematopoietic chimerism without aggressive lymphodepletion evolves as one of the seminal principles of the next generation of approaches to tolerance (Fig. 3). One way is further reduction of the intensity conditioning by TBI, costimulatory blockade, and depletion of selected CD4 or CD8 T-cell subsets [54, 55]. Another way is to substitute depleting with nondepleting antibodies against T-cell subsets [47, 56], which induce tolerance rather than sensitization through emergence of suppressor cells [57]. Other approaches divert T-cell behavior by enforced negative costimulation [50, 51], induction of immune privilege [58], and localized donor HSPC engraftment [59]. Newer perspectives suggest that depletion of host HSPC with c-kit antibodies and diversion of myeloid responses by inhibition of CD47 attains effective cytoreduction in immunocompetent rodents [60].

Mechanisms of Tolerization by Hematopoietic Chimerism

Robust transplant tolerance in mixed chimeras is based on evolution of stable multilineage reconstitution with immune progeny

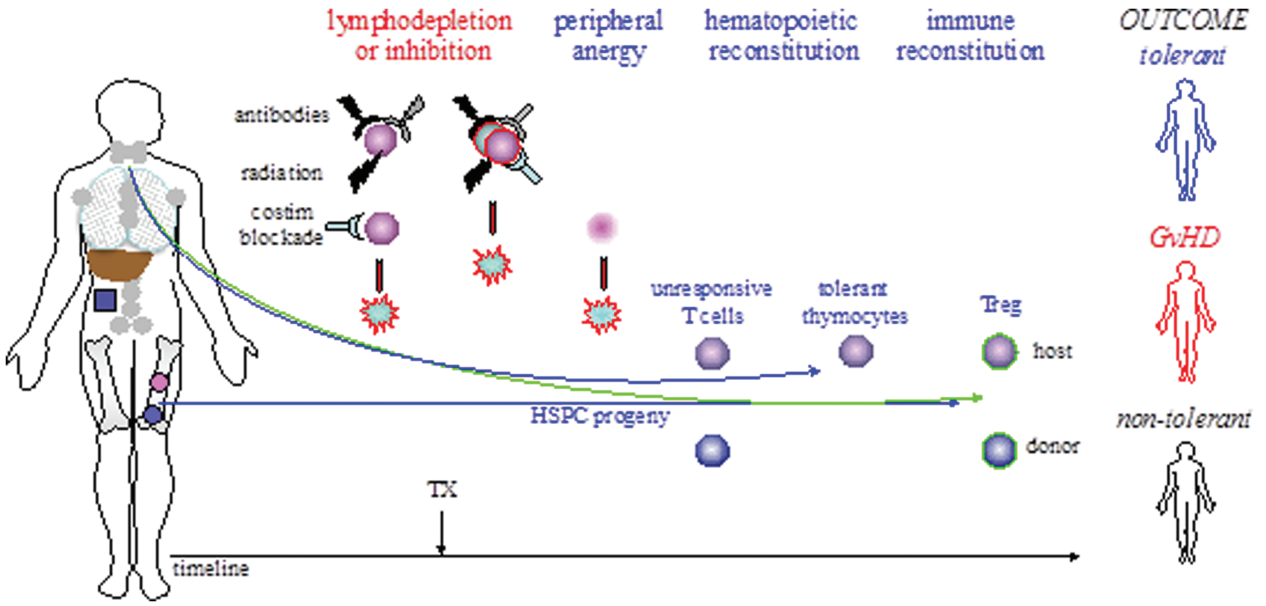

Figure 4. Stages of immune reconstitution for induction of transplant tolerance by hematopoietic chimerism. Following induction of peripheral host anergy by immunosuppressive therapy, stepwise immune reconstitution from the grafted donor and residual host progenitors generates mutually tolerant T cells. Delayed recovery of the thymus and reconstitution of suppressor subsets (Treg) contribute to maintenance of the state of tolerance. Peripheral and central mechanisms are closely interrelated and the relative impact within the tolerizing process varies according to the nature of preparatory conditioning and the quality of immunohematopoietic reconstitution. The possible outcomes in reference to the goal of the procedure and potential complications range from optimal tolerance without graft versus host disease (GVHD) to worst case scenario of nontolerant state with severe GVHD. Abbreviation: HSPC, hematopoietic stem and progenitor cells.

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 straight forward and apparently most important activity of donor T cells involves direct deletion of residual host T cells that

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acquire alloreactivity [77, 78] using canonical mechanisms of apoptosis such as Fas-ligand (FasL), tumor necrosis factor-α (TNFα), 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 TNFα [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].

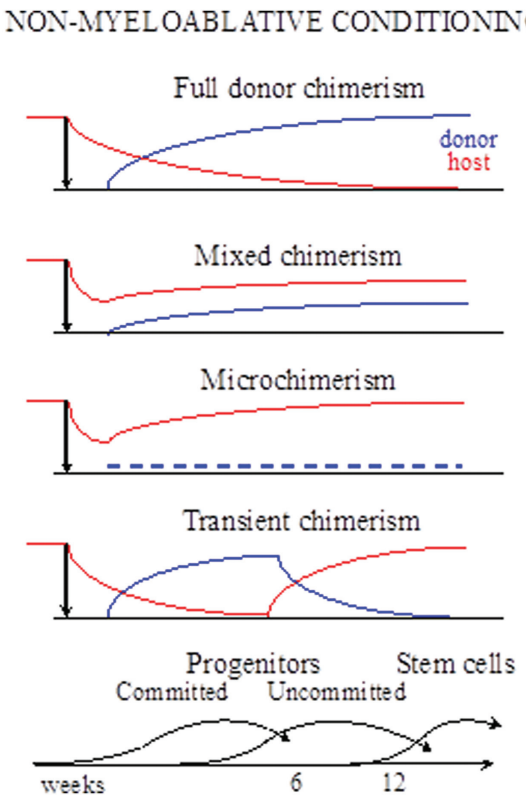

Figure 5. Variability in types of chimerism compatible with induction of transplant tolerance as detected in peripheral blood. Donor chimerism may replace or coexist at variable ratios with host immunohematopoietic system, make a small or transient contribution of immune and hematopoietic reconstitution. The lower panel provides a rough time scale for sequential donor-derived reconstitution from different subsets of progenitor and stem cells.

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 alloantigens 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 alloantigens 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 alloantigens 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 alloantigens 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 alloantigens [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 naïve and effector/memory T cells [121]. Quite paradoxal 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 reinstituted thymic function as a source of naturally occurring Treg (nTreg) or peripheral interconversions of naïve 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

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required in initial stages of graft acceptance. For example, Treg suppress early CD8 T-cell responses [102] under nonchimeric and chimeric costimulatory blockade [136] and consistently, elimination of CD8 T cells obviates the need for peripheral Treg-mediated suppression [137]. Evolution and function of Treg is rather dispensable in sustaining tolerance following preparatory conditioning by costimulatory blockade [138] and TLI in combination with monoclonal antibodies [139]. Consequently, depletion of CD4 T cells (including nTreg) several months after transplantation does not abolish tolerance [140] and reciprocally, transfer of mixed splenocyte preparations from chimeric mice into immunodeficient recipients does not confer tolerance to donor grafts [105]. The differential roles of Treg in induction and/or maintenance of transplant tolerance are reconciled by distinct activities in reference to the mode of conditioning, the tempo of immune-hematopoietic reconstitution and the nature of the secondary tissue graft. In essence, Treg contribution to peripheral tolerance closes a circuit of involvement of the thymus as the main source of newly generated nTreg in mixed chimeras and underlines the interrelationship between central and peripheral mechanisms of tolerance.

The Effector-Suppressor Cell Equilibrium. The overall intensity of transplant-associated immune reactions reflects a homeostatic equilibrium between effector and suppressor forces: downsizing the effector arm generally reduces the dependence on active suppression. For example, depletion of alloreactive T cells obviates the dependence of the tolerogenic state on protracted Treg activity [141] and nonresponsiveness of T cells from recipients of combined HSPC and kidney grafts often persists after Treg depletion [134]. Unfortunately, the inherent mode of Treg-mediated suppression involves functional suppression without depletion and/or induction of T-cell responses [142]—it is therefore essential to sustain their activity [129] or reinforce their capacity to delete effector cells [143].

Peripheral and Central Tolerance

Dissociation between mechanisms of peripheral and central tolerance is essentially based on the mode of ablation or suppression of host alloresponses, and different strategies are likely to accentuate distinct pathways of immune nonresponsiveness. In fact, peripheral and central tolerance are closely related and often mechanistically intercalated under various experimental and clinical conditions.

From Central to Peripheral Tolerance. Focus on central tolerance mediated by evolution of tolerant APC from the hematopoietic graft that cause preemptive deletion of reactive clones in the thymus [103, 104] is gradually switching to a peripheral paradigm of tolerance [116, 144]. First, thymic emigrants are prone to continued education in the periphery, a physiological process that starts during evolution of adaptive immunity in the neonate [117, 145, 146] and persists in later life [147]. The capacity of T cells to undergo programing decays and disappears in the process of peripheral T-cell maturation, irrespective of the maturity of the organism [12]. The basis for the critical dependence of the state of tolerance on steady alloantigen exposure and reversal of tolerance by antigen withdrawal [63, 95] is progressive deletion of alloreactive T cells in the periphery that occurs as a consequence of repetitive encounters and recurring TCR engagement [69]. Second, the process of thymic clonal deletion is often accompanied by a reversible state of peripheral clonal anergy mediated by functional inactivation of potentially self-reactive T cells [148], while preserving memory of the foreign antigens without executing active immune attack [149]. For example, extrathymic deletion of alloreactive T cells is mandatory to establishment of tolerance following hematopoietic cell transplantation using costimulatory blockade [51, 66, 103]. Third, naturally occurring suppressor subsets originating from the thymus and operating in the periphery play a central role in induction and maintenance of tolerance [110, 150].

From Peripheral to Central Tolerance. Robust tolerance may be achieved by peripheral deletion [43–45, 54, 55, 151] or inhibition [47, 56, 57] of selected T-cell subsets using monoclonal antibodies, through presentation of alloantigens in conjunction with apoptotic ligands [152] and costimulatory blockade [51, 61, 137]. Transition from peripheral to central tolerance is not always an easy and straightforward process [116, 153] and mandatory persistence of the alloantigens is not always sufficient to induce and sustain tolerance even if suppressor subsets are operative [154]. Occasionally, kidney and liver grafting is associated with egress of cellular components from the graft, creating systemic microchimerism that contributes to tolerization (possibly central) and allowing discontinuation of immunosuppressive therapy [33, 97, 155]. In general, implantation of tissues and organs at a remote site under the shield of immune privilege does not readily convert into tolerance unless systemic immunomodulation is applied [58, 152, 156], such as a localized bioreactor of donor hematopoiesis within a limited bone marrow compartment that confers indefinite acceptance of tissue/organ grafts at remote sites [59].

Characteristics of Tolerance by Mixed Chimerism

Failure to Induce Tolerance

The Case of Split Tolerance. Stable tolerance is best achieved by durable multilineage donor hematopoietic chimerism [22, 92], however, the relationship between chimerism and tolerance is not always compulsory [116, 157]. Split tolerance refers to selective acceptance of either hematopoietic cell or tissue graft from the same donor and rejection of the other. In the case of durable acceptance of the hematopoietic graft, rejection of donor-matched tissues may be caused by differential stimulation against polymorphic tissue-specific antigens and variable sensitivity of cells and tissues to effector immune mechanisms [158]. In the case of selective tissue/organ acceptance, donor hematopoietic progeny may be undetected and/or transient despite a state of dominant tolerance [29]. Altogether, this phenomenon emphasizes involvement of distinct tolerizing mechanisms in acceptance of hematopoietic and tissue/organ grafts.

Resistance to Induction of Tolerance. One of the barriers of induction and maintenance of tolerance is persistent activity of heterogeneous subsets of host natural killer [159] and effector/memory cells, which are insensitive to AICD-type negative regulation [160] and costimulatory blockade [161]. Distinct characteristics endow residual host effector/memory T cells with the capacity to convert into cytotoxic T cells, mediate resistance to engraftment of mismatched hematopoietic progenitors [162] and

infiltrate grafts even in the absence of elaboration in professional 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 alloreactivity 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 naïve 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 interacts with and affects the transition to the other stages.

The nature and intensity of preparative 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 reinstitution 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

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capacity to apply hematopoietic cells for induction of indefinite tolerance to tissue/organ grafts.

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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.

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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