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Mechanisms and biomarkers of immune quiescence in kidney transplantation

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Abstract

This review discusses the current understanding of biomarkers of immune quiescence based on reviews of published literature in kidney transplant operational tolerance and mechanistic studies based on a better characterization of the stable, well-functioning renal allograft.

Keywords

Immune tolerance; Tolerant footprint; Molecular biomarkers; Regulatory dendritic cells; Regulatory T cells

1. Introduction

The concept of transplant tolerance encompasses the presence of a well-functioning graft, lacking histological signs of rejection, in the absence of any immunosuppressive (IS) drugs, in an immunocompetent host [1,2]. Most reports use a cut-off point of 1 year after IS withdrawal to see if stable (or metastable) tolerance has been achieved [1–3]. Spontaneous operational tolerance has incidentally been found in patients, who are either non-adherent or are under physician-directed IS minimization at the time of clinically evident over IS, such as in the context of malignancy and severe infections [3,4]. On the contrary, induction of deliberate tolerance has occasionally been observed in humans; for example, with induced mixed chimerism seen after adoptive transfer of tolerogenic regulatory cells [4–6]. Selecting which patient will achieve this state and when drugs should or can be withdrawn safely for deliberate tolerance induction, remains difficult, as no single tolerance specific biomarker has been validated sufficiently for clinical use [4]. Benefits from IS withdrawal are very attractive, such as less IS-related complications, lower drug costs, and resulting in a better quality of life [7]. Therefore, considerable interest has been garnered in the community for

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Conflicts of interest

None.

detection of marker “states” for kidney transplant tolerance, so as to identify the patient and the timing for IS withdrawal, rather than the current ad hoc, trial and error approach [8].

Stable transplant tolerance requires both a state of donor-specific hyporesponsiveness and active immune regulation [9], inclusive of suppression or apoptosis of donor-reactive inflammatory cells and expansion in the number/activation state of regulatory cells. Harnessing the pathophysiology and clinical definitions of transplant tolerance to develop diagnostic biomarkers of metastable tolerogenic states, as surrogate biomarkers of immune quiescence, has been one approach to better assess and detect a state of ongoing/active immune acceptance, that would be amenable to IS manipulation and minimization, without rebound graft rejection. The process for development of these diagnostic markers faces challenges of patient selection, clinical phenotyping, sample numbers, false discovery rates during unbiased approaches, and difficulty in obtaining replicate or equivalent validation and cross-validation cohorts (Fig. 1). Additionally, assays and clinical development processes cannot translate into clinical benefit without continued support from funding agencies and clinical collaborations. Finally, during the clinical development phase, multi-step trials are needed to be approved by regulatory agencies before applying these discoveries back to the clinic, where they can be used to change practice guidelines, and support acquisition of reimbursement, and development of new or revised ICD-9 codes (Fig. 1).

2. How do we define immune quiescence?

An unanswered, yet important, a question is to re-evaluate our understanding of immune quiescence and its actual definition. A lack of coherence for this definition among clinical and research groups results in misleading results from different studies. The definition of immune quiescence, in the context of the kidney allograft, faces challenges from insensitive clinical diagnosis (with the redundancy of the serum creatinine for detecting early injury), the variability of tissue sampling by biopsy, the invasiveness of the biopsy, and the high inter-intraobserver variability in pathological diagnoses [10–12]. Our group and others have shown that normal “clinical” graft function cannot be quarantined from subclinical tissue injury and normal histology cannot entirely preclude patchy inflammatory molecular changes in the same kidney [13–16]. Thus, a clinical diagnosis of non-rejection is not necessarily a lack of inflammation; and stable graft function is not necessarily immune quiescence.

As the majority of genomic studies in kidney transplant tolerance have used a clinical diagnosis for stable graft function [17–23], it is likely that incorrect input phenotype diagnoses in those studies may be another reason why inconsistent gene signature patterns were found in different microarray analysis [17–23]. Before moving forward, the first hurdle to overcome is the lack of standardized molecular testing in order to discriminate stable graft function, or a control group, from a rejection group and other injuries. We would suggest that the *absence* of any of the validated biomarkers for graft injury and rejection from blood, such as donor-derived cell-free DNA, and the monocyte-specific 17 gene-set called the kidney solid organ response test, or kSORT, will support selection of stable transplant patients and more precise phenotyping of patients to be included in tolerance studies for finding the most sensitive and specific biomarkers for immune quiescence.

3. The kidney: resistant to tolerance induction

The kidney is vulnerable to immune injury from many events as seen in immune-mediated glomerular diseases, which are common causes of end-stage failure [24]. Even under IS therapy after transplantation, the kidney graft carries a high risk of immune injury which gates graft life expectancy. When compared with the liver graft, the most tolerogenic transplanted organ, with 20–42.6% being tolerant after deliberate IS withdrawal [25–30], the rates of operational tolerance observed in kidney transplantation are closer to 7% [8,31]. Studies also indicate that the kidney graft is more likely to be resistant to tolerance induction [32]. Some kidney transplant trials have found that T cell depletion results in the subsequent repopulation of activated memory T cells which are resistant to suppression by regulatory T cells [32,33].

4. Understanding pathways in human studies of induced, deliberate transplant tolerance: clues for immune monitoring for graft accommodation

Successful tolerance induction in animal models have been reported through several combinatorial mechanisms including hematopoietic mixed chimerism [34,35], regulatory cell transfer [36–38], depleting antibodies [34], and costimulatory blockades; with some reported success in selected human trials [39–41]. However, stable mixed chimerism has been more difficult to achieve in the human setting [32], and usually requires some modification of the recipient immune environment either by myeloablative [34] or non-myeloablative IS protocols [42,43] (Fig. 2). Though $CD34^+$ monocyte stem cells may be important in some types of tolerance induction strategies [34,44], the nature of engraftment can still be unpredictable. Therefore, using samples from patients who are undergoing various tolerance induction protocols, with varying degrees of success, can be problematic, as a potential biomarker for stable tolerance will be difficult to define due to the underlying heterogeneity of induction, approaches, and likely mechanisms that underlie the tolerogenic process. Additionally, as there are very few patients that actually achieve “success” in each designated protocol, there are insufficient sample numbers to develop regimen specific biomarkers.

Development of transplant tolerance can be categorized into three different phases: as an induction phase, as a metastable phase, and lastly, a stable phase [45]. In the induction phase, donor-reactive clones are depleted from the recipient reticuloendothelial (RE) system providing a space for donor-derived stem cells [45,46]. Antigen recognition by the adaptive immune system is declined by intensified conditioning protocols [46]. Antigen presentation to naïve T cells by immature dendritic cells ends up with incomplete T cells activation on the grounds of insufficient pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-6 (IL-6), interleukin-1β (IL-1β) or prostaglandin E2 (PGE2) [47]. By bidirectional communication, activated T cells provide an activation signal for dendritic cell maturation and survival via CD-40/CD-40 ligand and Tumor necrosis factor (TNF)-related activation-induced cytokine (TRANCE)/TRANCE receptor interactions [48–50]. In the tolerogenic milieu (Fig. 2, legend 2A), incompletely activated T cells go to an anergic state or undergo apoptosis while immature dendritic cells fail to develop [45,48].

Immature dendritic cells can be activated by anti-inflammatory cytokines like interleukin-10 (IL-10) which are released from the apoptotic T cells, adapting them to a regulatory phenotype [51,52]. The immunoregulatory dendritic cells mediate graft acceptance by decreased cytokine production, a loss of capacity to stimulate $CD4^+$ and $CD8^+$ T cells in response to donor alloantigen [53,54]. Plasmacytoid dendritic cells (pDCs) also indirectly drive regulatory T cells (Tregs) differentiation [55]. A small number of leukocytes that are contained within kidney graft migrate into the recipient and establish microchimerism concurrent with an immunologic ignorance in the metastable phase [45]. The factors that lead to the outcome of this chimerism whether they go to rejection or tolerance have as yet to be fully determined [56]. Immune quiescence from the previous phase cannot ensure stable tolerance because some peripheral donor-reactive lymphocytes can escape from the induction process and the mature immune system can repopulate new alloreactive cells [45]. To obtain life-long tolerance, active immunoregulation is required to overcome newly developed donor-reactive clones [45,46]. The regulatory phenotypes of T cells, B cells, macrophages, and dendritic cells identified from human and animal models are further discussed below for their potential roles in the development of tolerance. Monitoring of different cell subsets, though feasible, has been difficult to apply for clinically relevant monitoring, given the different mechanisms involved in different tolerance induction protocols and the small patient numbers enrolled in each of them. Nevertheless, we discuss some of the important cell subsets involved in tolerance mechanisms based on published studies.

Tregs are naturally occurring or can be induced [57] (Fig. 2, legend 2B). The suppressive effects of Tregs include mechanisms involved in secretion of anti-inflammatory cytokines (IL-10, IL-35), tumor growth factor-β (TGF-β) mediated inhibition T cell activation, exosome carried-microRNA (miRNA) production to silence T cell genes specific to cytokine production, and contact-dependent suppression with CD80/CD86 on antigen-presenting cell (APC) by Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA4) on Tregs to signal T cell inhibition. In addition Tregs are also involved in induction of apoptosis by multiple pathways via granzyme A/B and perforin, inducible cyclic adenosine monophosphate (cAMP) early repressor (ICER), TNF-related apoptosis-inducing ligand (TRAIL), the Fas/Fas-ligand pathway, the galectin-9/T cell immunoglobulin, and the mucin domain-3 (TIM3) pathway [58].

Our understanding of the role of regulatory B cells (Bregs) in human transplant tolerance is evolving [59]. These Bregs are IL-10 producing B cells and can be $CD19^+CD24^{hi}CD38^{hi}$ transitional B cells or $CD19^+CD5^+CD1d^{hi}$ B cells [60] (Fig. 2, legend 2C). Bregs provide IL10 dependent suppression of differentiation of naïve T cells into T helper 1(Th1) and Th17 cells, promote conversion of $CD4^+CD25^-$ T cells into Tregs, downregulate antigen presentation by macrophages, dendritic cells, and monocytes, and suppress production of proinflammatory cytokines such as IL-1, IFN-γ, and TNF-α by $CD4^+$ T cells, monocytes, and macrophages [60]. On the other hand, IL-10 independent mechanisms can also inhibit $CD4^+$ T cell functions via CD40-CD40 ligand and CD80/CD86-CD28/CTLA-4, inducing T cell apoptosis via the Fas/Fas-L cascade [61], and suppression T cells by granzyme B, Indoleamine 2,3-dioxygenase (IDO), adenosine (ADO), 5′ adenosine monophosphate (5′ AMP) and TGF-β [60].

Another immunoregulatory cell in kidney transplantation is the macrophage. Regulatory macrophages (Mregs) have shown promising results in pilot human clinical trials, namely the transplant acceptance-inducing cell II trial (TAIC-II) [62]. In mice, Mregs have shown deletion of cocultured allogeneic T cells via phagocytosis and inhibit T cell activity in vitro via inducible nitric oxide synthase (iNOS) [63]. In human, Mregs have shown potent suppressive effects on T cells proliferation via interferon (IFN)-gamma induced indoleamine 2,3-dioxygenase (IDO) activity and contact-dependent deletion of activated T cells [38] (Fig. 2, legend 2D).

Kidney transplantation concurrent with hematopoietic stem cell transplantation or tolerogenic cell therapy permits an opportunity for us to explore the tolerance atmosphere and define possible intracellular signaling and cell surface biomarkers (Fig. 1), for instance: forkhead box protein 3 ($FoxP3^+$), $CD4^+CD^{25hi}FOXP3^+$ cells, FOXP3/α-1,2-mannosidase ratio [21], Perforin-Granzyme A/B, IL-10 producing B cells, $CD19^+$ cells, $IgD^-CD38^{+/-}CD80^+$ memory B cells [64], CD32a/CD32b ratio [64], microRNA (miRNA)-142-3p [65], CD20 messenger RNA (mRNA) [20], and dehydrogenase/reductase 9 (DHRS9) [66].

5. Biomarkers for immune quiescence in spontaneous, operational transplant tolerance

Transcriptional studies have been applied for an unbiased, hypothesis-generating approach to identify novel signature gene transcripts in peripheral blood as putative new biomarkers to detect operationally tolerant patients, and by extension, apply these to monitor for graft immune quiescence. Single center and collaborative research groups in the US and EU (Immune Tolerance Network, ITN) and Europe (Indices of Tolerance, IOT) [67]) have worked together to focus on developing transplant tolerance gene footprints from peripheral blood samples. Conceptually, the process is to find the targeted gene which shows differential expression in the tolerance group compared to different control groups- the target genes differ based on which control group is selected- either stable graft function, acute rejection, chronic rejection, or healthy non-transplant volunteers. The most common process to identify such genes are microarray gene profiling, quantitative polymerase chain reaction (qPCR) validation, and immunophenotyping [17–23] (Fig. 1). As the gene lists differ, based on the control group selected, the challenge remains as to how to choose the best controls, and how to normalize the analysis across multiple clinical confounders, particularly the confounding effect of immunosuppression, so as to select the best gene-set that will be most clinically informative for immune quiescence monitoring for transplant patients on standard maintenance IS therapy.

Reports from ours and other groups have identified signature gene assays from tolerant kidney recipients [17–23] and have highlighted roles for immature B cells and myeloid and plasmacytoid dendritic cells in operational tolerance, but given the small numbers of patients with either induced or operational tolerance, national and international collaborations across patient and physician groups are imperative to advance the field. Publicly accessible online gene expression datasets of tolerance, such as Gene Expression

Omnibus (GEO) datasets or Array Express, are also an indirect form of collaborations which provide more sources for validation sets (https://www.ebi.ac.uk/arrayexpress/search.html?query=kidney+transplantation+tolerance). Nonetheless, the nature of multi-center studies brings about a variety of clinical characteristics of samples and laboratory incongruity. Different clinical and IS variables are important confounding factors that can limit the identification of successful and reproducible biomarkers [68]. Recently, the meta-analysis of multiple microarray studies in kidney transplant tolerance has shown, when the controls group consists of stable transplant patients on maintenance IS, B cell-related genes are a center-point of tolerance gene signatures [22]. From five studies, there were 14 common genes recognized (0.08%). Neither those 14 genes nor the unique gene lists from the different five studies were able to discriminate tolerance from stable graft function in the pooled dataset. The author selected the top 20 genes from statistical analysis for validation and significant discrimination between the tolerance phenotype, and stable graft phenotype was found, as expected. In a recent publication, B cell signatures of tolerance associated genes, IGKV1D-13 and IGKV4-1, were persistently found in a multi-center prospective cohort of kidney transplant recipients on IS maintenance therapy over a two-year period [69]. Further validation studies will be needed to refine and confirm these findings. It is currently unknown how if these gene sets can actually be used to minimize or withdraw IS in patients showing high scores for operational tolerance- to date, this has not been tested in kidney transplant patients and would need the conduct of a carefully designed, regulated, and adaptive designed clinical trial.

Variations in experimental platform probe design and non-uniform computational analysis among tolerance microarray studies may be another reason for inconclusive results of multiple tolerance studies [17–23]. The differences in fold change and p-value cutoffs also affect microarray interpretation [70]. Furthermore, how one narrows the group of genes is critical for candidate gene selection in the assay development process. Key genes with low expression level may be removed by analytic processes that use simple fold changes.

6. Conclusions

Advanced genomics and transplantomics have gained insights in the tolerance niche, but further work needs to be done to develop a clinically validated and reliable immune quiescence biomarker that can also identify the acquisition of operational tolerance, while a patient is on stable maintenance IS with a well-functioning graft. Though different research groups have tried to identify signature gene assays from tolerant kidney recipients, results have found only a few overlaps among these gene patterns. We have discussed in our review that some of these results can be explained by many factors such as limited eligible tolerant cases, various criteria for phenotype selection, clinical confounders, and inconsistent analytic methodologies that contribute to variations in gene discovery. In addition to focusing on biomarkers of immune quiescence, also including the typing of samples for biomarkers of graft injury and rejection, and noting the absence of the latter, would better classify a stable, immune quiescent allograft. A combination of biomarkers of rejection and quiescence would provide the best biomarker combination to support IS customization and minimization trials.

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Abbreviations:

5′AMP	5′ adenosine monophosphate
ADO	adenosine
DSAs	anti-donor specific antibodies
APC	antigen-presenting cell
CXCL-9	chemokine (C-X-C motif) ligand 9
cAMP	cyclic adenosine monophosphate
CTLA4	cytotoxic T-lymphocyte associated protein 4
ELISAs	enzyme-linked immunosorbent assays
Fas/Fas-L	fas cell surface death receptor, fas cell surface death receptor-ligand
FoxP3+	forkhead box protein 3
GEO	gene expression omnibus
IS	immunosuppressive
IOT	indices of tolerance
IDO	indoleamine 2,3-dioxygenase
ICER	inducible cyclic adenosine monophosphate (cAMP) early repressor
iNOS	inducible nitric oxide synthase
IFN	interferon
IL-1β	interleukin -1β
IL-10	interleukin-10
IL-35	interleukin-35
IL-6	interleukin-6
kSORT	kidney solid organ response test
mRNA	messenger RNA
miRNA	microRNA

pDCs	plasmacytoid dendritic cells
PGE2	prostaglandin E2
qPCR	quantitative polymerase chain reaction
Bregs	regulatory B cells
Mregs	regulatory macrophages
Tregs	regulatory T cells
RE	reticuloendothelial
TIM3	T cell immunoglobulin and mucin domain-3
Th	Helper T cells
CLIA	the clinical laboratory improvement amendments
FDA	the food and drug administration
ITN	the immune tolerance network
TAIC-II	the transplant acceptance-inducing cell II trial
TRAIL	TNF-related apoptosis-inducing ligand
TGF-β	tumor growth factor-β
TNF	tumor necrosis factor
TRANCE	tumor necrosis factor (TNF)-related activation-induced cytokine

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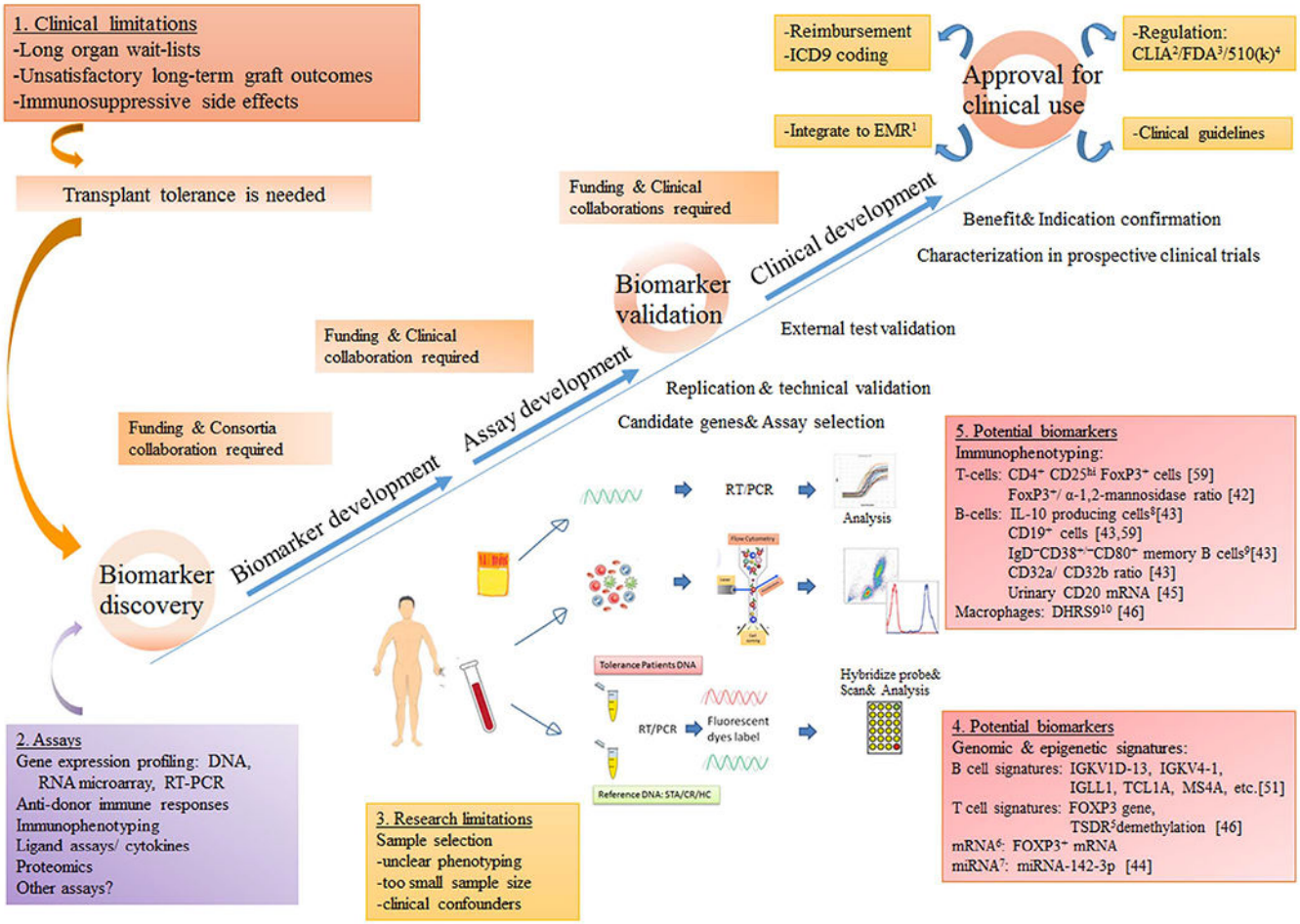

Fig. 1.

A summary of different components of successful biomarker discovery and validation for transplant tolerance. Abbreviation: [1]EMR: electronic medical record, [2]CLIA: The Clinical Laboratory Improvement Amendments, [3]FDA: the Food and Drug Administration, [4]510(k): section of the Food, Drug and Cosmetic Act requires device manufacturer who must register, to notify FDA of their intent to market a medical device at least 90 days in advance., [5]TSDR: Regulatory T cells-specific demethylated region (TSDR), [6]mRNA: messenger ribonucleic acid, [7]miRNA: micro-ribonucleic acid, [8]IL-10: Interleukin-10, [9]IgD: immunoglobulin G, [10]DHRS9: dehydrogenase/reductase 9. References numbers: provided in the brackets.

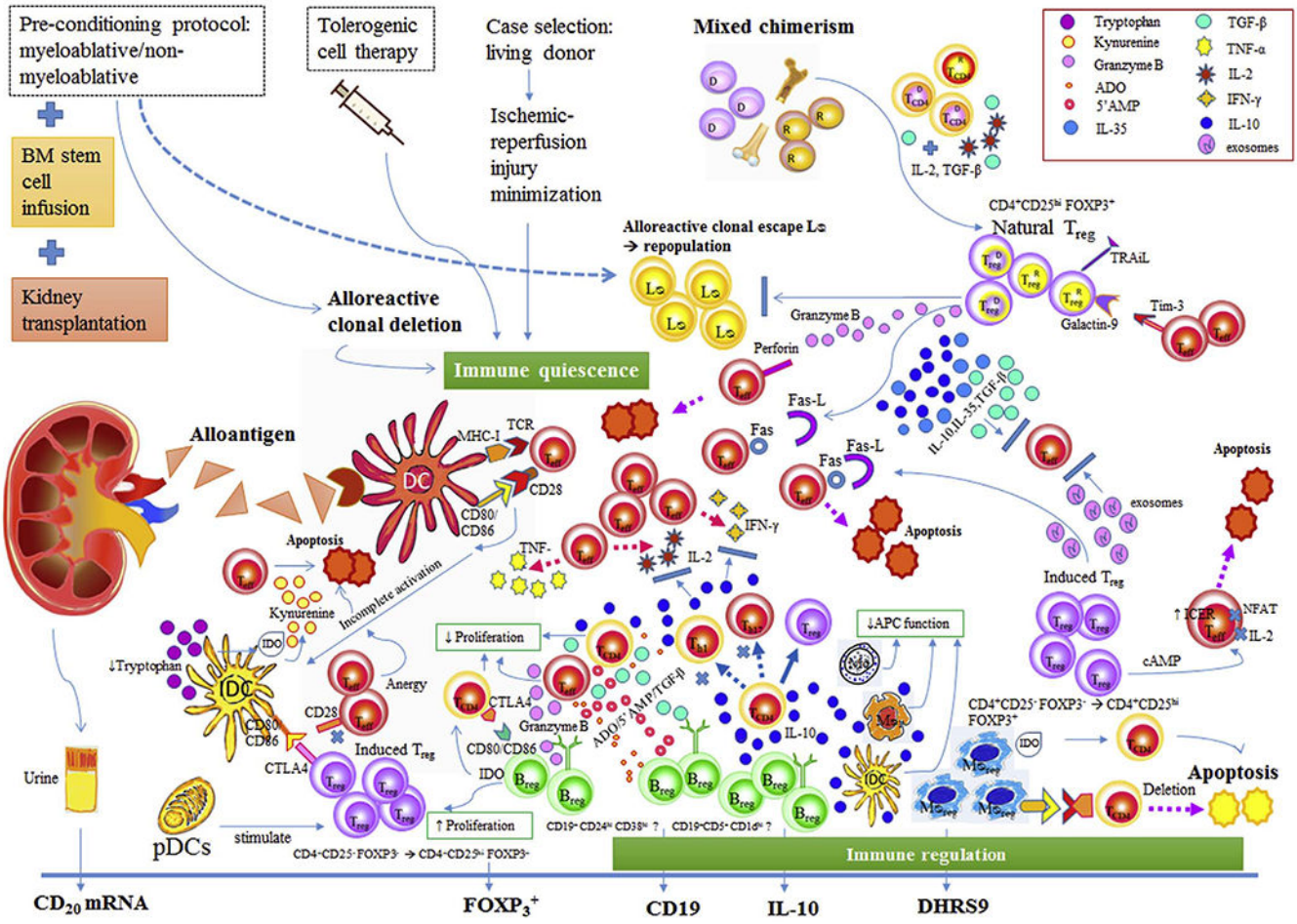

Fig. 2.

A summary of potential pathways to suppress donor-specific responsiveness by apoptosis of donor-reactive inflammatory cells and expansion of regulatory cells. 2A: In tolerance milieu, incomplete activated T cells go to anergy state or apoptosis while immature dendritic cells fail to develop maturity [45,48]. The immature dendritic cells can be activated to regulatory phenotype by IL-10 which are released from the apoptotic T cells [51,52]. Tregs modulate dendritic cell activation via CTLA-4 which binds CD80 (and CD86) with high affinity and resulting in the secretion of the enzyme IDO which catalyzes the breakdown of the amino acid tryptophan into N-formyl-kynurenine [52]. Tryptophan depletion leads to profound T lymphocyte apoptosis [52]. Another dendritic cells mechanism is through pDCs which indirectly operate Tregs differentiation [55]. 2B: Tregs suppressor effects include (1) anti-inflammatory cytokines secretion (IL-10, IL-35, TGF-β) to inhibit T cell activation, (2) exosome carried-miRNA production to silence T cells gene specific to cytokine production, (3) contact-dependent suppression with CD80/CD86 on APC by CTLA4 on Tregs to signal T cell inhibition, and (4) induction of apoptosis by multiple pathways such as via granzyme A/B and perforin, ICER, TRAiL, the Fas/Fas-ligand pathway, the galectin-9/TIM3 pathway [58]. 2C: Bregs mechanisms in tolerance milieu via IL-10 are suppression the differentiation of naive T cells into Th1 and Th17, promotion $CD4^+$ $CD25^-$T cells conversion into Tregs, downregulation of antigen presentation by macrophages, dendritic cells and monocytes,

suppression of production of proinflammatory cytokines by $CD4^+$ T cells, monocytes, and macrophages. In addition, IL-10 independent mechanisms are inhibiting $CD4^+$ T cell functions via CD80/CD86-CD28/CTLA-4, induce T cell apoptosis via Fas/Fas-L cascade [61], suppress T cells by granzyme B, IDO, ADO, 5′ AMP and TGF-β. 2D: Mregs have shown potent suppressive effects on T cells proliferation via IFN-γ induced IDO activity and contact-dependent deletion of activated T cells. Abbreviation: T_{reg}: Regulatory T cells, B_{reg}: Regulatory B cells, $MØ_{reg}$: Regulatory macrophages, T_{eff}: Effector T cells, T_{CD4} $CD4^+$ T cells, IDC: immature dendritic cells, pDCs: Plasmacytoid dendritic cells, LØ: lymphocytes, Th1: Helper 1T cells, Th17: Helper 17T cells, CTLA-4: Cytotoxic T lymphocyte antigen-4, TNF-α: Tumor necrotic factor-α, IL-2: Interleukin-2, IFN-γ: Interferon-γ, D: Donor, R: Recipient, TCR: T cell receptor, MHC-I: Major histocompatibility complex class I, FOXP3: Forkhead box protein 3, IDO: Indoleamine 2,3-dioxygenase, ADO: Adenosine, 5′AMP: 5′ Adenosine monophosphate, TGF-β: Transforming growth factor-beta, IL-10: Interleukin-10, Fas, Fas-L: Fas cell surface death receptor, Fas cell surface death receptor-ligand, Mo: Monocyte, $MØ_1$: Classical activated macrophages, APC: Antigen presenting cells, TRAiL: TNF-related apoptosis-inducing ligand, Tim-3: T cell immunoglobulin and mucin domain-3, NFAT: Nuclear factor of activated T-cells, cAMP: Cyclic adenosine monophosphate, ICER: Inducible cyclic adenosine monophosphate (cAMP) early repressor, mRNA: messenger ribonucleic acid, DHRS9: dehydrogenase/reductase 9.