

# Fifty Shades of Transplantation Tolerance: Beyond a Binary Tolerant/Non-Tolerant Paradigm

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## Abstract

**Purpose of Review** It has long been considered that tolerance in a transplant recipient is a binary all-or-none state: either the graft is accepted without immunosuppression identifying the recipient as tolerant, or the recipient rejects the graft and is not tolerant. This tolerance paradigm, however, does not accurately reflect data emerging from animal models and patients and requires revision.

**Recent Findings** It is becoming appreciated that there may be different gradations in the quality of transplantation tolerance based on underlying cellular mechanisms of immunological tolerance, and that individuals may enhance the robustness of their state of transplant tolerance by strengthening or combining different cellular mechanisms. Furthermore, evidence suggests that even if tolerance is lost, the loss may be only temporary, and in some circumstances, tolerance can be restored.

**Summary** Shifting our focus from an all-or-nothing tolerance paradigm to one with many shades may help us better understand how tolerance operates, and how this state may be tracked and enhanced for better patient outcomes.

**Keywords** Tolerance · Transplantation · Robustness · Erosion

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## Introduction

Tolerance is a polysemic term: it can refer to alterations in immune cells so that they fail to enact a rejection response—be it to self, tumors, allergens, or transplanted organs/tissues. In addition, tolerance can be used to describe a transplant recipient whose allograft remains functional following withdrawal of pharmacological immunosuppression, irrespective of underlying immunological mechanisms. Achieving tolerance in transplanted patients has been a goal of the transplant field for many years. Conventional immunosuppression has allowed organs to be transplanted in patients that are genetically distinct from their donor, but these treatments must be taken for a patient's lifetime to prevent graft rejection and have many undesirable side effects. As a result, non-adherence to immunosuppression regimens is one of the major risk factors for graft rejection and loss [1]. Transplantation might be more widely used to benefit a greater number of patients if transplant tolerance could be routinely induced, following a short-term treatment that permanently prevents the immune system from rejecting the organ while maintaining immune competence to other antigens. Tolerance, induced in animal models and identified in rare patients who have ceased immunosuppression without rejecting their allografts, as well as in patients in whom tolerance has been prospectively induced, offers a starting point to discover the conditions necessary to make inducible and stably maintained tolerance a feasible goal for all patients. Increasingly, evidence from tolerance studies in animals and humans is revealing that tolerance is not an all-or-none state, and that even if tolerance is robustly induced initially, it may not be sustained long-term. Strategies to induce more robust states of tolerance may rely on the induction of multiple redundant cellular mechanisms that make it more resilient and less metastable. Furthermore, if tolerance is not a fixed state, but may fluctuate in response to

environmental cues, new strategies to monitor the state of tolerance and the cellular mechanisms that maintain it, as well as approaches to boost them, will need to be developed to ensure the preservation of the transplanted organ for the life of the tolerant patient.

In this review, we discuss a new conceptual model of transplantation tolerance that is graded, rather than binary. This model arises from data in animal systems and in patients that revealed that some states of tolerance are more resilient than others, and that tolerance can be eroded over time. Moreover, if transplantation tolerance is broken after it has been stably induced, it may spontaneously return, albeit not always at the same level as before its transient abrogation. This graded model of transplantation tolerance supports the hypothesis that multiple cellular mechanisms are necessary for sustaining a robust state of tolerance, and that each of these may be susceptible to its own type of reversal signals. In support of this new graded tolerance model, we have summarized results showing that different mechanisms of cellular tolerance maintain transplantation tolerance, as well as highlighted studies in which robust states of transplantation tolerance are lost only if multiple underlying cellular tolerance pathways are broken.

### Animal Models that Reveal the Robustness and Resilience of Transplantation Tolerance

Our group has shown that transplantation tolerance induced with a short-term treatment of anti-CD154 (anti-CD40L) and donor splenocyte transfusion (DST) is donor-specific and robust. Importantly, its maintenance phase is resistant to many inflammatory insults, including half-lethal doses of systemic Toll-like receptor (TLR) agonists (Alegre et al. unpublished and [2]). In addition, increasing the frequency of alloreactive T cells by injecting  $10^5$  alloreactive TCR-transgenic T cells, depleting regulatory T cells (Tregs), or blocking PD-1/PD-L1 interactions, either as single or pairwise interventions, all failed to precipitate transplant rejection in stably tolerant hosts [3•]. Only the combination of all three of these interventions was able to break tolerance [3•], underscoring the redundant/additive effects of controlling alloreactive T cell numbers, and regulating their function in both cell-extrinsic and cell-intrinsic manners. However, transferring greater numbers of alloreactive naïve T cells ( $> 2 \times 10^6$  cells) was ultimately sufficient to break stable tolerance. This high-dose transfer resulted in a modest increase in the numbers of endogenous alloreactive T cells producing IFN $\gamma$  in the graft, which was sufficient to overwhelm the local tolerance mechanisms and trigger graft rejection [3•]. Another intervention that has led to cardiac allograft rejection in costimulation-blockade-mediated tolerance in mice is lymphodepletion resulting from antibody-mediated T cell depletion or irradiation during the maintenance period of tolerance [4]. Indeed, the residual effector T

cells not only expanded faster than the remaining regulatory cells but they also took on a memory phenotype and were resistant to Treg suppression [4]. Finally, established transplantation tolerance could also be abrogated with the administration of agonistic anti-CD40 mAb [2], as could tolerance to a malignancy in a model of acute myeloid leukemia [5]. Agonistic anti-CD40 likely provides surrogate help to alloreactive CD8 $^+$  T cells, as well as activates dendritic cells that then present donor-derived antigens indirectly to CD4 $^+$  T cells [6].

Infection with *Listeria monocytogenes* has also been shown to break established anti-CD154/DST-induced transplantation tolerance [7]. *Listeria* infection, but not LCMV or *Staphylococcus aureus* infections, has been the only acute infection identified to date that can prevent not only the induction of tolerance when it occurs at the time of transplantation, but also break tolerance when experienced later, during the maintenance phase [7–10]. *Listeria* infection results in a systemic increase in IL-6 and type I interferon (IFN) that act to suppress Treg function and promote alloreactive T cell proliferation and IFN $\gamma$  production, respectively [7]. *Listeria* infection in stably tolerant recipients can cause the complete cessation of cardiac allograft heartbeat and loss of tolerance in a subset of animals, or it can induce a rejection crisis, defined by a slowing heartbeat that does not progress to complete heartbeat cessation in another. Recovery from *Listeria*-induced rejection crisis was marked by an erosion in tolerance, which could then be completely abrogated by antibodies that disrupt the PD-1/PD-L1 inhibitory checkpoint pathway [11••]. Thus, eroded tolerance after infection required PD-1/PD-L1 interactions, whereas in uninfected tolerant mice, the combination of simultaneous PD-1/PD-L1 blockade, adoptive transfer of alloreactive T cells, and Treg depletion [3•] was necessary to precipitate the rejection of the stably accepted allograft.

Abrogation of tolerance, defined as the complete loss of cardiac allograft function, is not an invariably permanent state. In tolerant mice that fully rejected their allografts following *Listeria* infection, tolerance was spontaneously restored as early as 1 week after the rejection event, as evidenced by the ability of these mice to accept secondary donor-matched allografts without the need for additional immunosuppression [12••]. While this spontaneous return of tolerance permitted the acceptance of the second graft, the new tolerant state was not as robust as before the infection. Post-infection mice were prevented from accepting second donor-matched heart grafts if Tregs were depleted, an intervention that failed to precipitate rejection when the second hearts were transplanted into non-infected tolerant mice [12••]. The erosion of tolerance following *Listeria* infection is likely due to an increase in alloreactive T cells and/or reversal of their cell-intrinsic tolerance

mechanisms following the *Listeria* infection, resulting in a greater dependence on Tregs for tolerance maintenance.

A list of interventions that have been used to disrupt tolerance, their effects, and impact on graft outcomes is summarized in Table 1. These data illustrate that anti-CD154+DST treatment induces a robust transplantation tolerance, because it takes multiple simultaneous disruptions of different cellular/molecular tolerance mechanisms to break it. We posit that if some of these cellular/molecular tolerance mechanisms are only transiently disrupted, and revert back to their original tolerant state, transplantation tolerance can be spontaneously restored. However, if not all cellular mechanisms are restored, the resulting transplantation tolerance might not be as robust as it once was (Fig. 1). Thus, depending on the cellular mechanisms of maintenance of tolerance elicited by specific therapeutic regimens, and the types of inflammatory pathways triggered by particular infections, as well as the duration of their impact, one could theorize that certain infections might have no significant effect on transplantation tolerance while others might erode tolerance, or precipitate a reversible or irreversible loss of tolerance.

Transplantation tolerance is robust not only when induced with anti-CD154+DST, but also when induced with non-depleting anti-CD4 and anti-CD8 [14–16]. The latter is also resistant to breaking by individual interventions, such as increasing the numbers of naïve alloreactive T cells, or allowing T cells to homeostatically proliferate, or providing additional alloantigen and ischemia-reperfusion inflammation via giving a new transplant, or depleting Tregs [14–16]. Additionally, Schroeder et al. showed that established tolerance induced with anti-CD4 alone was resistant to breakage with FTY720, an intervention that is capable of preventing the induction of tolerance [17]. This observation suggests that recirculation of new immune cells to the graft is not necessary for maintenance of tolerance, but is necessary for its induction.

Transient cyclosporine treatment induced a robust tolerance in a MHC-I-mismatch transplant model in swine that was resistant to the removal of regulatory cells via a leukapheresis protocol, and to retransplantation [18]. Only the combination of both procedures precipitated rejection [18]. Robust transplantation tolerance was also induced in a combined bone marrow/costimulation blockade chimerism model, in which treatment with exogenous IL-2 or depletion of CD25<sup>+</sup> cells did not break tolerance to the bone marrow transplant [19]. In contrast, while Treg depletion or anti-CTLA-4 treatment did not break established tolerance induced by anti-CD3 mAb in an islet transplantation model [20], anti-PD-L1 alone could precipitate rejection [21], suggesting tolerance in this model may be less robust. PD-1/PD-L1 blockade was also sufficient to break tolerance of cardiac allografts induced with CTLA-4-Ig [22], and of long-term single-mismatched skin grafts in synchimeric mice induced with anti-CD154 and CTLA-4-Ig [23]. Similarly, spontaneous kidney

transplantation tolerance that occurs in select strain combinations in mice may be less robust, as it was susceptible to Treg depletion alone [24]. Collectively, these observations are consistent with our overall model of transplantation tolerance existing in different states of robustness and resilience.

The return of transplantation tolerance after its transient abrogation following infection of tolerant mice is not unique to this animal model. In a non-human primate model of combined bone marrow/kidney transplantation, a similar transient breaking of established tolerance was reported with high dose IL-2 administration that resulted in the expansion of alloreactive effector T cells, primarily memory T cells [25•]. While the IL-2 also expanded Tregs, this was not sufficient to prevent the increase in IFN $\gamma$  production by conventional T cells, which precipitated acute cellular rejection. Strikingly, after IL-2 was withdrawn, graft function recovered [25•]. In contrast, when bone marrow transplantation was combined with costimulation blockade in mice, providing exogenous IL-2 did not break tolerance to the bone marrow transplant [19], suggesting a more robust state of tolerance. In a study by de Vries et al., costimulation-blockade-induced tolerance was broken following mast cell degranulation that resulted in Treg egress from the graft and impaired Treg expression of suppressor molecules such as IL-10, TGF $\beta$ , and granzyme B [2]. Though not formally tested, a return of tolerance in this setting is likely, as the changes in Tregs were only transient [2].

### Transplantation Tolerance in Patients—Deliberately Induced or Spontaneously Acquired

In a few patients, renal transplantation tolerance has been prospectively induced successfully by promoting donor hematopoietic cell engraftment through combined bone marrow/kidney transplantation [26–28]. This mixed bone marrow chimerism approach harnesses mechanisms of central tolerance, primarily through the elimination of donor-reactive T cell clones. Indeed, evidence of deletion has been identified in one group of patients [29]. Other mechanisms of cellular tolerance besides T cell deletion may also play a role in maintaining allograft tolerance in these patients, as animal models of mixed chimerism had suggested [19, 30]. Relevant to the stability and robustness of transplantation tolerance, several of these patients have been followed long term. Two of eight tolerized patients were diagnosed at 5 and 7 years post-transplantation with chronic rejection, and one patient experienced acute cellular rejection just 3 weeks after an acute pyelonephritis [31]. These data suggest that even chimerism-induced tolerance may not always be permanent, and may be susceptible to breaking, perhaps by select infections that trigger the right inflammatory pathways.

In other patients, transplantation tolerance has emerged spontaneously after the withdrawal of immunosuppression.

**Table 1** Multiple mechanisms of tolerance need to be compromised to result in cardiac graft rejection in anti-CD154+DST-induced robust tolerance

Intervention during the maintenance phase of tolerance	↑Cell # <sup>a</sup>	Treg brake removed <sup>b</sup>	PD-1 brake removed <sup>c</sup>	IFNγ <sup>d</sup>	↑Cell # +IFNγ <sup>e</sup>	Rej? <sup>f</sup>	Loss of tolerance score <sup>g</sup>	Ref
Listeria	+	+	–	+	+	✓	4	[7]
10 <sup>5</sup> TCR75	+	–	–	–	–	–	1	[3•]
> 2 × 10 <sup>6</sup> TCR75	+	–	–	+	+	✓	3	[3•]
Lymphodepletion	+	+	–	+	+	✓	4	[4]
αCD25	–	+/-	–	-?	-?	–	0.5	[3•, 13]
αPD-L1	–	–	+	-?	-?	–	1	[3•]
αCD25 + 10 <sup>5</sup> TCR75	+	+/-	–	-?	-?	–	1.5	[3•]
αPD-L1 + 10 <sup>5</sup> TCR75	+	–	+	-?	-?	–	2	[3•]
αCD25 + αPD-L1	–	+/-	+	-?	-?	–	1.5	[3•]
αCD25 + αPD-L1 + 10 <sup>5</sup> TCR75	+	+/-	+	+? <sup>h</sup>	+? <sup>h</sup>	✓	4.5	[3•]
αPD-L1 post-Listeria	+	–	+	+? <sup>h</sup>	+? <sup>h</sup>	✓	4	[11••]
αCD25 post-Listeria 2nd ♥	+	+	–	+? <sup>h</sup>	+? <sup>h</sup>	✓	4	[12••]
αCD25 no Listeria 2nd ♥	–	+	–	+/-?	-?	–	1.5	[12••]

♥ The heart symbol represents a transplanted heart; “?” denotes the absence of a conclusive result

<sup>a</sup> Increased cell number; “+” given for known increase in alloreactive T cells that occurs post-Lm and for increasing the numbers of alloreactive cells with adoptive transfer. “–” no known increase in cells, or not experimentally determined

<sup>b</sup> Treg brake removed; “+” given for known reduction in Treg percentages in the graft or for αCD25 given prior to a 2nd donor-matched graft. “+/-” given for αCD25 treatment with a pre-existing graft because depletion of Tregs is less efficient in tissues “–” given for no known change in Treg percentages, or not experimentally determined

<sup>c</sup> PD-1 brake removed; “+” given for αPD-L1 “–” given for no αPD-L1 given and high PD-1 expression intact, but some values were not experimentally determined

<sup>d</sup> IFNγ production “+” given for Lm, high dose TCR75 transfer, and lymphodepletion based on experimental data. “+/-” given for post-Lm day 14 (having recently expressed IFNγ and potentially poised to do so again with inflammation) or with 2nd donor-matched transplant as a source of inflammation (combined two (+/-) values (each 0.5) = 1 = +). “–” given for no known change in IFNγ; gray values not experimentally determined

<sup>e</sup> If a given treatment resulted in an increase in allospecific T cells and IFNγ, the same value was given in this column as what was in the IFNγ column to give additional weight to what is likely causing rejection (an increased number of IFNγ-producing T cells)

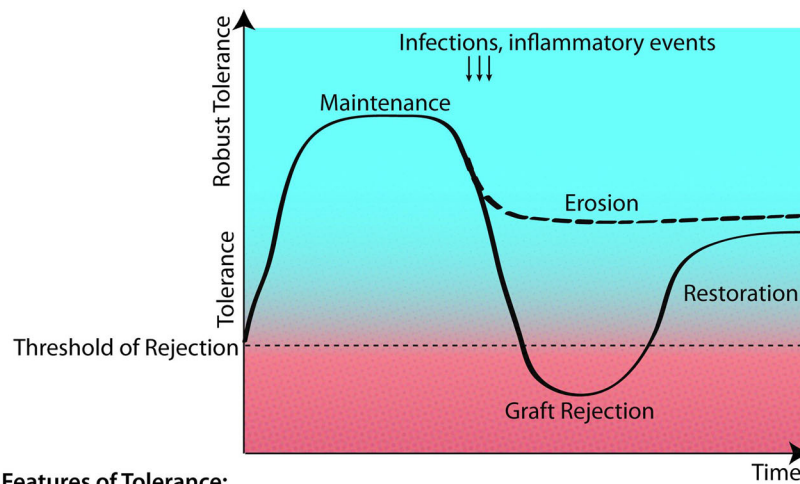
<sup>f</sup> “✓”=rejection in the majority of recipients, “–”= no rejection

<sup>g</sup> The sum of each value across a row was used to determine the loss of tolerance score with each “+” = 1, “+/-” = 0.5 and “–” = 0. The rejection column was not used

<sup>h</sup> “+” values were given based on the hypothesis that having increased alloreactive Tconv cells with an additional brake removed would result in the induction of IFNγ-producing T cells that mediate rejection. This hypothesis needs to be experimentally verified

In rare, operationally tolerant kidney transplant patients (estimated to occur at a frequency of less than 5 out of 10,000 transplants) [32], a B cell signature has been identified in peripheral blood [33, 34], as well as evidence of a signature of reduced T follicular helper cells [35] and increased memory Tregs [36]. Although this B cell signature has been attributed to tolerance, recent findings suggest that it may instead be representative of a signature of an absence of immunosuppression, arising from the comparison of tolerant individuals with stable patients on immunosuppression [37–39]. In spontaneously tolerant liver transplant recipients, an intra-graft iron metabolism signature was predictive of tolerance [40]. Interestingly, in both groups of patients, prior acute rejection episodes did not preclude these patients from later developing tolerance [41•, 42••], suggesting that tolerance can still be induced or restored following rejection. In addition, in a

cohort of kidney transplant patients that were followed long-term post-immunosuppression withdrawal, there was a higher incidence of infections that preceded a loss of tolerance than in those that retained functional grafts [42••]. These data suggest that tolerance in certain individuals may not be robust enough to withstand some infections to stably persist for the life of the patient. Successful weaning of conventional immunosuppression in liver transplant recipients has been correlated with the time elapsed since transplantation, recipient male gender, and age at the time of transplantation. Indeed, in patients at > 10 years post-transplantation, successful weaning was observed in 79.2% (n = 19 of 24) of patients. Determining whether liver tolerance is robust and persistent in patients will require long-term follow up, as rejection has been reported in these tolerant patients after many years of drug discontinuation [43, 44].



#### Features of Tolerance:

Treg/Tconv ratio:	High	Low	High
Numbers of allo-Tconv:	Low	Higher?	Higher?
PD-1 dependence:	Yes	?	Sometimes
Low alloreactivity:	Yes	No	Yes

**Fig. 1** Threshold model of tolerance and mechanisms involved. Tolerance is not an all-or-none state but rather exists as a gradient of robust tolerance resulting from the number of combined mechanisms of T cell tolerance. If multiple mechanisms are simultaneously compromised, this may result in dipping below the threshold necessary to prevent rejection (solid line). If individual mechanisms are disrupted, this may result in an erosion of tolerance (dashed line). Tolerance mechanisms may later recover resulting in a restoration of tolerance. Whether all tolerance mechanisms are fully restored or not will determine whether the restored transplantation tolerance state is as robust as the initial tolerance, or if it is eroded. The example shown above lists different tolerance mechanisms examined and how their strength changes during a *Listeria*-triggered loss of tolerance and post-

infection return of tolerance. Graft-specific Tregs are expanded or induced following tolerance induction, but these cells can be overwhelmed transiently by alloreactive conventional T cells (allo-Tconv) and *Listeria*-specific T cells in the graft. Tolerant mice maintain low numbers of allo-Tconv, similar to naïve mice, but these cells expand and stay elevated post-*Listeria* infection. Many tolerant T cells express the negative regulator PD-1, and in certain circumstances, such as post-infection, eroded tolerance becomes susceptible to PD-1/PD-L1 blockade. Naïve T cells have the potential for high alloreactivity but tolerance induction can result in inhibition of their cytokine production either extrinsically or intrinsically. Alloreactivity transiently increases during infection-mediated rejection, but approaches baseline levels during the restoration of tolerance

## Cellular Mechanisms of Tolerance—Their Potential Reversibility

Individual immune cellular pathways of tolerance may undergo transient or permanent reversal of tolerance under certain circumstances. Loss of regulatory T cell suppressive function, levels of intrinsic T/B cell dysfunction, and/or expansion of alloreactive T and B cells during inflammatory insults may individually and additively contribute to a loss of tolerance.

### T Cells

Chronic inflammation including inflammatory cytokines such as IL-6 and TNF have been shown to promote the instability of the Treg lineage and impair Treg function [45], or diminish the susceptibility of conventional T cells to Treg suppression [46, 47]. PD-1/PD-L1 blockade can result in an increase in effector functions including increased production of IFN $\gamma$  [20, 23]. Homeostatic proliferation has been shown to drive self-tolerant CD8<sup>+</sup> T cells temporarily out of a cell-intrinsic dysfunctional state [48, 49•], but if these cells are left to quiesce, they can revert to their previous tolerant state as they

appear to have an epigenetic imprinting of their hypo-responsiveness [49•].

### B Cells

If CD4 T cell tolerance is broken, it may allow these cells to provide additional help to B cells. Increasing the multivalency of B cell antigens and providing B cells with additional CD4 help allowed self-tolerant B cells to reverse their anergic state [50]. If this occurred in a transplant setting, it might result in alloantibody production and chronic graft rejection.

### NK Cells

NK cells can become tolerant/anergic in the long-term presence of MHC I-deficient cells because of chronic stimulation via activating receptors, unopposed by the inhibitory signals normally induced by self-MHC engagement. NK cell anergy could be overridden with high levels of IL-12, IL-18, or a mutant IL-2 “superkine” to provide control of MHC-I-deficient tumors [51].



## Dendritic Cells

Dendritic cell maturation has been shown to be impaired upon immunization with a self-antigen in a Treg-, IL-10R-dependent manner [52, 53]. Maturation markers were restored, however, in the presence of pro-inflammatory TLR3 signals [52], suggesting this type of DC tolerance could be overridden during an infection if necessary. Other TLR ligands such as CpG or co-administration of viruses and dendritic cells have helped override established T cell tolerance to tumors [54, 55].

## Conclusion

In this review, we have proposed a new working model explaining outcomes of transplantation tolerance versus rejection, one that is based on a gradient rather than a binary state (Fig. 1). Robust tolerance has multiple fail-safes to prevent rejection from occurring. Only when several mechanisms are simultaneously disrupted does rejection ensue. The notion that transplantation tolerance can exist in different states of robustness is reminiscent of the recent findings of graded mechanisms of tolerance to tissue-restricted self-antigens. Legoux et al. reported that ubiquitously expressed self-antigens induced T cell deletion, and that tissue-restricted self-antigens expressed in the lung and intestine enhanced thymically-derived FoxP3<sup>+</sup> Tregs, while pancreatic-restricted self-antigens were ignored by T cells [56•]. Importantly, regulatory tolerance exhibited limited durability and could be reversed with repeated antigen rechallenge while deletional tolerance was more robust. Likewise, Malhotra and colleagues reported that self-peptide expression patterns in the thymus determined the mechanisms of self-tolerance, with peptides presented uniformly inducing clonal T cell deletion, those with limited thymic expression inducing partial clonal deletion, impaired effector T cell but enhanced Treg potentiation, and those excluded from the thymus having no impact the T cell repertoire [57]. The existence of graded states of tolerance has profound implications for the search of tolerance biomarkers, and for the design of therapeutic approaches targeting multiple cellular tolerance mechanisms to preserve allografts for the life of the transplant recipient.

The concept that tolerance can be overridden transiently during inflammatory events, but then resurfaces when the inflammation resolves (Fig. 1), may also have wide-reaching clinical implications. It may help explain why certain transplant patients can be successfully weaned of immunosuppression revealing a state of operational tolerance despite having experienced prior acute rejection events [41•, 42••]. Moreover, several autoimmune diseases are known to undergo phases of relapse and stages of remission. It is possible that disease relapse is triggered by pro-inflammatory events that

overwhelm an already suboptimal self-tolerant state in individuals genetically predisposed to autoimmunity. With the quiescence of inflammation, regulation may dominate again to explain disease remission. In a similar fashion, initial immune-dependent regression of tumors can be followed by tumor recurrence. Tumor elimination by anti-tumor T cells may be aided by bystander inflammation, while tumor recurrence may be facilitated by activated Tregs [58]. In fact, *Listeria monocytogenes* is currently being used in clinical trials to improve anti-tumor immunity [59] suggesting that similar mechanisms of tolerance in the context of cancer as in the transplant model may be overcome by infection. A better understanding of the loss and spontaneous restoration of antigen-specific tolerance can have wide clinical applicability for therapeutic approaches to transplantation, autoimmunity and cancer.

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## Compliance with Ethical Standards

**Conflict of Interest** Anita Chong and Maria-Luisa declare grants from the National Institutes of Health during the conduct of this study.

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**Human and Animal Rights and Informed Consent** Reported studies/experiments with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards (including the Helsinki declaration and its amendments, institutional/national research committee standards, and international/national/institutional guidelines).

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