Recent Progress in Tolerance Induction through Mixed Chimerism

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Key Words

Transplantation · Tolerance · Chimerism · Costimulation blockade

Abstract

Organ transplant recipients require life-long treatment with immunosuppressive drugs. Currently available immunosuppression is associated with substantial morbidity and mortality, and is ineffective in inhibiting chronic rejection and graft loss. Therefore, a permanent state of donor-specific tolerance remains a primary goal for transplantation research. The induction of mixed hematopoietic chimerism is an attractive concept in this regard. Hematopoietic chimerism modulates the immunologic repertoire by extending the mechanisms of self-tolerance to donor-specific allotolerance. Despite recent progress in developing nontoxic bone marrow transplantation protocols for rodents, translation to large animals has remained difficult. Here, we outline the concept of tolerance via mixed chimerism, and review recent progress and remaining challenges in bringing this approach to the clinical setting.

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Introduction

Historical Perspective

To date, the only way to overcome rejection processes in the clinical setting is drug-induced nonspecific immunosuppression. For more than 20 years, calcineurin inhibitors (CNIs) have been the backbone of posttransplant immunosuppression, used most commonly in combination with newer immunosuppressive drugs. However, while being very effective in preventing acute rejection episodes, nonspecific chronic immunosuppression is accompanied by severe morbidity and mortality and does not effectively prevent chronic graft loss. Diabetes mellitus, hypertension, neurotoxicity, malignancies, infections and severe nephrotoxicity are all associated with CNIs [1–4]. Morbidity and a high risk of chronic graft failure could be avoided with the induction of specific tolerance towards donor antigens.

The finding that chimerism is related to tolerance dates back to the 1940s when Owen and colleagues observed that acceptance of even fully major histocompatibility complex (MHC)-mismatched skin is possible between particular cattle twins [5, 6]. These so-called ‘freemartin cattle’ share a common placental circulation and are thus naturally occurring chimeras, tolerant to tissue antigens from each other. In 1954, tolerance was actively induced for the first time. Injection of allogeneic cells into neonates demonstrated that this chimeric state results in

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specific tolerance which could be manipulated and directed as desired [7]. Based on these observations, the first attempts to experimentally induce hematopoietic chimerism in adult mice were performed with lethal doses of total body irradiation (TBI) leading to global destruction of the hematopoietic repertoire in the host which was then completely reconstituted with donor bone marrow cells (BMC, termed 'full chimerism') [8].

The Concept of Mixed Chimerism and the Mechanism of Central Tolerance

Full chimerism is associated with a higher risk of graft-versus-host disease (GVHD) and somewhat reduced immunocompetence than 'mixed chimerism' [9, 10]. Mixed chimerism, which describes a balance of donor and recipient cells coexisting in the host, is thus preferred [11, 12]. Intrathymic (or central) clonal deletion provides a very robust form of tolerance in all chimerism approaches achieving acceptance of even the most immunogenic tissues, such as skin and small intestine [11, 13]. Central deletion is usually regarded as superior to regulatory or anergic mechanisms since clonal deletion physically eliminates T cells with a certain specificity. In regulation and anergy, cells remain in a nonreactive state in the individual, but may be susceptible to environmental influences that provoke reactivation.

Mixed chimerism ensures intrathymic T cell deletion of donor-reactive cells as long as chimerism persists, ideally lifelong. This is mediated mainly by bone marrow (BM)-derived dendritic cells which originate from the recipient and the donor [14, 15]. T cells are positively selected mainly by the thymus epithelia. Thus, development of recipient-MHC restriction renders full chimeras less effective to respond to foreign antigen presented by donor MHC in the periphery [12, 16]. Mixed chimeras, in contrast, provide antigen-presenting cells (APCs) of the donor and recipient in the periphery, preserving immunocompetence to antigen presented in peripheral tissues. This has been demonstrated, for instance, by rapid rejection of third party grafts. However, dissociation between chimerism and tolerance has been observed in some cases. Reliable chimerism, particularly in T cells, has been suggested to be a critical issue to successfully achieve tolerance. The level of chimerism that is induced may also have an influence on the robustness of tolerance [17]. Although several other experimental approaches have been suggested to induce tolerance, like donor-specific transfusion or costimulation blockade alone, mixed chimerism is generally regarded as the most promising, if not only, way of inducing 'true tolerance' in transplantation [18].

From Nonmyeloablative to Noncytoreductive Host Conditioning

Myeloablation, T Cell Depletion and Costimulation Blockade

When allogeneic BM is transplanted, preexisting donor-reactive recipient T cells are the primary immunological barrier that needs to be overcome in order to achieve engraftment. These are mature T cells in the periphery and without appropriate host conditioning, they cause acute allograft rejection. Thus, in the first steps for tolerance induction – before a state of hematopoietic chimerism is achieved – we face similar problems as in conventional organ transplantation, that is, adequate conditioning of the recipient to obviate acute alloimmune response. Secondly, a physiologic barrier resists donor cell engraftment in host tissues as demonstrated by only low-level and transient chimerism in syngeneic hosts that received no TBI or doses less than 1.5 Gy [19].

The first protocols for mixed chimerism were myeloablative (that is, they completely destroyed the recipient's hematopoietic system) and therefore quite toxic [11]. T cell-depleting antibodies – eliminating the main players of acute rejection and GVHD – helped to develop nonmyeloablative protocols (that is, they destroy only part of the recipient's hematopoietic system) [20–24]. Anti-CD4- and anti-CD8-depleting monoclonal antibodies (mAbs), plus a more restricted irradiation to the thymic region, prevented intrathymic alloreactivity and permitted mild doses of TBI [21]. Thymus irradiation could be eliminated by repeated administration of T cell-depleting antibodies [23]. When exposed to depleting antibodies, recipients are also protected against GVHD, since they remain in the circulation, additionally eliminating graft-derived T cells. T cell depletion in combination with thymic irradiation permitted the induction of chimerism and tolerance without TBI if very high doses of BM were transplanted [25]. However, irradiation could not be eliminated altogether even with very high doses of T cell-depleting mAbs [22].

The discovery that costimulation molecules (especially the CD28 and CD40 pathways) are necessary for cell signaling driving the immune reaction gave rise to the idea of biologically engineering blocking agents that compete with physiological receptor-ligand interactions. Such costimulation blockers have recently replaced global T cell destruction in mixed chimerism models. T cell receptor (TCR) binding to the MHC/antigen complex does not exert sufficient stimulation for fully activating T cells, so additional costimulatory signals are required.

Table 1. Experimental mouse models for the induction of tolerance via mixed chimerism

Year	Journal	Author	Ref.	Strain combination	Myelosuppression (TBI or cytotoxic drugs)	Recipient cell depletion
1998	J Exp Med	Wekerle et al.	28	recipient: C57BL/6; donor: B10.A	d0; 3 Gy TBI	x
1999	Transplantation	Wekerle et al.	27	recipient: C57BL/6; donor: B10.A	d0; 3 Gy TBI	d-5; anti-CD4 (1.8 mg), anti-CD8 (1.4 mg)
2000	Nat Med	Wekerle et al.	46	recipient: C57BL/6; donor: B10A	none	x
2000	J Immunol	Durham et al.	41	recipient: C57BL/6; donor: Balb/c	none	x
2000	Blood	Seung et al.	38	recipient: Balb/c; donor: C57BL/6	d0; 4 Gy TBI	x
2001	J Immunol	Adams et al.	29	recipient: C57BL/6; donor: Balb/c	d5; busulfan (20 mg/kg)	x
2001	J Immunol	Ito et al.	36	recipient: C57BL/6; donor: B10.A	d0; 3 Gy TBI	d-1; anti-CD8 (0.35 mg)
2001	Am J Transplant	Kurtz et al.	58	recipient: C57BL/6 CD40L-/-; donor: B10.A	d0; 3 Gy TBI	d-1; anti-CD8 (0.35 mg)
2001	Blood	Taylor et al.	30	recipient: C57BL/6; donor: Balb/c	d-1; 2 Gy TBI	x
2001	Blood	Quesenberry et al.	37	recipient: B6; donor: Balb/c	d0; 1 Gy TBI	x
2002	Blood	Kean et al.	45	recipient: C57BL/6; donor: Balb/c	d-1; busulfan 20 mg/kg	x
2003	J Clin Invest	Seung et al.	39	recipient: Balb/c; donor: C57BL/6	x	x
2003	Blood	Blaha et al.	40	recipient: C57BL/6; donor: Balb/c	d-1; 1 Gy TBI, d0-28; rapamycin (0.2 mg/kg), MP (10 mg/kg), MMF (20 mg/kg)	x
2005	Am J Transplant	Bigenzahn et al.	43	recipient: C57BL/6; donor: Balb/c	d-1; 3 Gy TBI	anti-CD25 or anti-IL-2
2005	Blood	Westerhuis et al.	51	recipient: C57BL/6; donor: Balb/c	none	d-5/-1; anti-NK1.1 (0.25mg), anti-CD8 (0.25mg)
2005	Transplantation	Blaha et al.	48	recipient: C57BL/6; donor: Balb/c	d0-27; rapamycin (0.2 mg/kg), MP (10 mg/kg), MMF (20 mg/kg)	x
2006	BMC Immunol	Graca et al.	50	recipient: CBA, Balb/c; donor: B10	none	first stage (4 weeks pre-BMT), second stage (d0/2/4); anti-CD4 (1 mg), anti-CD8 (1 mg) (nondepleting)

DST = Donor-specific transfusion; MR1 = clone name, anti-CD40L mAb; MP = methylprednisolone; MMF = mycophenolate mofetil.

DST	MR1 (anti-CD154 mAb)	CTLA4Ig	BMC unseparated	T cell-depleted BMC	Mechanistical study
x	d0; 0.45 mg	d2; 0.5 mg	15 × 10 ⁶ ; d0	x	
x	d0; 0.45 mg	d2; 0.5 mg	15 × 10 ⁶ ; d0	x	
x	d0; 0.45 mg	d2; 0.5 mg	200 × 10 ⁶ ; d0	x	
x	d0/2/3/6/14/38/ 60/90; 0.5 mg	x	20 × 10 ⁶ ; d0/2/3/6/ 14/38/60/90	x	
x	d0/3; 0.5 mg	x	25 × 10 ⁶ ; d0	x	
yes (initial BM dose)	d0/2/4/6; 0.5 mg	d0/2/4/6; 0.5 mg		20 × 10 ⁶ ; d0/6	
x	d0; 0.5 mg	x	20 × 10 ⁶ ; d0	x	CD8 cells are responsible for resistance to allogeneic BM engraftment under costimulatory blockade
x	x	x		20 × 10 ⁶	MR1 blocks interaction between CD40 and CD40L, anti-CD40L mAbs need not play a role in signaling/depletion
x	d-1/0/1; 0.2 mg	x	40 × 10 ⁶ ; d0	x	
d-10; 10 × 10 ⁶ spleen cells	d-10/-7/-3/0/3; 1.6 mg	x	40 × 10 ⁶ ; d0	x	
x	d0/2/4/6; 0.5 mg	d0/2/4/6; 0.5mg		20 × 10 ⁶ ; d0	
d-7; 10 × 10 ⁶ spleen cells	d-7/-4/0/3; 0.5 mg	d-7/-4/0/3; 0.5mg	50 × 10 ⁶	x	
x	d0; 1 mg	d2; 0.5 mg	15-20 × 10 ⁶	x	
x	d0; 1 mg	d2; 0.5 mg	15-20 × 10 ⁶ ; d0	x	peripheral deletion and regulation are essential in early phase of tolerance induction
x	d0; 0.5 mg	x	30 × 10 ⁶ ; d0	x	chimerism can be increased by NK cell depletion
x	d0; 1 mg	d2; 0.5 mg	50 × 10 ⁶ ; d0	x	
x	first stage (4 weeks pre-BMT), second stage (d0/2/4) 1 mg	x		10 × 10 ⁶ ; d0	

In the absence of costimulation, T cells are rendered anergic [26]. Anti-CD40L (CD154) mAb and the fusion protein CTLA4Ig to date provide the least toxic way of preventing alloreactive T cell response in mixed chimerism regimens [27–30]. Importantly, when given alone, ‘costimulation blockers’ do not induce true tolerance as demonstrated by rejection of skin grafts [31, 32] – the most stringent test for tolerance – but they can be very efficiently used as part of BM transplantation (BMT) protocols for tolerance induction through mixed chimerism [28–30].

While T cell tolerance is critically required to tolerate alloreactivity, it is important to achieve tolerance also among additional populations. In this respect, mixed chimerism has been demonstrated to achieve B cell and natural killer (NK) cell tolerance as well [33–35] and thus appears to be able to achieve robust tolerance in the most relevant effector cell populations.

Nonmyeloablative Costimulation Blockade-Based BMT Regimens

Recently developed BMT models are mainly based on costimulation blockers and nonmyeloablative doses of TBI or busulfan. Various dosing and timing regimens have led to an increasing number of BMT protocols.

First, protocols differ in the administration of costimulation blockers (table 1). Anti-CD40L is often used in combination with CTLA4Ig but anti-CD40L alone is used in other mixed chimerism protocols [28, 29, 36–39]. A wide range of doses of costimulation blockers has been used from 0.5 to 8 mg/mouse in single or multiple injections [28–30, 36, 37, 40, 41]. Delayed administration of CTLA4Ig (day 2) has been suggested to permit negative signaling through the physiologic CTLA4 receptor and therefore result in improved outcomes in some models [42].

Second, different strain combinations are used for tolerance induction (table 1). Some protocols involve MHC-mismatched animals on the same genetic background (i.e. MHC-congenic combinations, such as B10.A to B6) [28, 36], while others employ MHC mismatches plus minor mismatches (for example Balb/c to B6) [29, 39–41, 43]. A complete mismatch, also involving minor mismatches, would correspond to a more stringent and more clinically relevant situation. In addition, some strains per se constitute a very stringent setting, such as B6 mice which tend to be more resistant to costimulation blockade [31].

Third, TBI is an essential component in most protocols (table 1). It has been shown that radiation to restrict-

ed areas also allows BMC engraftment [44]. Instead of TBI, also nonmyeloablative doses of busulfan have been used in costimulation protocols [29, 45].

Nonmyelosuppressive Protocols under the Cover of Costimulation Blockade

It would be clinically desirable to completely avoid TBI due to its toxic effects. TBI may surmount physiological barriers by opening up niches in the BM and thereby creating space for donor stem cells accompanied by the induction of systemic cytokine release and enhanced cell migration [44]. But also nonspecific immunosuppressive effects play a role. Proof-of-principle studies have shown that mixed chimerism and tolerance can indeed be induced without any irradiation when transplanting the approximately 10-fold dose of allogeneic BM under the cover of costimulation blockade [41, 46–48]. However, these high numbers of BMC are clinically not feasible.

Granulocyte colony-stimulating factor-mobilized peripheral blood stem cells (PBSCs) could pose a solution to the limited availability of BMC from a single donor. Murine PBSCs have recently been shown to engraft in an essentially syngeneic system [49]. However, allogeneic PBSCs are more immunogenic and less tolerogenic in murine mixed chimerism models [Wekerle et al., unpubl. data]. Another very recent approach for nonmyelosuppressive host treatment involves clinically relevant numbers of BMC (approximately 5×10^7 BMC/kg), but, additionally to anti-CD40L costimulation blockade, coreceptor blockade of CD4 and CD8 through repeated injections of nondepleting antibodies is started 4 weeks prior to BMT [50].

Depletion of NK cells allows transplantation of reduced BMC numbers for successful chimerism induction. The elimination of NK cells through mAb-mediated depletion had a positive effect on BM engraftment in anti-CD40L-based protocols [51].

Compatibility of Costimulation Blockade with Immunosuppressant

Alternatively to high-dose BMT protocols, the minimally required dose of TBI can be reduced by short-term treatment with certain conventional immunosuppressive agents. Since only transient, their administration would be clinically acceptable provided that true tolerance can be achieved thereafter. A 4-week course of rapamycin-based immunosuppression allows a reduction in the dose of TBI from 3 Gy down to 1 Gy in a costimulation blockade-based model [40], plus it helps to reduce the minimally required dose of BMC required for BMT without

Fig. 1. Schematic illustration of the CD28 and CD40 costimulation pathways and the therapeutic blockade thereof. **a** Interaction of a T cell with an APC. Antigens are presented via the MHC to the TCR, co-stimulation mediated by B7.1/2-CD28 and CD40/CD154 interactions induce activating signals, whereas ligation of CTLA4 by B7.1/2 induces an inhibitory signal to the T cell. **b** Mechanisms of costimulation blockers anti-CD154 mAb and CTLA4Ig. The activating signal CD28 (and the inhibitory signal CTLA4) is inhibited by CTLA4Ig and the activating CD40 signal is inhibited by anti-CD154 mAb, while TCR-MHC interactions are allowed to occur. + = Activating signal; - = inhibitory signal; ? = unknown/controversial.

any TBI [48]. However, CNIs (cyclosporine A or tacrolimus) prevent tolerance induction in this protocol [40]. Investigating the compatibility of mixed chimerism protocols with immunosuppressive drugs is important, since clinically a short-term immunosuppression is ethically indispensable. Negative effects of cyclosporine A were also shown in another anti-CD40L protocol which was improved with administration of sirolimus [52]. A study using nonmyeloablative busulfan plus costimulation blockade consisting of anti-CD40L and CTLA4Ig suggests the compatibility of CNIs and corticosteroids [53]. Seemingly contradicting results of (mechanistic) in-depth studies in chimerism protocols are probably related to the specifics of the models studied, including the differences of the host conditioning regimens employed (table 1).

Mechanisms of T Cell Tolerance in the Induction Phase after BMT with Costimulation Blockade

Mechanisms of Anti-CD40L in BMT Protocols

Anti-CD40L mAb is an indispensable component in costimulation-based mixed chimerism protocols. It efficiently binds CD40L expressed on activated T cells (fig. 1) and prevents CD40L from binding to its receptor, CD40, which is expressed on the APC. CD40 blockade prevents activation of APC, that is upregulation of MHCs, costimulatory molecules and cytokines. Additionally, a T cell-depleting effect has been ascribed to anti-CD40L in some

systems [54–56]. There is also some evidence for a direct CD40L signaling pathway to the T cell as CD40L ligation can induce immunomodulatory cytokines and/or apoptosis of allograft-specific T cells [57]. Some studies in CD40L knockout mice, however, seem to contradict the suggested, active mechanism for tolerance induction in mixed chimerism protocols [58]. The predominant effect seems to be blocking the CD40 signal. Furthermore, when CD40 ligation is prevented, the impairment of antigen presentation will, in turn, decrease T cell response.

Mechanisms of CTLA4Ig in BMT Protocols

Simultaneous blockade of the CD28 pathway with the CD40 pathway has been shown to have synergistic effects in graft prolongation [59]. CD28 is a positive costimulator, constitutively expressed on T cells, which promotes expansion and survival of the T cell if engaged with B7 molecules on the APC (fig. 1). However, its homologue CTLA4, which is a negative regulator, inducible on activated T cells and constitutively expressed on regulatory T cells, binds B7 with a much higher affinity than CD28, thereby preventing the activation of the CD28 signaling pathway. Thus, physiologic CTLA4 was used for construction of the competitive costimulation blocker CTLA4Ig [60]. But apart from outcompeting CD28 and hence decreasing CD28 signals, CTLA4 is also suggested to actively antagonize to CD28 signaling by inducing anergy [61]. In BMT protocols a delayed administration of CTLA4Ig seems beneficial, probably to allow physiologi-

cal CTLA4 engagement with B7 for negative T cell signaling. Besides, recent findings have demonstrated that surface CTLA4 and the fusion construct CTLA4Ig can also act through induction of indoleamine-2,3-dioxygenase (IDO), a tryptophan-catabolizing enzyme [62]. Although CTLA4Ig was shown to depend on the tryptophan-depleting enzyme IDO in pancreas islet transplantation [63], IDO proved to have no critical role in a CTLA4Ig-based BMT regimen for tolerance induction [64].

Peripheral Deletion after BMT plus Costimulation Blockade

As mentioned above, the fundamental mechanism of long-term tolerance in mixed chimeras is central deletion of newly developing alloreactive T cells. Deletional mechanisms are also important in the induction phase, as progressive, peripheral deletion of preexisting, mature, alloreactive T cells occurs after BMT with costimulation blockade. It has been shown that a reduction in the clone size of alloreactive T cells is a general prerequisite for allowing tolerance induction across MHC barriers [65, 66] and that numbers of superantigen-reactive T cells are starting to decrease in the first week after BMT, even when using thymectomized recipient mice [28]. Using recipients that code for a transgenic TCR further confirmed the peripheral, thymus-independent deletion of truly alloreactive T cells early after BMT [67, 68]. Experiments with Fas-deficient and Bcl-X_L-transgenic mice revealed that passive cell death, which occurs under the lack of costimulation, and activation-induced cell death, which is usually promoted by repeated antigen stimulation and IL-2, both seem to play a role in early deletional mechanisms [69].

Regulatory Mechanisms

Although early, extrathymic deletion is a crucial mechanism, tolerance can already be observed before peripheral deletion is complete as demonstrated by mixed leukocyte reactions and skin graft acceptance [46, 58]. This encouraged investigating anergic and regulatory mechanisms in recent years. In a mixed chimerism model using 3 Gy TBI, fully mismatched BMC, CTLA4Ig and anti-CD40L mAb, the administration of recombinant IL-2 (which can restore proliferation in classical anergy) did not prevent tolerance induction. Neutralization of IL-2 using an anti-IL-2 antibody, in contrast, prevented tolerance induction in this model. Early depletion of CD25⁺ cells, which have been reported to have major regulative properties in the immune response, led to skin graft loss of all mice, whereas late treatment with anti-CD25 did

not disturb established skin graft tolerance in long-term BM chimeras. Adoptive transfer experiments further demonstrated that CD4⁺ cells exert regulatory functions early after BMT [43]. Thus, in this model regulation has an important role in the induction but not the maintenance of tolerance.

However, regulatory mechanisms have been shown to play no role in models involving T cell depletion. It might also be the level of chimerism that is decisive or the degree of deletion [70, 71]. Protocols inducing lower levels of chimerism are thought to be more dependent on regulatory mechanisms due to a lack of sufficient donor APCs that drive negative selection processes in the thymus. Likewise, in protocols for nonhuman primates which induce only transient rather than stable macrochimerism (see below), regulation is thought to play a more important role [72].

Using Regulatory Mechanisms in New Approaches

Additionally to targeting alloreactive T cells to prevent acute rejection, it has been suggested to use or promote expansion of 'facilitating cells' or 'regulatory cells' for BMC engraftment that could potentially replace TBI [73–77]. Plasmacytoid precursor dendritic cells have been shown to be responsible for successful chimerism induction [73]. Another approach seeks to stimulate CD4⁺CD25⁺ regulatory T cells by anti-CD3 mAb or their growth factor IL-2. However, stimulation of these cells in a costimulation protocol consisting of low-dose TBI, CTLA4Ig and anti-CD40L did not promote chimerism induction [Wekerle et al., unpubl. data]. NK T cells represent another type of regulatory cells which can be stimulated by α -galactosylceramide (α -Gal). Administration of α -Gal to stimulate NK T cells in the mixed chimerism protocol mentioned above, however, revealed the opposite effect. Further experiments using T cell depletion in NK T knockout mice demonstrated that rejection can occur in the absence of T cells. It seems that rejection is promoted by NK T cells indirectly, via activation of NK cells [Wekerle et al., unpubl. data].

Mixed Chimerism Protocols for Large Animals and (Pre)Clinical Trials

Large Animals

In contrast to murine protocols which are very efficiently inducing high levels of chimerism and robust tolerance with very mild BMT regimens, large animals and nonhuman primates require more extensive protocols. A

severe problem for translating chimerism protocols from rodents to (pre)clinical application involves the reduced frequency of memory cells in specific pathogen-free-housed animals. Memory cells are less dependent on costimulation and thus relatively resistant to costimulation blockade. Moreover, they can show cross-reactivity to alloantigen. It has been suggested that 'immune history' as well as ongoing infections pose a major obstacle to overcome for clinical success of tolerance [78]. Additionally, costimulation blockade can prevent an ongoing immune defense to persistent viral infections [79]. Despite these hurdles, chimerism and tolerance can be achieved in large animals. A miniature swine protocol achieved chimerism and renal allotolerance without TBI and thymic irradiation, using depleting antibodies and short-course cyclosporine A [80]. CTLA4Ig administered from day 1 to day 7 prior to TBI has been shown to be capable of reducing TBI down to 1 Gy in a canine transplantation protocol, employing donor PBMC infusions and posttransplant short-term immunosuppression with mycophenolate mofetil and cyclosporine [81].

Nonhuman Primates

Tolerance to MHC disparate grafts in nonhuman primates can be achieved through nonmyeloablative TBI, antithymocyte globulin (ATG), thymic irradiation, splenectomy and simultaneous transplantation of donor BM and kidney followed by a 4-week administration of cyclosporine [82]. Transient mixed chimerism can be achieved using this protocol resulting in long-term renal graft survival despite cessation of immunosuppression. Tolerance was demonstrated in vivo and in vitro by mixed leukocyte reaction and skin transplantation. Moreover, a prolongation of heart allografts and long-term survival of pancreatic islet have been demonstrated [83, 84]. Attempts at reducing conditioning, for example by omission of splenectomy, failed in the regimen mentioned above since splenectomy seemed to be crucial in the establishment of B cell tolerance but a delayed kidney transplantation was possible [85]. When ATG was replaced with anti-CD2 antibody, NK cell tolerance failed [86]. Additionally using the costimulation blocker anti-CD40L mAb in the basic protocol, chimerism levels could be well improved and splenectomy could be eliminated, but attempts to avoid thymic irradiation failed [87]. Preliminary studies with costimulation blockade that targets the CD28 plus the CD40 pathway, the use of rapamycin, nonmyeloablative doses of busulfan and a short-course treatment with anti-IL-2R have been reported to induce transient macrochimerism levels of more than 50% [88].

Clinical Application

The induction of tolerance via mixed chimerism has been proven possible in humans. Prospective pilot trials for chimerism induction and organ tolerance have been performed in patients suffering from multiple myeloma and end-stage renal failure. The recipients were conditioned with cyclophosphamide, ATG and thymic irradiation and simultaneously received BM and a kidney from a human leukocyte antigen-identical sibling. Cyclosporine was given up to 77 days. Transient chimerism led to long-term acceptance of renal grafts even after withdrawal of immunosuppressant [89–91].

Thus, these results are proof-of-principle that tolerance can be induced through chimerism in the clinical setting. However, toxicities of the regimen (in particular GVHD) render the approach not widely applicable.

Remaining Challenges – from the Clinic Back to Basics

One major impediment to the development of clinically routinely applicable mixed chimerism protocols is the lack of clinically available costimulation blockers. In particular, anti-CD40L mAb was associated with unexpected thromboembolic side effects in humans [92–94] and its clinical development has thus been suspended [95]. Solutions to this problem are a high priority in the field and could theoretically be provided by RNA interference [96] or by targeting alternative costimulation molecules [97, 98]. The second costimulation blocker, CTLA4Ig (abatacept), which has been extensively tested in animal models, exhibited no unexpected toxic side effects in humans and has recently been approved for the treatment of rheumatoid arthritis [99, 100]. However, the original CTLA4Ig construct was not sufficiently potent to be developed as immunosuppressant in organ transplantation. Therefore, a second-generation CTLA4Ig (belatacept, LEA29Y) with improved affinity has been developed [101]. Belatacept is currently being evaluated in renal transplantation with the intent to replace CNIs [102]. The available data from the phase II trial show that belatacept is similarly effective as cyclosporine A in preventing acute rejections after renal transplantation (in combination with Simulect, CellCept and steroids).

The initial pilot trial of mixed chimerism [89, 90] and the growing experience with this approach in nonhuman primates [103] are the platforms to further develop this concept. In particular, advances are necessary to achieve lasting chimerism with less recipient conditioning. Besides, measures avoiding the risk of GVHD – which would be unacceptable in a transplant recipient without

concomitant malignancy – are a prerequisite for widespread use. Furthermore, tolerance assays need to be developed that are suitable for clinical use so that they can serve as endpoints and can help in guiding clinical therapy during tolerance trials.

A Future for Molecular Chimerism or Xenograft Tolerance?

Molecular chimerism is a gene therapy approach that seeks to selectively introduce the disease-relevant antigen(s) into autologous (syngeneic) hematopoietic stem cells which are then transplanted back to the individual [104–106]. Indeed, molecular transfer of MHC molecules achieved allogeneic tolerance to MHC class I and class II molecules [107, 108]. Robust tolerance to skin allografts was shown to rely on deletion of CD8⁺ T cells, demonstrated by mice expressing a transgenic TCR to the transduced MHC class I molecule [109]. When molecular chimerism was established in mice that express a transgenic TCR on CD4⁺ cells reactive to MHC class I-derived peptides presented through the indirect pathway, adoptive transfers of CD4⁺CD25⁺ positive cells together with effector T cells into immunodeficient mice prevented skin graft rejection, arguing for a regulatory type of cells in this model [110]. The authors suggest that alloantigen density in the indirect pathway may be lower than that in the direct pathway. Therefore indirect allo-recognition may present too low amounts of antigen to mediate deletion of CD4⁺ transgenic T cells. In contrast, the higher antigen density in the direct pathway favors deletion of CD8⁺ transgenic T cells.

As a long-term objective, the induction of molecular chimerism is supposed to offer a helpful tool for xenotransplantation which, if safe and feasible, would solve the problem of organ shortage [106]. In contrast to the allogeneic situation, in which mainly T cells have to be tolerated, xenograft approaches (most relevantly pig to human) have to deal with the induction of B cell tolerance as well, especially towards carbohydrate epitopes [111]. GalT^{-/-} knockout mice which, like humans, develop natural anti-Gal antibodies, are used as recipients in studies indicating that mixed chimerism can, indeed, induce both T and B cell tolerance [112–114]. When applying mixed chimerism approaches, tolerance develops to pre-existing and newly developing anti-Gal B cells by mechanisms of anergy in the early phase and deletion or receptor editing in the late phase [115]. Tolerance can also be established in presensitized animals when administering

high amounts of BMC [116]. B cells from early chimeras transplanted into GalT^{-/-} B cell-deficient mice revealed that preexisting anti-Gal B cells are rendered anergic in the early phase when the antigen is present. Newly developing B cells could not be found in spleens of late chimeras, suggesting deletion and/or receptor editing [117]. Still, engraftment barriers across species are difficult and might be overcome via retroviral gene therapy [104, 118].

Molecular chimerism of alpha-Gal was indeed shown to induce xenograft tolerance in mice since immunization with pig cells did not stimulate antibody production. Anti-Gal antibodies that naturally occur in non-chimeras as IgM antibodies seem to switch to anti-Gal IgG antibodies [117].

Interestingly, gene therapy with MHCs can also be useful for autoimmune diseases, as a protective MHC molecule proved a beneficial outcome in a diabetes mouse model [119]. However, in transplantation molecular chimerism approaches will require tolerization of all donor antigens which might be achieved through infectious tolerance [120, 121].

Concluding Remarks

In principle, the mixed chimerism approach emulates the mechanisms of self-tolerance, leading to a robust and long-lasting form of tolerance. Mixed chimerism and tolerance can be achieved not only in rodents but also in large animals and even in humans in highly selected cases. The use of the costimulation blockers anti-CD40L and CTLA4Ig has led to the establishment of very mild and effective protocols in rodents which still need to be fully translated into large animals. Advanced BMT protocols are a remaining major research goal which could lead to the full realization of the potential of the mixed chimerism approach in clinical transplantation.

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