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Hematopoietic chimerism and transplantation tolerance: a role for regulatory T cells

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The immunosuppressive regimens currently used in transplantation to prevent allograft destruction by the host's immune system have deleterious side effects and fail to control chronic rejection processes. Induction of donor-specific non-responsiveness (i.e., immunological tolerance) to transplants would solve these problems and would substantially ameliorate patients' quality of life. It has been proposed that bone marrow or hematopoietic stem-cell transplantation, and resulting (mixed) hematopoietic chimerism, lead to immunological tolerance to organs of the same donor. However, a careful analysis of the literature, performed here, clearly establishes that whereas hematopoietic chimerism substantially prolongs allograft survival, it does not systematically prevent chronic rejection. Moreover, the cytotoxic conditioning regimens used to achieve long-term persistence of chimerism are associated with severe side effects that appear incompatible with a routine use in the clinic. Several laboratories recently embarked on different studies to develop alternative strategies to overcome these issues. We discuss here recent advances obtained by combining regulatory T cell infusion with bone-marrow transplantation. In experimental settings, this attractive approach allows development of genuine immunological tolerance to donor tissues using clinically relevant conditioning regimens.

Keywords: transplantation tolerance, hematopoietic chimerism, regulatoryT lymphocytes, passive tolerance, active tolerance, chronic rejection

INTRODUCTION

The immunosuppressive regimens developed since the discovery of cyclosporine A showed ever increasing efficiency in reducing the severity and occurrence of acute rejection episodes. Recently, a systematic analysis of the literature firmly identified acute rejection events as a bad prognosis factor for long-term graft survival (Wu et al., 2009). Since immunosuppressive drugs efficiently control acute rejection, this explains how they significantly improved allograft survival over the past 40 years despite failing to have a direct impact on chronic rejection. The failure of current treatments to control chronic rejection processes combined with their deleterious side-effects urgently call for development of novel therapies against allograft rejection (Kahan, 2003; Meier-Kriesche and Kaplan, 2011).

During lymphocyte development in primary lymphoid organs, and due to the random rearrangement of genes encoding the antigen receptor, many autospecific T and B cell precursors arise. Since such cells would cause devastating autoimmune pathology, the natural mechanisms involved in the induction of self-tolerance play a crucial role in the survival of the species (Waldmann, 2010). Self-tolerance is defined as a state in which autoimmune attack is either prevented or deviated to non-detrimental responses (Walker and Abbas, 2002; Hogquist et al., 2005). It allows development of protective immunity and is therefore very specific. It appears very attractive to manipulate the mechanisms involved in self-tolerance in order to make them prevent allograft rejection. If successful, this would allow for indefinite survival of grafts.

TOLERANCE-INDUCTION BY CELLS OF HEMATOPOIETIC ORIGIN: PROOF OF PRINCIPLE

Several layers of complementary mechanisms ensure tolerance to self-antigens. Interestingly, considerable insight into these mechanisms was obtained through transplantation models and by manipulating the development of the immune system early in life, during embryogenesis or in neonates. Owen (1945) first observed that dizygotic twin cattle, that almost invariably develop placental anastomosis, "have identical blood types" as adults and he concluded "the critical interchange is of embryonal cells ancestral to the erythrocytes". Later, Billingham, Medawar, and colleagues showed that these chimeric twins "accepted" each other's skins when grafted later in life (Billingham et al., 1952). In a 1953 landmark paper, the same group showed that skin allograft survival could be substantially prolonged by injecting a single-cell suspension of donor tissues *in utero* or into neonates (Billingham et al., 1953). Such treatment led to varying levels of hematopoietic chimerism, which was later shown to be critically involved in allograft survival (Lubaroff and Silvers, 1973; Wood and Streilein, 1982; Wren et al., 1993; Alard et al., 1995).

In the two systems described above, lymphocytes developed in the presence of (and thus learned to be tolerant to) donor antigens. However, in adults the situation is more complicated as, in addition

to developing lymphocytes, preexisting donor-specific mature cells would also need to be rendered tolerant. To bypass this concern, several laboratories decided to deplete the pool of mature T cells (Main and Prehn, 1955; Trentin, 1956; Brocades Zaalberg et al., 1957). These groups first experimented this approach through the elimination of all hematopoietic cells. Recipient mice were lethally irradiated or treated with cytotoxic drugs, reconstituted with donor bone marrow, and grafted with skin. These strategies invariably led to substantially increased survival of homo- and xenografts. More recently, Ildstad and Sachs (1984) definitely validated these observations by inducing long-term survival of allogenic and xenogenic skin grafts using a comparable approach. Similar results were obtained in the rat for heart and skin grafts (Colson et al., 1995b; Orloff et al., 1995). Combined, these observations clearly demonstrated that hematopoietic chimerism leads to prolonged survival of allografts.

CELLS OF HEMATOPOIETIC ORIGIN INDUCE T CELL TOLERANCE BY INDUCTION OF APOPTOSIS AND ANERGY

To address the question of how cells of hematopoietic origin induce tolerance, researchers needed a means to identify T cell precursors specific for a given antigen. Kappler et al. (1987b) showed that practically all T cells expressing the variable TCR segment Vβ17a, representing up to 15% of the T cell repertoire in certain mouse-strains, recognized the MHC class II molecule I-E. Given that they had developed an antibody against this Vβ domain, the mechanisms involved in T cell tolerance to I-E could now be analyzed. It was shown in I-E expressing mice that $V\beta17a^+$ T cell precursors were eliminated at an immature stage during thymic development (Kappler et al., 1987a). The following year, the same authors further characterized this mechanism and showed that clonal deletion requires the expression of the negatively selecting ligand by thymic cells of hematopoietic origin (Kappler et al., 1988). Many other illustrations of clonal deletion of T cells expressing given TCR Vβ segments by endogenous or exogenous superantigens have since been published (MacDonald et al., 1988b; Luther and Acha-Orbea, 1997).

Could thymic elimination of reactive clones also be involved in the neonatal induction of tolerance to alloantigens? This question was addressed by MacDonald et al. (1988a) who showed that the transfer of superantigen-expressing spleen cells into neonates lead to the intrathymic deletion of superantigen-reactive T cells. Similar conclusions were rapidly drawn by others (Streilein, 1991). Later, intrathymic deletion of donor-specific precursors was also reported in adult mixed hematopoietic chimeras using TCR transgenic cells as a tracer population (Manilay et al., 1998).

Thus, thymic cells of hematopoietic origin are involved in deletion of autospecific T cell-precursors and mixed hematopoietic chimerism leads to deletion of alloreactive cells. Using thymic organ cultures to analyze the involvement of different stromal cells, it was shown that dendritic cells (DC) are critically involved in this process (Matzinger and Guerder, 1989; Jenkinson et al., 1992; Anderson et al., 1998). This was further confirmed using a transgenic mouse model in which TCR ligand-expression was essentially restricted to DC using the CD11c promoter (Brocker et al., 1997). Among the thymic DC subtypes, both $Sirp\alpha^+$ and $Sirp\alpha^-$ conventional DC have been implicated in central tolerance-induction by deletion (Wu and Shortman, 2005; Baba et al., 2009). However, other populations of hematopoietic cells may also play a role in this process, including $CD4^+CD8^+$ thymocytes, thymic macrophages and B cells (Pircher et al., 1992, 1993; Kleindienst et al., 2000), and circulating peripheral DC (Bonasio et al., 2006).

Combined, the data discussed thus far showed that DC, and potentially other cells of hematopoietic origin, contribute to tolerance induction by elimination of developing thymocytes. Using conditioning regimens that totally deplete host T cells before bone-marrow transplantation, it was proposed that this mechanism was necessary and sufficient for maintenance of tolerance and that peripheral mechanisms do not contribute to this process (Khan et al., 1996). However, other mechanisms could be involved when less aggressive regimens are used. In normal mice, it has been proposed that deletion of autospecific T cells that escaped thymic selection could also occur in peripheral lymphoid organs and could be involved in the maintenance of self-tolerance (Russell, 1995). To test if similar mechanisms were involved in induction of tolerance to alloantigens following bone-marrow transplantation, Wekerle et al. (1998) tracked T cells specific for a given superantigen in thymectomized mice transplanted with allogeneic bone-marrow under cover of CTLA4-Ig and antibody to CD154 ("co-stimulatory blockade"). They observed a rapid deletion of donor-specific host T cells from the peripheral $CD4^+$ compartment. This observation was later confirmed using a TCR transgenic mouse model (Kurtz et al., 2004) and it was further shown that peripheral deletion relies essentially on two types of mechanisms: activation-induced cell death, a Fas-dependent process that can be promoted by IL-2 and that leads to apoptosis of activated T cells when restimulated with high doses of antigen (Lenardo, 1991; Ju et al., 1995; Russell, 1995); and passive cell death or death "by neglect," a Fas-independent process that can be prevented by overexpression of Bcl-2 or Bcl-x_L and that leads to T cell apoptosis when stimulated with low dose of antigen and/or in the absence of co-stimulatory signals (Boise et al., 1995; Van Parijs et al., 1996; Wekerle et al., 2001). It was also shown that in addition to DC, other populations of hematopoietic cells such as B cells have the capacity to delete allospecific precursors from the peripheral T cell compartment (Fehr et al., 2008a,b). Finally, hematopoietic cells can also cause T cell tolerance by inducing a non-responsive state called clonal anergy (Rammensee et al., 1989; Tomita et al., 1994; Hawiger et al., 2001). Combined, the cited reports clearly show that cells of hematopoietic origin can induce "passive" tolerance (i.e., apoptosis and anergy).

CAN HEMATOPOIETIC CELLS INDUCE ACTIVE REGULATORY MECHANISMS?

T lymphocytes from chimeric mice in which radioresistant cells express MHC molecules but hematopoietic cells do not, vigorously react to self-antigens *in vitro* (van Meerwijk and MacDonald, 1999) and in some well-defined experimental conditions *in vivo* (Hudrisier et al., 2003). Combined with the observations listed above, this shows that hematopoietic cells play a central role in the deletion and/or functional inactivation of self-reactive precursors. However, passive mechanisms are not sufficient to fully control self-reactivity. Individuals carrying a mutated FOXP3 gene

develop the rapidly lethal autoimmune syndrome immuno dysfunction, polyendocrinopathy, enteropathy, X-linked (IPEX). This is explained by the fact that Foxp3 is required for the programming of a population of regulatory $CD4^+$ T lymphocytes (Treg) that inhibit and/or divert innate and adaptive immune responses, mainly those directed against self-antigens. Genuine tolerance to self, and consequently probably to non-self-antigens, therefore requires Treg (Fontenot and Rudensky, 2005; Sakaguchi et al., 2006; Shevach et al., 2006).

Given their central (though not exclusive) role in the control of autoimmune responses, it was probably not a very surprising finding that the Treg repertoire is strongly enriched in autospecific cells (Romagnoli et al., 2002; Hsieh et al., 2004). Development of self-antigen-specific Treg in the thymus depends on interaction of developing precursors with MHC/self-peptide ligands expressed by thymic epithelial cells (Bensinger et al., 2001; Romagnoli et al., 2005; Ribot et al., 2006, 2007; Aschenbrenner et al., 2007). Moreover, the transplantation of allogeneic thymic anlagen (i.e., the initial cluster of pluripotent embryonic cells from which the thymus will develop) into mice induces Treg-mediated tolerance to subsequent skin grafts of the same donor, again showing that thymic epithelial cells can select antigen-specific Treg (Le Douarin et al., 1996). However, the capacity to trigger Treg differentiation in the thymus is not a property restricted to epithelial cells as it has been reported that thymic DC are also involved in this process (Watanabe et al., 2005; Proietto et al., 2008; Wirnsberger et al., 2009). Moreover, induction of Treg differentiation by DC has also been reported in peripheral lymphoid organs under certain carefully controlled experimental conditions (reviewed by Romagnoli et al., 2008). It may therefore be hypothesized that hematopoietic chimerism can lead to differentiation and/or expansion of Treg specific for donor antigens and thus to the development of dominant tolerance. However, in experimental systems where the conditioning regimen used to induce mixed hematopoietic chimerism involved the total deletion of host T cells, transfer of syngeneic naïve $CD4^+$ T cells into the recipient leads to bone-marrow rejection and to the concomitant loss of donor-specific transplantation tolerance (Wren et al., 1993). This result clearly demonstrated that hematopoietic chimerism *per se* is insufficient for induction of dominant tolerance to alloantigens. Given the non-redundant role of Treg in maintenance of self-tolerance, hematopoietic chimerism therefore appears unlikely to be sufficient for permanent survival of allografts.

Active tolerance mechanisms are not limited to those mediated by Treg. "Immune deviation" from a harmful Th1 to a less detrimental Th2 response has also been shown to play a role in control of immune responses (Rocken, 1996; Walker and Abbas, 2002). Alloreactive Th2 cytokine producing T cells have been observed after neonatal injection of lymphohematopoietic cells (Streilein, 1991) and immune deviation by IL-4 was shown to play a critical role in tolerance to alloantigens (Donckier et al., 1995). The hematopoietic (micro-)chimerism induced in this experimental model, which is critically required for the allograft tolerance (Lubaroff and Silvers, 1973), therefore appears to induce an active regulatory mechanism. However, this mechanism appears insufficient for induction of full immunological tolerance to alloantigens (see below). Stem-cell transplantation under cover of cyclophosphamide can induce tolerance to MHC-matched skin allografts in mice. It was shown that NKT cells, another immunoregulatory population, play a central role in this phenomenon (Iwai et al., 2006). Regulatory T cell populations other than $Foxp3^+$ cells may therefore be induced by hematopoietic chimerism but their activity appears insufficient for prevention of chronic allograft rejection.

DOES HEMATOPOIETIC CHIMERISM INDUCE GENUINE TOLERANCE TO ALLOGRAFTS?

As discussed above, hematopoietic chimerism is thought to be sufficient for induction of tolerance to allografts. The mechanisms involved include central and peripheral clonal deletion and anergy. After the initial reports on allograft tolerance in dizygotic cattle twins that had shared blood circulation during embryonic life, it became clear that most skin grafts were rejected in the long term (Stone et al., 1965, 1971). Second skin grafts from the same donor survived less long than the first grafts, but substantially longer than third party organs, showing that the tolerance mechanism had not waned away.

Also neonatal injection of allogeneic splenocytes, leading to hematopoietic microchimerism, is thought to induce tolerance to subsequent skin grafts. However, this procedure appeared to work only in a limited number of donor/host combinations. Importantly, most of the reported donor/host combinations concerned MHC congenic strains (i.e., expressing distinct MHC haplotypes on an identical genetic background) and chronic rejection was not systematically studied (Streilein and Klein, 1977). Moreover, even when acceptance of skin allografts was achieved, it did not correlate with immunological unresponsiveness (Streilein, 1991; Donckier et al., 1995).

In adult mice, lymphoablation was achieved using lethal total body irradiation or depleting antibodies to, e.g., CD4 and CD8. Myeloablation, required for induction of hematopoietic chimerism, was induced by the irradiation or administration of myeloablative drugs. Subsequent transplantation of allogenic or xenogenic bone marrow led to persistent chimerism (reviewed in Wekerle and Sykes, 1999; Cosimi and Sachs, 2004). Skin grafts from the bone-marrow donors could survive for prolonged periods, but success-rates were often well below 100% and chronic rejection was not studied. In some host/donor combinations, hematopoietic chimerism failed to prevent acute rejection of skin allografts (Boyse et al., 1970), and T cell reactivity to skin-specific antigens not expressed by hematopoietic cells was responsible for this observation (Scheid et al., 1972; Boyse et al., 1973). Also the survival of cardiac allografts was favored by hematopoietic chimerism (Steinmuller and Lofgreen, 1974). However, histological analysis of surviving hearts revealed frequent chronic rejection (Russell et al., 2001). Also in the rat, myelo- and lymphoablation followed by induction of hematopoietic chimerism was reported to prolong survival of skin, heart, and renal allografts (Slavin et al., 1978; Colson et al., 1995b; Orloff et al., 1995; Blom et al., 1996). However, chronic rejection was seldom adequately studied. It appears therefore that immunological tolerance to allografts is not systematically achieved by induction of hematopoietic chimerism in lymphoablated recipients.

Hematopoietic chimerism can also be induced with non-lymphoablative regimens. Surprisingly, under certain of these conditions, allografts appear to do better than when lymphoablative conditioning is used (**Table 1**). Blocking the T cell co-stimulatory molecule CD28 with an Ig-fusion protein of its CTLA4-ligand (CTLA4-Ig), combined with inhibition of the CD40/CD40L (i.e., CD154) pathway involved in activation of antigen presenting and B cells, substantially prolongs heart and skin allograft survival (Larsen et al., 1996). However, histological signs of chronic rejection of cardiac allografts was observed in all thus conditioned mice (Shirasugi et al., 2002). When co-stimulatory blockade was combined with induction of hematopoietic chimerism, heart, skin, and also intestine allografts survived substantially longer and no chronic rejection was observed (Wekerle et al., 1998, 2000; Adams et al., 2001; Shirasugi et al., 2002; Guo et al., 2003). Transplantation tolerance in such settings was dominant and depended on Treg, at least during early stages (Bigenzahn et al., 2005; Domenig et al., 2005).

Combined, these data indicate that hematopoietic chimerism *per se* appears insufficient for induction of transplantation tolerance. However, when combined with conditioning regimens that allow for development of dominant tolerance, prevention of chronic rejection can be achieved.

INDUCTION OF HEMATOPOIETIC CHIMERISM: TOWARD THE CLINIC

Given the very encouraging results obtained with mixed hematopoietic chimerism in rodents, several groups have attempted to induce hematopoietic chimerism and transplantation tolerance in large animal models (Wekerle and Sykes, 1999; Cosimi and Sachs, 2004; Horner et al., 2006). Experimental protocols are necessarily more complex than in rodents since adult recipients were used and high dose whole body irradiation is associated with a too high level of morbidity. A combination of immunosuppressive drugs and antibodies, as well as lower levels of irradiation or irradiation limited to lymphoid organs, was therefore used as conditioning regimen (**Table 2**). In miniature swine, a preconditioning of T cell depletion, low dose total body irradiation, thymic irradiation, and splenectomy, followed by bone-marrow and skin transplantation, led to persistent hematopoietic chimerism in five out of six animals. Four of these animals were transplanted with donor skin. Half of these animals appeared to accept, but the other half rejected the skin allografts (Fuchimoto et al., 2000). Also using a milder conditioning regimen, persistent chimerism was obtained in miniature swine and one out of two skin grafts appeared to be permanently accepted (Fuchimoto et al., 2000). When the latter protocol was used for kidney transplantation, four out of four allografts survived more than 100 days (Fuchimoto et al., 2000, 2001). Therefore, as observed in rodents, persistent hematopoietic chimerism led to an incomplete level of allograft tolerance that appeared efficient for protection of poorly immunogenic organs such as kidney but fails to prevent rejection of highly immunogenic skin allografts.

In Cynomolgus monkeys, a preconditioning regimen was used that consisted of T cell depletion, low dose total body irradiation, thymic irradiation, and splenectomy, followed by bone-marrow and kidney transplantation (Kawai et al., 1995, 2002, 2004; Kimikawa et al., 1997b). Only transient hematopoietic chimerism was observed, but nevertheless 8 out of 15 grafts did not show signs of rejection (**Table 2**). An acute cellular rejection process led to the loss of the other grafts (Kimikawa et al., 1997a; Kawai et al., 1999). A similar preconditioning regimen was used for monkeys that received a cardiac allograft. Three out of five animals developed transient chimerism, but all five hearts were eventually lost by a rejection-process characterized by cellular infiltrates (Kawai et al., 2002). The observation that kidney allografts were more likely to be accepted than heart allografts confirmed earlier data on transplantation in miniature swine that, interestingly, also showed that kidneys can play an important role in tolerance to heart allografts (Madsen et al., 1998; Mezrich et al., 2003a,b). Taken together these data highlight the difficulty to obtain an efficient and persistent engraftment of hematopoietic stem cells in large animal models. When only transient, hematopoietic chimerism induces tolerance mechanisms that are probably different from and less efficient than those induced in hosts with long-term persistence of hematopoietic donor cells.

INDUCTION OF HEMATOPOIETIC CHIMERISM: IN THE CLINIC

Based on the promising results in monkeys, induction of hematopoietic chimerism for prevention of allograft rejection has also been performed in humans (**Table 3**). Infusion of donor bone-marrow showed some beneficial effect in renal allograft recipients (Monaco, 2003). Interestingly, in an early report in which large numbers of patients were described, infusion of donor bone marrow, leading to transient chimerism, inhibited acute but not chronic rejection (Barber et al., 1991; McDaniel et al., 1994). One of the first reported cases of long-term allograft survival achieved by induction of hematopoietic chimerism concerned a woman with end-stage renal disease secondary to multiple myeloma (Spitzer et al., 1999). The patient received an immunosuppressive but non-myeloablative conditioning regimen. HLA-matched bone marrow and kidney from the patient's sister were transplanted and the immunosuppressive drug cyclosporine A administered for 73 days. Whereas the hematopoietic chimerism disappeared after discontinuation of immunosuppression, the kidney remained functional for at least another 7 years (Fudaba et al., 2006). In total, six multiple myeloma patients receiving this treatment have been reported and all maintained renal function after discontinuation of immunosuppression for 2–7 years (Spitzer et al., 1999; Buhler et al., 2002; Fudaba et al., 2006). Stem-cell transfusion was also shown to have a beneficial effect in liver transplantation (Donckier et al., 2004). Another example concerned a patient with end-stage renal disease who received an HLA-matched kidney graft. The conditioning regimen, which included total lymphoid irradiation, immunosuppression, and a graft of mobilized $CD34^+$ stem cells, led to persistent hematopoietic chimerism. At the time of publication, the renal graft had remained functional for 34 months (Scandling et al., 2008).

Induction of hematopoietic chimerism followed by kidney transplantation was also performed with HLA single haplotype mismatched grafts (Kawai et al., 2008), a clinically important setting. Five patients with end-stage renal disease received an immunosuppressive but non-myeloablative preparative regimen

Table 1 | Combined bone-marrow and organ transplantation in the mouse: non-myelo- and lymphoablative procedures.

BM/SC graft => host	Organ/tissue graft	Conditioning[a]	Hematopoietic chimerism[b]	Allograft survival	Reference
ANTIBODIES					
No BM, C3H host	BALB/c heart	αCD154 and CTLA4-Ig		7/7 > day 70 (no chronic rejection at d63)	Larsen et al. (1996)
	BALB/c skin			15/15 > day 50 (no chronic rejection at day 50)	
B10.A => B6	B10.A skin	αCD154 and CTLA4-Ig	Persistent	8/8 at day 145	Wekerle et al. (2000)
			Transient	1/5 at day 145	
B10.A => B6	B10.A skin	αCD154 and CTLA4-Ig, sublethal TBI	Persistent	7/9 at day 160	Wekerle et al. (1998)
BALB/c => B6	BALB/c skin	αCD154 and CTLA4-Ig, BUS	Persistent	7/7 at day 250	Adams et al. (2001)
BALB/c => B6	BALB/c heart	αCD154 and CTLA4-Ig	Undetectable	8/9 at day 180, chronic rejection at day 300 in 8/8 hosts	Shirasugi et al. (2002)
		αCD154 and CTLA4-Ig, BUS	Persistent	5/5 at day 180, no chronic rejection at day 300	
BALB/c => B6	BALB/c intestine	αCD154 and CTLA4-Ig, BUS	Persistent	5/7 at day 92	Guo et al. (2003)
BALB/c => B6	BALB/c skin	αCD154 and CTLA4-Ig, Rapa, Treg	Persistent?	7/7 at day 170	Pilat et al. (2010)
BALB/c => B6	BALB/c skin	low dose TBI, αCD154	Persistent	0/4 at day 60	Luo et al. (2007)
		B6.C-H-2^d skin		4/4 > day 180	
BALB/c => B6	BALB/c skin	αCD154 andαLFA-1	Persistent	4/7 > day 270	Metzler et al. (2004)
		αCD154 and Rapa		4/7 > day 226	
		αCD154 and BUS and various		22/24: chronic rejection	
	BALB/c heart			4/24: mild chronic rejection	
	BALB/c skin	αLFA-1 and Rapa		0/6 day 117	
BALB/c => B6	BALB/c pancreatic islets	Rapa, low dose TBI	Persistent	6/6 > day 100	Luo et al. (2005)
B10.BR => CBA	B10.BR skin	αCD4 andαCD8(nd)	Persistent	8/8 at day 240	Qin et al. (1990)
B10 DST => C3H	C3H heart	αCD4(nd)	N/D	7/7 at day 100	Pearson et al. (1992)
DRUGS					
BALB/c => B6	BALB/c skin	SC, CP, αThy1	Persistent	10/15 at day 159	Mayumi and Good (1989)
BALB/c => C3H	BALB/c skin			7/8 at day 165	
B6 => C3H	B6 skin			4/9 at day 185	
C3H => B6	C3H skin			0/19 (chronic rejection)	
B10.BR or BALB/c => B10	B10.BR or BALB/c skin	Sublethal TBI, CP	Persistent	9/10 at day 60	Colson et al. (1995a)

(Continued)

Table 1 | Continued

BM/SC graft => host	Organ/tissue graft	Conditioning[a]	Hematopoietic chimerism[b]	Allograft survival	Reference
BALB/c => B10	BALB/c heart			6/6 > day 420	
B10.A(5R) => B10	B10.A(5R) skin	SC, CP	Transient	4/10 at day 200	Tomita et al. (1990b)
C3H => AKR	C3H skin	SC, CP	Transient	5/8 at day 100=>	Tomita et al. (1990a)
B10.BR => AKR	B10.BR skin			5/6 at day 100	
AKR => C3H	AKR skin			5/8 at day 100	
B10.BR => C3H	B10.BR skin			4/8 at day 100	
B10.D2 => BALB/c	B10.D2 skin			5/6 at day 100	
B10 => AKR	B10 skin			0/6 at day 12	
B6 => C3H	B6 skin			0/6 at day 13=>	
B6 => AKR	B6 skin			0/6 at day 12	
AKR SC => C3H	AKR skin	CP	Persistent	9/10, day 120	Eto et al. (1990)
AKR SC => C3H	B10.BR skin			0/5 at day 13	
B10.BR SC => C3H	B10.BR skin			10/10 at day 120	
B10.BR SC => C3H	AKR skin			0/5 at day 14	
DBA/2 => BALB/c	DBA/2 skin			8/10 at day 80	
DBA/2 => BALB	B10.D2 skin			0/5 at day 13	
DBA/2 SC => BALB/c wt	DBA/2 skin	CP	Persistent	6/6 at day 100	Iwai et al. (2006)
DBA/2 SC => Vα14 NKT KO			0/6 at day 50		
B10.A => B6	B10.A skin	αCD4(d) and αCD8(d), CP, TI, TBI	Persistent	6/6 at day 100	Mapara et al. (2001)

[a] *α, antibody to; BUS, busulfan; CP, cyclophosphamide; nd, non-depleting; SC, CD34+ stem cells; TBI, total body irradiation; TI, thymic irradiation; Treg, CD4+CD25+ regulatory T cells.*

[b] *N/D, not detected.*

and a combined bone-marrow/renal allograft. All developed a transient multi-lineage chimerism. Whereas one patient lost the allograft by acute humoral rejection 10 days post transplantation, four out of the five patients, treated with a combination of immunosuppressive drugs for up to 14 months, maintained renal function for up to 1400 days thereafter. Renal biopsies showed normal tissue for three of these patients, with some minor signs of chronic rejection for the fourth. *In vitro* studies suggested specific absence of T cell-responses to directly presented alloantigens. However, two out of the four patients later developed alloantibodies, one showing complement depositions in the graft (Porcheray et al., 2009). It needs to be emphasized that T cell reactivity to indirectly presented donor antigens is required for alloantibody-production by host B-lymphocytes. The apparent absence of T cell response to directly presented alloantigens and the production of alloantibodies are therefore not in contradiction. Combined, these studies suggested that long-term acceptance of (though not genuine immunological tolerance to) kidney allografts can be obtained by a therapy including induction of transient hematopoietic chimerism and therefore represented a major step forward in transplantation medicine.

Less promising results were obtained in a study in which HLA-mismatched pancreatic islets were transplanted into type I diabetes patients (Mineo et al., 2008). The conditioning regimen used was very mild but nevertheless led to transient hematopoietic chimerism. However, all four patients that initially adhered to immunosuppressive therapy lost graft-function rapidly after drug weaning.

A ROLE FOR REGULATORY T CELLS IN HEMATOPOIETIC CHIMERISM-ASSOCIATED TOLERANCE?

At this point, one might wonder if more work is warranted to obtain tolerance to (and therefore permanent acceptance of) organ allografts. When considering the very promising results obtained with kidney allografts in humans, one has to keep in mind that this organ might represent a special case. The human islet study failed, and the monkey and swine studies gave substantially less satisfying results with skin and heart allografts than with renal transplants. Moreover, in miniature swine it was shown that kidney allografts induced tolerance to heart allografts (Madsen et al., 1998). The thymus and Treg may play a role in this phenomenon (Yamada et al., 1999; Mezrich et al., 2003a).

To induce genuine immunological tolerance to donor tissues, hematopoietic chimerism needs to persist in the long term to continuously induce tolerance of newly developing lymphocytes in primary lymphoid organs. Indeed, if hematopoietic chimerism is only transient, mature allospecific lymphocytes will develop and, in the absence of dominant tolerance mechanisms, will eventually destroy the graft. Long-term hematopoietic chimerism has

Table 2 | Combined bone-marrow and organ transplantation in large animals and non-human primates.

Species	Organ	Conditioning[a]	Immuno-suppression	Hematopoietic chimerism[c]	Allograft survival[d]	Reference
"Cattle"	Skin	Co-twins	None	Persistent	30% at 2 years	Stone et al. (1971)
	Body skin	Co-twins	None	Persistent	0/10 at day 68	Emery and McCullagh (1980)
	Auricular skin				5/12 > day 60	
Dog	Heart	TLI, donor BM	±ATG, ±MTX, ±CsA	N/A	0/29 at day 329	Strober et al. (1984)
	Kidney	ALS, donor BM	None	N/A	>14[b], >17[b], >38[b], >78[b] d, ≤ 4/24	Caridis et al. (1973)
	Kidney	ALS, donor BM	None	N/A	0/13 at day 300	Hartner et al. (1986)
Miniature swine	Kidney	Lethal TBI ± CP	None	Persistent	>200, >200, >200, >200, 75	Guzzetta et al. (1991)
	Skin	αCD3-DT; TBI; TI; donor BM	30 days CsA	Persistent	45, 50, >50, >235 days	Huang et al. (2000)
	Skin	αCD3-DT; TI; donor PBSC	30 days CsA	Persistent	>300, 45 days	Fuchimoto et al. (2000)
	Kidney				>120, >180, >100 days, "long term"	Fuchimoto et al. (2000, 2001)
Rhesus monkey	Kidney	ATG, donor BM	None	N/A	20% at day 240	Thomas et al. (1983, 1987)
Cynomolgus monkey	Kidney	ATG; TBI; TI; splenectomy; donor BM	4 weeks CsA	Transient	>3478, >2569, >834[e], >771[e], >405[e], 260, >198[e], >196[e], >137[e], 72, 44, 40, 37, 40, 37 days	Kawai et al. (1995), Kimikawa et al. (1997b), Kawai et al. (2002, 2004)
				N/D	**14, 175** days	
	Heart			Transient	**509, 428, 138** days	
				N/D	**56, 43** days	
	Kidney	ATG; TBI; TI; donor BM	4 weeks CsA	Transient	**117, 95, 43**	
	Kidney	ATG; TBI; TI; donor BM; aCD154	4 weeks CsA	Transient	>1710, >1167, 755, 206, 837, 401, 373, 58	Kawai et al. (2004)

[a] *α, antibody to; αCD3-DT, anti-CD3 antibody coupled to diphtheria toxin; ALS, anti-lymphocyte serum; ATG, antithymocyte globulin; BM, bone marrow; CP, cyclophosphamide; PBSC, peripheral blood stem cells; TBI, total body irradiation; TI, thymic irradiation; TLI, total lymphoid irradiation.*

[b] *Renal allografts that were not rejected but were lost for other reasons.*

[c] *N/A, not analyzed; N/D, not detected.*

[d] *Animals that rejected their allografts are indicated in bold.*

[e] *Renal allografts that were not rejected but were lost for other reasons.*

been achieved with very aggressive conditioning regimens inducing total host T cell depletion. However, the level of toxicity and the severe immunosuppression associated with this type of treatment do not allow their use in the clinic. Alternative conditioning regimens have been envisaged to avoid rejection of donor bone marrow while allowing for survival of part of the host T cells. They included the injection of non-depleting antibodies to block T cell co-stimulatory pathways and the injection of antibodies specific for some T cell markers upregulated upon activation. As described throughout this review, these methods gave very promising results in rodents. However, induction of a permanent chimerism was far more difficult to achieve in large animals. This observation might be largely responsible for the less satisfying results obtained with heart and skin allograft in miniature swine and primates. Moreover, antibody-based therapies can also generate unpredicted side effects that complicate translation into the clinic. For example, the use of anti-CD154 antibody in a non-human primate renal allograft model led to severe thromboembolic complications (Kawai et al., 2000; Koyama et al., 2004) due to CD154 expression on activated platelets and to CD40 expression on the vascular endothelium (Henn et al., 1998; Slupsky et al., 1998). The use of anti-CD154 has also been associated with impaired humoral immunity against influenza in a heart allograft model (Crowe et al., 2003). Antibodies targeting other T cell surface markers also present limitations as targeted molecules can be expressed by other populations, e.g., CD25 on Treg. While inhibiting the allogeneic

Table 3 | Combined bone-marrow and organ transplantation in humans.

Organ	Conditioning[a]	Immuno-suppression[a]	Hematopoietic chimerism[c]	Allograft survival[b]	Reference
Kidney (HLA-matched)	ALG; CP ALG; CP; donor BM	Maintenance CsA; azathioprine; prednisone	N/A	**13**/54 rejected **3**/57 rejected	Barber et al. (1991)
Kidney	Donor BM	2 weeks ALG + maintenance	Persistent microchimerism Transient/ND	21/23 at 1 year (but "chronic rejection") 1/7 at 1 year	McDaniel et al. (1994)
Kidney (haplocompatible)	TBI, ARA-C, CP, ATG (splenectomy)	10 months CsA, Pred	Persistent	>15 months	Sorof et al. (1995)
Kidney (related donors)	Not specified, prior BM transplantation to treat hematological disorders	None	Persistent	>15, >30, >3 months	Butcher et al. (1999)
Kidney (HLA-matched)	CP; ATG; TI; donor BM	2 months CsA	Transient/persistent	>7.3, >5.3, >4.3, >3.5, >2.8, >2 years	Spitzer et al. (1999), Buhler et al. (2002), Fudaba et al. (2006)
Kidney (HLA-matched)	ATG; TLI; donor PBSC	6 months CsA	Persistent	>34 months	Scandling et al. (2008)
Kidney (HLA-mismatched)	CP; αCD2; TI; donor BM	≤14 months CsA/Rapa	Transient	>1932, >1666, **10 days**, >1050, >707 days; donor-specific antibodies	Kawai et al. (2008), Porcheray et al. (2009)
Pancreatic islet (HLA-mismatched)	High dose HSC	1 year "Edmonton" (FK506, Rapa)	Transient	**451, 480, 178, 471, 158, 510 days**	Mineo et al. (2008)
Liver	ATG; CP; donor HSC	28–90 days FK506, Rapa	Transient/ND	>240, >290	Donckier et al. (2004)

[a] *α, antibody to; ALG, anti-lymphocyte globulin; ARA-C, arabinofuranosyl cytidine; ATG, antithymocyte globulin; BM, bone marrow; CsA, cyclosporin A; CP, cyclophosphamide; HSC, CD34+ hematopoietic stem cells; PBSC, peripheral blood stem cells; Pred, prednisone; Rapa, Rapamycin; TBI, total body irradiation; TI, thymic irradiation; TLI, total lymphoid irradiation.*

[b] *Patients that rejected their allografts are indicated in bold.*

[c] *N/A, not analyzed; N/D, not detected.*

response, such approaches could therefore prevent the establishment of regulatory mechanisms important for graft survival. They also non-specifically inhibit T cell-dependent immunity, including protective responses against pathogens. Finally, most of the protocols used in large animals or currently tested in the clinic require strong initial immunosuppressive treatments that induce major qualitative and quantitative modifications of the immune system that last for years. In conclusion, while highly promising, these strategies still need to be optimized before going into the clinic.

To overcome the issues listed above, several laboratories embarked on studies to evaluate the potential of Treg to promote allograft protection (reviewed by Li and Turka, 2010). The capacity of naturally occurring Treg to control allogeneic responses was already highlighted by Sakaguchi et al. (1995) landmark paper. Using *in vivo* activated polyclonal Treg with irrelevant specificity, Karim et al. (2005) first induced tolerance to allogeneic skin graft in lymphopenic Rag-deficient hosts reconstituted with naïve CD45RB^{high} CD4^{+}T cells (Karim et al., 2005). Similar results were obtained in another lymphopenic system with *in vitro* expanded donor-specific Treg (Golshayan et al., 2007). Interestingly, this approach also significantly prolonged skin allograft survival in unmanipulated wild-type hosts. In a transplantation-model across minor histocompatibility antigens, another group protected male skin graft from rejection by syngeneic female hosts using Foxp3-transduced male antigen-specific TCR transgenic CD4^{+} T cells (Chai et al., 2005). In another study, a genetically manipulated Treg population with direct and indirect alloantigen-specificity substantially prolonged skin allograft survival and delayed chronic rejection of heart allografts when co-injected with anti-CD8 antibody (Tsang et al., 2008). More recently, prevention of transplant arteriosclerosis and long-term survival of skin allograft were achieved with *in vitro* expanded naturally occurring CD127^{low}CD25^{+}CD4^{+} human Treg in a chimeric humanized mouse system (Issa and Wood, 2010; Nadig et al., 2010). Combined, these reports demonstrated the capacity of Treg to delay rejection processes.

Based on the large body of literature on transplantation tolerance through hematopoietic chimerism and on the immunosuppressive potential of Treg, several laboratories decided to combine Treg infusion with bone-marrow transplantation (**Figure 1**). This method is expected to allow the establishment of complementary tolerance mechanisms, thus mimicking the complex network of checkpoints and regulatory systems naturally involved in maintenance of self-tolerance (**Figure 2**). Moreover, in addition to their general modulatory effects on the reactivity of the immune system, Treg expressing the transcription factor Foxp3 have the capacity to establish an immune-privileged niche for allogeneic hematopoietic stem cells after transplantation into non-irradiated recipients

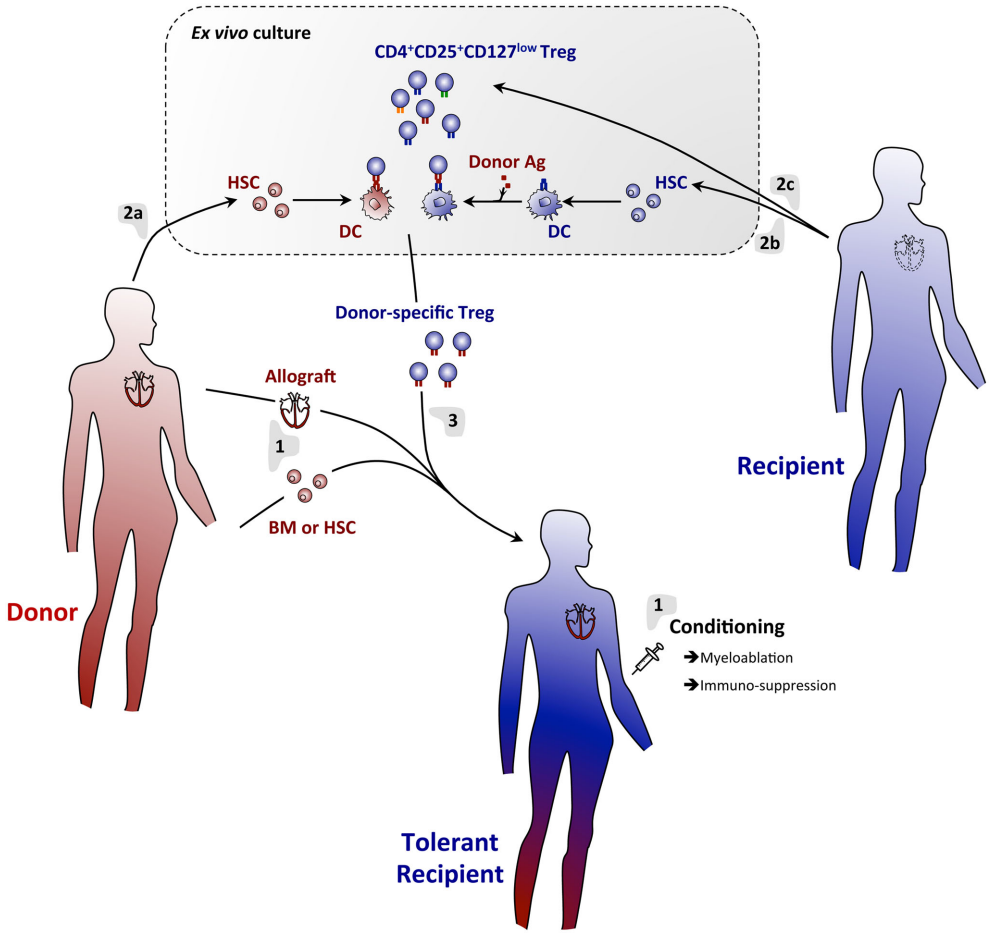

FIGURE 1 | A regulatory T cell/hematopoietic chimerism-based protocol for induction of transplantation tolerance. (1) The allograft (e.g., heart) will be transplanted with concomitant infusion of donor BM or HSC into conditioned hosts. Rejection of the grafts will temporarily be prevented using an immunosuppressive regimen. (2) Donor (a) and host (b) BM will be cultured *in vitro* under conditions allowing for differentiation of DC. Host DC will be pulsed with donor antigen to assure indirect presentation of these antigens. Thus generated DC will then be co-cultured with host-derived Treg (c), allowing for expansion of Treg specific for directly and indirectly presented donor antigens. (3) Thus generated donor-antigen-specific Treg will then be infused into the host. Immunosuppression may temporarily be continued using drugs that do not affect Treg (e.g., Rapamycin). Using this protocol, full tolerance to donor-tissue will be achieved and chronic rejection effectively prevented.

(Fujisaki et al., 2011). The co-injection of Treg with allogeneic bone-marrow should therefore promote its engraftment. Administration of donor-specific Treg prevented rejection of bone-marrow allografts in preconditioned mice (Joffre et al., 2004; Joffre and van Meerwijk, 2006). Promising results were later obtained using polyclonal donor Treg (Hanash and Levy, 2005). However, similar protocols failed to substantially prolong survival of skin and heart allografts (Joffre et al., 2008; Tsang et al., 2008; Pilat et al., 2010). In contrast, when combined with bone-marrow transplantation, administration of a single dose of Treg fully prevented rejection of skin and heart (Joffre et al., 2008). Whereas Treg specific for directly presented donor-antigens allowed for survival of bone-marrow allografts, they failed to prevent chronic rejection of skin and heart. In contrast, Treg specific for indirectly presented donor-antigens fully prevented chronic heart and skin allograft rejection (Joffre et al., 2008). These results firmly demonstrated the clinical potential of Treg infusion in induction of bone-marrow chimerism and in the subsequent prevention of acute and chronic allograft rejection.

TREG AND HEMATOPOIETIC CHIMERISM-BASED STRATEGIES: SOME LIMITATIONS TO OVERCOME

The data described above constitute a proof of principle that combining Treg and bone-marrow infusion can lead to subsequent tolerance to allogeneic tissues, even in very stringent donor/host combinations and for highly immunogenic tissues such as the skin. However, 5 Gy total body irradiation was required in that protocol (Joffre et al., 2008). This dose appears not suitable for clinical use as it is associated with severe temporary leukopenia. Interestingly, the group of Wekerle recently induced hematopoietic chimerism in mice using a comparable approach, but without or with very limited cytoreductive conditioning (Pilat et al., 2010, 2011). Treatment with costimulation-blocking agents, a short course of rapamycin, and injection of polyclonal Treg allowed for

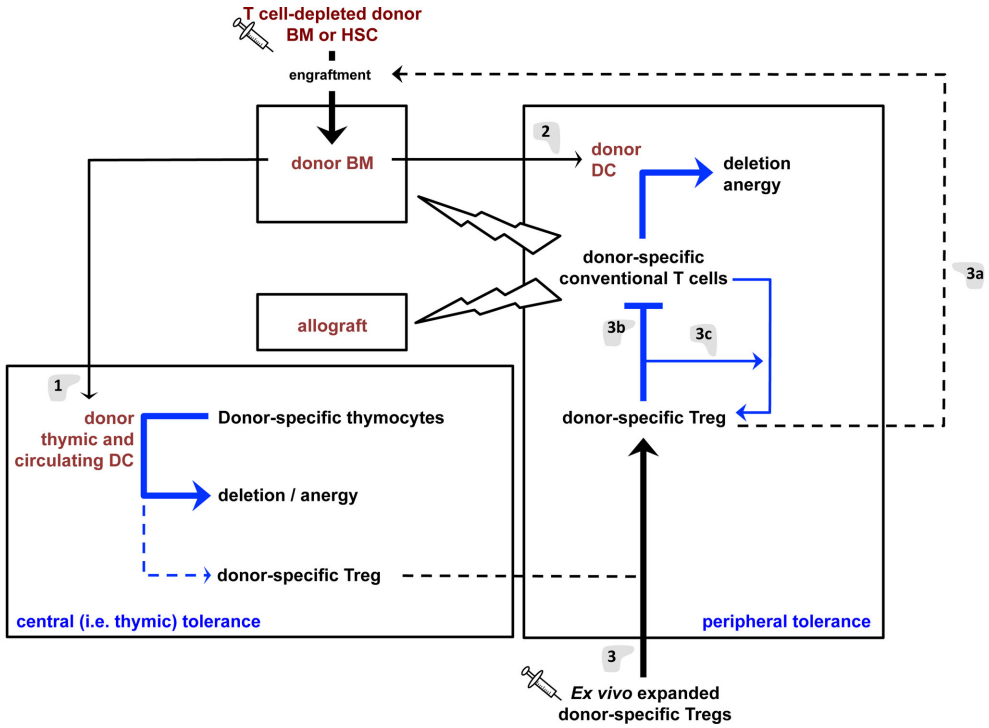

FIGURE 2 | Tolerance mechanisms induced by the proposed regulatory T cell/hematopoietic chimerism-based protocol for induction of transplantation tolerance. (1) Hematopoietic cells (e.g., DC) derived from the grafted BM will colonize the recipient's thymus and induce deletion and anergy (i.e., "recessive tolerance") of developing donor-specific host T lymphocytes. DC may also promote limited differentiation of donor-specific Treg that will contribute to transplantation tolerance. (2) Donor DC will also induce recessive tolerance of mature peripheral donor-specific T lymphocytes. These cells may, to a limited extent, directly induce donor-specific Treg. However, the dominant tolerance (i.e., Treg) induce by hematopoietic chimerism in (1) and (2) appears insufficient to durably prevent most notably chronic allograft rejection. (3) Infusion of donor-specific Treg will aid in engraftment of grafted donor BM/HSC (a) and inhibit the reactivity of mature peripheral donor-specific T lymphocytes (b), thus favoring graft-acceptance. They will also allow the differentiation of donor-specific conventional T lymphocytes into Treg (c), thus assuring persistence of tolerance and preventing chronic allograft rejection.

induction of hematopoietic chimerism. Skin grafts transplanted on these mice survived for more than 160 days, without signs of rejection or appearance of donor-specific antibodies (Pilat et al., 2010). More recently, this group raised similar conclusions using polyclonal host $CD4^+$ lymphocytes previously transduced with a retroviral vector containing Foxp3 and a drug-free conditioning regimen where 1 Gy total body irradiation replaced the short course of rapamycin (Pilat et al., 2011). These protocols represent a major step forward to the clinic. However, they still rely on anti-CD154 treatment and this antibody is presently not usable in patients (see above). Different non-mutually exclusive strategies can be envisaged to avoid co-stimulatory-blockade. The use of hematopoietic or mesenchymal stem cells instead of bone-marrow cells could be an option, as this will significantly reduce the immunogenicity of the graft. Another strategy would be to improve the efficiency of the immunosuppression by injecting donor-specific Treg. Alloantigen-specific T cells survived longer and in several transplantation models gave substantially better results than polyclonal Tregs with irrelevant specificity (Joffre et al., 2004, 2008; Nishimura et al., 2004; Golshayan et al., 2007).

Another aspect that still needs to be tested before translating Treg-based strategies into the clinic is represented by the antigen-specificity of the treatments. Indeed, if Treg activation is antigen specific, these cells exert their suppressor effector function in a non-antigen-specific-manner *in vitro* (Thornton and Shevach, 2000). If true *in vivo*, infused Treg may therefore inhibit protective immunity. However, it has been shown that hematopoietic chimerism/Treg-based therapy against allograft rejection is (at least) donor specific. A related issue is that tolerance to donor-antigen may be broken by (e.g., viral) infection (Welsh et al., 2000; Williams et al., 2001; Forman et al., 2002). It therefore also needs to be verified to what extent Treg-based therapies are resistant to infection. Experimental work will need to be performed to clarify these important issues.

Finally, infused Treg do not necessarily survive indefinitely and tolerance may therefore wane away with time. In other transplantation models, however, it was shown that a tolerant T cell population can render naïve T cells tolerant and even tolerogenic (Waldmann, 2010). Very recently it was shown that this so-called "infectious tolerance" depends on Treg that induce novel Treg required for persistence of tolerance to allografts (Kendal et al., 2011). Even if it remains to be shown that also infused Treg can cause infectious tolerance, it appears therefore that Treg can induce life-long tolerance to allografts.

CONCLUSION

The data discussed here indicate that induction of persistent hematopoietic chimerism combined with infusion of Treg with appropriate specificity efficiently leads to life-long tolerance to allografts in experimental animal models (**Figure 1**). Thus, and not very surprisingly so, the mechanisms involved in the maintenance of tolerance to self antigens also appear required for tolerance to donor antigens (**Figure 2**). More work will need to be performed to establish conditioning regimens compatible with clinical constraints and to assess immunocompetence of grafted animals. The validity of these conclusions for non-human primates and humans remains to be studied. Very substantial progress has been made in recent years in the induction of authentic immunological tolerance to allogeneic organ grafts, and transplant recipients may soon be able to live a life free of the fear of losing the graft and of the severe side effects of immunosuppressive drugs.

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