

Induction of Mixed Chimerism for Reversal of Autoimmunity in Type 1 Diabetes

Jeremy Racine^{1,2,3} and Defu Zeng^{1,2,3*}

¹Irell and Manella Graduate School of Biological Sciences, City of Hope, Duarte, CA, USA

²Department of Diabetes Research, Beckman Research Institute, City of Hope, Duarte, CA, USA

³Department of Hematology and Hematopoietic Cell Transplantation, Beckman Research Institute, City of Hope, Duarte, CA, USA

Abstract

Type 1 diabetes (T1D) results from autoimmune attack of insulin-producing pancreatic islet β cells. T1D autoimmunity is associated with particular MHC or HLA types in mouse or humans. T1D autoimmunity arises from defects in both central negative selection and peripheral regulation of autoreactive T cells as well as intrinsic defects of B cells. Current therapies that target at improving peripheral tolerance of autoreactive T cells or depleting B cells have not yielded significant therapeutic effects in reversal of autoimmunity in T1D patients. Induction of mixed chimerism with bone marrow cells from non-autoimmune donors has been recently indicated to be a curative therapy for reversal of autoimmunity in T1D. In this review, we have summarized and discussed T1D related abnormalities in the hematopoietic compartment, regimens that induce mixed chimerism in the T1D animal model of non-obese diabetic (NOD) mice, and how mixed chimerism corrects central negative selection and peripheral tolerance of autoreactive T and B cells.

Keywords: Chimerism; Autoimmunity; Type 1 diabetes; Hematopoietic cell transplantation; Thymic negative selection; Anti-CD3; Peripheral tolerance

Introduction

Type 1 diabetes (T1D) results from autoimmune destruction of insulin-secreting β cells [1-3], and the autoimmunity is associated with particular major histocompatibility complex (MHC) II I-A^{b7} in mouse and human leukocyte antigen (HLA)-DQ in humans [4-6]. These genetic backgrounds contribute to central and peripheral tolerance defects [7-12]. Foxp3⁺ regulatory (Treg) and natural killer (NK) T cells play important roles in maintaining peripheral tolerance, and have been shown to be quantitatively and qualitatively abnormal in non-obese diabetic (NOD) mice [9,10,13-15] and humans [16,17]. *In vivo* activation and expansion of Treg via anti-CD3 or anti-CD3 plus insulin vaccine were shown to prevent T1D development and reverse new-onset T1D in NOD mice as well as ameliorate new-onset T1D in humans [18-22], but the efficacy in humans only lasts about 18 months [21]. Similarly, activation and expansion of NKT cells in NOD mice were shown to prevent T1D [23-26], but therapy in humans yielded minimum effect [27]. In addition, many other immunomodulatory therapies yield little effect in humans [28,29]. It is not yet clear why immunotherapies that modulate peripheral tolerance do not reverse autoimmunity in T1D patients. It is possible that the simultaneous correction of both central and peripheral tolerance defects is required for curing autoimmunity in T1D. Recent studies indicate that induction of mixed chimerism can be a curative therapy for reversal of autoimmunity in T1D [30-34]. In this review, we will summarize the scientific basis for induction of mixed chimerism as a curative therapy for autoimmunity in T1D, regimens that induce mixed chimerism, and the mechanisms wherein mixed chimerism reverses autoimmunity in T1D.

T1D autoimmunity is associated with defects in hematopoietic compartments

In the late 1980s, a series of studies with NOD mice revealed that autoimmune T1D could be transferred into non-autoimmune mice via hematopoietic stem cell transplantation (HCT) [4,35-38], and

cells from NOD mice could destroy non-autoimmune islet β cells as well [37]. The observation that bone marrow (BM) grafts could lead to diabetes in previously non-diabetic patients has also been observed clinically [39]. These observations make it clear that type 1 diabetes arises from defective immune cells in the hematopoietic compartment.

T1D autoimmunity is associated with defects in hematopoietic cell-derived antigen presenting cells that mediate thymic negative selection

T cell development and “education” occurs in the thymus. Immature thymocytes enter the cortex, and begin the process of rearranging their T cell receptor locus. Not every rearrangement produces functional T cell receptors (TCR) that are capable of interacting with MHC on antigen presenting cells. In order to test whether a functional TCR has been created, developing thymocytes interact with cortical epithelial cells (cTEC) to determine whether a TCR signal can be delivered by the MHC of the host, a process known as positive selection [40]. A thymocyte that has passed the positive selection checkpoint enters the medulla, where it encounters both bone marrow derived DCs, as well as medullary epithelial cells (mTECs) that present self-antigens. The thymocytes that express TCR with high affinity for self-antigens are induced to go through apoptosis (negative selection); the thymocytes with intermediate affinity for self-antigens can be induced to up-regulate expression of Foxp3 and become regulatory T cells; the thymocytes with low-affinity for self-antigens survive and are exported

*Corresponding author: Defu Zeng, MD, The Beckman Research Institute of the City of Hope (Gonda Building R2017), Duarte, CA 91010, USA, Tel: 626-359-8111 (ext 62587); Fax: 626-301-8136; E-mail: dzeng@coh.org

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to the periphery [41]. Upwards of 50-80% of positively selected T cells can be negatively selected in the medulla [42].

The self-antigens that drive the negative selection checkpoint can arise in the thymus or periphery. First, mTECs are directly capable of expressing tissue-specific antigens (TSAs), a process largely mediated by autoimmune regulator (AIRE) [43-46]. Mutations in AIRE can lead to massive systematic autoimmunity [43], but there are antigens expressed in mTECs that are expressed in an AIRE-independent manner [43,47]. Second, thymic dendritic cells can process and present antigen expressed initially in mTECs [48]; dendritic cells can capture blood-borne antigens that enter the thymus [49], or can physically encounter the antigen in the periphery and bring the antigen back into the thymus [50].

The greatest contributing factor for development of diabetes is certain susceptible alleles of the MHC II molecules [4-6]. NOD mice lack expression of the MHC II molecule I-E [5,51], and have a three amino acid substitution on the β -chain of I-A [52], thus, called I-A^{E7}, a mutation which is similar to susceptible human DQ alleles [53]. This mutation gives rise to several different altered functions for the I-A^{E7} molecule. First, I-A^{E7} has been described as a promiscuous peptide binder [54], with the three amino acid difference from I-A^d leading to increased peptide binding repertoire when compared to I-A^d. Second, many of the T cells that respond to insulin have been found to respond to epitopes that actually bind very poorly to I-A^{E7} due to changes in the P9 pocket of I-A^{E7} [55,56]. With the improper binding of peptide, and increased dissociation of peptide binding, properly timed interaction with antigen specific T cells is decreased [57], likely leading to decreased ability to induce negative selection in the thymus [7,8,58]. However, some studies have found that the negative selection defects observed in NOD are actually T cell intrinsic signaling defects that prevent their apoptosis when signaled with MHC II-self-antigen complex [59].

A more recent report showed that negative selection of certain autoreactive clones (i.e. BDC2.5 T cells) in NOD mice is actually not defective. Instead, a selective defect in the activation of Erk 1/2 downstream of NOD TCR $\alpha\beta$ receptor signaling led to enhanced positive selection and higher numbers of CD4⁺CD8⁺ thymocytes. Due to the limitation of negative selection niches, more autoreactive T cells escape from negative selection and were exported to the periphery [60]. Interestingly, another report showed that TCR $\alpha\beta$ ⁺ T cells in NOD mice had defective β -selection and defective generation of CD4⁺CD8⁺ thymocytes [61]. Thus, further work dissecting altered negative and

T1D autoimmunity is associated with defects in peripheral regulation

Even in healthy individuals, a small amount of autoreactive T cells do escape from thymic negative selection and migrate into the periphery. However, these cells are controlled by peripheral tolerance mechanisms. Peripheral tolerance can be an active process maintained by regulatory cell populations such as Foxp3⁺ Treg cells, NKT cells, and tolerogenic dendritic cells. There have been some conflicting reports as to whether there is a defect in Foxp3⁺ Treg cells in T1D. Some groups have reported decreased numbers and function of Treg cells in NOD mice and in human subjects [16,62], while others have seen normal numbers and function of the Treg cells [63,64]. One complicating factor could be age of patients in analysis, as age associated declines in Treg cell numbers or function have been observed in NOD mice and diabetic patients [9,65]. Defective peripheral tolerance in T1D could be

also due to the fact that the effector T cells have a defective response to the Treg suppressive signals [66]. This observation is important in light of other studies showing that NOD thymus is actually very efficient in generating natural Tregs (nTreg) when compared to other strains of mice [67]. Dendritic cells in NOD mice have been found to lose their tolerogenic features because they have reduced indoleamine dioxygenase-2,3 (IDO) production [68] and increased IL-12 production [69].

T1D Autoimmunity is associated with defects in B cell tolerance

B cells have been shown to be important for the expansion of an immune response in their role as an antigen presenting cell [70,71]. For T1D development, B cells are a crucial antigen presenting cell (APC) [72-75], as B cell knockout mice are largely protected from diabetes development [76,77]. Re-introduction of B cells into B cell deficient NOD indicated that B cells were required for initial priming of glutamic acid decarboxylase (GAD) reactive T cells [78], indicating that B cells were a necessary antigen presenting cells in T1D development. Patients treated with anti-CD20 had improved C-peptide levels compared to placebos, but C-peptide levels continued to decline, indicative of only a partial protective effect [79]. While it was reported to be effective in reversing overt diabetes [80], and preventing diabetes development [80,81], the effect was incomplete. Recent evidence reveals a potential reason for this conflicting data, as anti-CD20 becomes ineffective when auto-antibodies are already present, due to intra-pancreas B-cell phenotypic changes [82].

During B cell development, tolerance processes take place in the bone marrow as well as in the spleen. Immature B cells in the spleen can be divided into transitional 1 (T1) and T2 B cells [83]. Immature T1 B cells in the spleen of NOD B cells are less susceptible to apoptosis when encountering soluble self-antigens, as compared to non-autoimmune mice [84]. T2 B cells, which are less susceptible to antigen induced apoptosis [85,86] and marginal zone B cells, an important antigen presenting B cell population [87], are both expanded in NOD mice compared to non-autoimmune mice [84,87,88]. In addition, anergy of self-reactive B cells in NOD mice can be more readily reversed [84] and anergic autoreactive B cells in NOD mice are still capable of maturation, have increased marginal zone (MZ) tropism, and effectively serve as antigen presenting cells [89].

Hematopoietic cell-derived thymic APC expression of protective MHC II restored thymic negative selection of autoreactive T cells and prevented T1D development

As mentioned above, autoimmunity in NOD mice is associated with a defective thymic negative selection of autoreactive T cells [7,8], which was shown to be associated with expression of particular MHC II by BM-derived APCs but not by thymic epithelial cells [90,91]. Thymic negative selection can be restored by making the BM-derived APCs that express disease susceptible MHC II (i.e. I-A^{E7}) to co-express disease-resistant MHC II molecules (i.e. I-A^b) through transgenic expression or backcrossing in many studies [90-96], although not in all [97,98].

For example, it was reported that I-A^{E7} restricted β cell-reactive transgenic CD4⁺V β 11⁺ T cells in transgenic NY-4.1 NOD mice were completely deleted by BM-derived thymic APC co-expression of I-A^b, I-A^f/I-E^k, I-A^{nb1} or I-E^{nb1}, and partially deleted by the co-expression of I-A^g or I-A^k alone, but not depleted by the co-expression of I-A^s, via back-crossing NY-4.1 NOD mice to mice expressing different MHC II [92]. Follow up studies dissected the hematopoietic cells required

for deletion. Protective alleles of MHC II on dendritic cells and macrophages, but not on B cells, were required [93]. Additionally, the bone marrow-derived APCs that express protective I-A^b were involved only in the deletion of I-A^{g7} restricted autoreactive T cells in the thymic medulla, but not in the positive selection of the autoreactive T cells in the thymic cortex or autoantigen presentation in the periphery [90]. This suggests that the autoreactive NY-4.1 transgenic T cells express a promiscuous TCR that can interact with both I-A^{g7} or I-A^b MHC II, and the interaction with I-A^b but not I-A^{g7} mediates negative selection of the autoreactive T cells in thymic medulla.

On the other hand, I-A^{g7} MHC II-restricted transgenic autoreactive CD4⁺Vβ4⁺ T cells that specifically recognize chromogranin A from BDC2.5-NOD mice were found not to be deleted in the thymus of heterozygous I-A^{g7}/I-A^b mice, after back-crossing to I-A^b C57BL/6, although heterozygous I-A^{g7}/I-A^b mice showed no insulinitis and homozygous I-A^{g7}/I-A^{g7} mice showed severe insulinitis [99,100]. The disease prevention in heterozygous I-A^{g7}/I-A^b mice was associated with expansion of non-autoreactive CD4⁺ T cells with endogenous α chains [97], likely due to the presence of I-A^b in the cortex.

Thymic APC-expression of protective MHC II can also mediate thymic deletion of autoreactive CD8⁺ T cells. For example, BALB/c (H-2^d) mice that express both transgenic influenza virus hemagglutinin (HA) on islet β cells and transgenic TCR that specifically recognize H-2K^d-restricted HA epitope on CD8⁺ T cells spontaneously develop insulinitis and diabetes. The autoimmune diabetes was prevented in H-2^{b/d} haplotype. Interestingly, the autoreactive CD8⁺ T cells in the thymus of H-2^{b/d} haplotype mice was deleted at CD4⁺CD8⁺ stage in a MHC II molecule dependent manner [94].

Thymic BM-derived APC expression of H-2^{nb1} (K^b, A^{nb1}, E^k, D^b) was also shown to delete H-2K^d-restricted, diabetogenic transgenic CD8⁺ T cells in AI-4-NOD mice (K^d, A^{g7}, E^{null}, D^b) [96,101]. AI-4 CD8⁺ T cells, which were originally isolated from islets of NOD mice, and partake in the earliest phases of β-cell destruction [102], can mediate insulinitis without CD4⁺ T cell help [103]. Surprisingly, AI-4 CD8⁺ T cells can also be deleted in MHC-matched background, as B6.H-2^{g7} mice harboring this TCR transgenic T cell clone has a massive negative selection of the clone in the thymus. Interestingly, this clone is *more* aggressive in mediating insulinitis in B6.H-2^{g7} than in NOD mice, despite the negative selection that does occur in the thymus of B6.H-2^{g7} mice. A few AI-4 CD8⁺ T cells that escape from the thymus negative selection can still induce severe insulinitis [104]. Further studies are needed to distinguish the MHC molecules that mediate thymic selection and peripheral tolerance.

Bone marrow stem cells transfected with protective MHC II restored thymic negative selection and prevented T1D development

To translate the concept that thymic APC co-expression of protective MHC II molecules can restore thymic negative selection and prevent T1D development into clinical application, investigators have been searching for ways to make BM-derived APCs to express protective MHC II molecules for the prevention of T1D. It was reported that retroviral transfection of autoimmune NOD marrow stem cells with MHC II genes that encode I-Aβ chain molecules results in thymic deletion of autoreactive T cells as indicated with deletion of BDC15-tetramer⁺ CD4⁺CD8⁺ thymocytes, as well as prevention of insulinitis and diabetes development [105]. Transfection of NOD marrow stem cells with autoantigen genes (i.e. insulin) were also reported to prevent T1D development in NOD mice [106]. These experiments were performed

with ~ 4 weeks old NOD mice conditioned with myeloablative TBI [105,106]. These approaches are not ready for clinical application due to the concern about the long-term uncertainty of retroviral transfected stem cells [107] as well as the requirement for high-dose TBI conditioning of recipients [108], which has long term side effects [109].

Induction of “mixed” chimerism in myeloablative and non-myeloablative TBI-conditioned NOD recipients prevented T1D development

Chimerism is defined as engraftment of allogeneic donor lympho-hematopoietic system in a recipient. Full chimerism is the complete replacement of the host-type lympho-hematopoietic system with the donor-type; and mixed chimerism is the co-existence of donor- and host-type lympho-hematopoietic system in a recipient. Chimerism is usually measured with the presence of donor-type lymphocytes among blood or peripheral lymphoid tissues (i.e. spleen and lymph nodes) mononuclear cells. Although the persistent presence of the small percentage of multiple lineage of donor-type cells including T, B, macrophage, and granulocyte cells in the periphery can be a good demonstration of engraftment of donor-type lympho-hematopoietic system and defined as mixed chimerism, the presence of the small percentage of host-type lymphocytes cannot be defined as mixed chimerism, because radiation-resistant residual host-type memory lymphocytes can last for a long-time. In this case, true mixed chimerism needs to be confirmed with the presence of de novo developed host-type CD4⁺CD8⁺ immature thymocytes in the thymus as well as de novo developed immature B220⁺ B cells and Mac-1⁺/Gr-1⁺ macrophage/granulocyte cells in the bone marrow.

Induction of mixed chimerism with allogeneic BM cells was proposed to be an effective approach to deliver the protective MHC to autoimmune recipients [110,111]. It was reported that induction of “mixed” chimerism in recipients conditioned with lethal TBI reversed T1D [32,112,113]. However, we recently found that the “mixed” chimerism in those high-dose TBI-conditioned recipients lacked de novo generation of host-type CD4⁺CD8⁺ thymocytes in the thymus or B220⁺ B cells or Mac-1⁺/Gr-1⁺ macrophage/granulocyte cells in the bone marrow [30]. Therefore, those “mixed” chimeras were in fact full chimeras.

Although non-myeloablative TBI-conditioning in combination with administration of anti-CD40L were shown to induce mixed chimerism in non-autoimmune recipients [114], similar regimen resulted in complete chimerism in autoimmune NOD mice [115]. Diabetic NOD mice conditioned with depleting antibodies (anti-CD4, anti-CD8, anti-Thy) and non-myeloablative TBI (400 cGy) and treated with co-stimulatory blockade of anti-CD40L were shown to develop “mixed chimerism” with final presence of ~8% CD4⁺ and ~1% CD8⁺ host-type T cells among total T cells in the periphery of the chimeric recipients, but the presence of host-type CD4⁺CD8⁺ thymocytes in the thymus or B220⁺ B cells in BM was not evaluated [34]. Thus, those host-type T cells are likely the residual mature T cells existed before HCT, and those recipients may not be truly mixed chimeras with de novo developed host-type T and B cells after HCT. Complete chimeras established with high-dose TBI-conditioning have the potential to develop graft vs host disease (GVHD) and immune-deficiencies [33,116]. True mixed chimerism with co-existence of donor and host immune cells has a better immune function, with less chances of infection after HCT [33,117]. Mixed chimerism has been shown in humans not to cause GVHD [118,119]. High-dose TBI-conditioning

is not acceptable to T1D patients. Thus, a radiation-free conditioning regimen that can induce mixed chimerism is preferred for reversal of autoimmunity in T1D patients.

Induction of mixed chimerism with radiation-free anti-CD3/CD8 conditioning regimen prevented and reversed T1D in prediabetic and overt diabetic NOD mice

We have recently reported a radiation-free and GVHD preventative anti-CD3/CD8-conditioning regimen that can induce mixed chimerism in both prediabetic as well as late-stage diabetic NOD mice [30,120-122]. In brief, recipient mice were conditioned with anti-CD3/CD8 mAbs to partially deplete host T cells and down-regulate the TCR-CD3 complex on residual host T cells. The host T cells did not re-express their TCRs until ~ 10 days after antibody injection. Seven days after anti-CD3/CD8 conditioning, donor BM and CD4⁺ T-depleted spleen cell (CD8⁺ T cells) were transplanted. Transplantation at 7 days after antibody conditioning could avoid the negative impact of anti-CD3/CD8 mAb on donor CD8⁺ T cells, because antibodies were undetectable at that time. The latter cells can kill the disarmed host T cells to facilitate donor cell engraftment. Under this radiation-free anti-CD3/CD8-conditioning regimen, both non-autoimmune and autoimmune mice developed mixed and complete chimerism dependent on the dose of infused donor CD4⁺ T-depleted spleen cells, and neither mixed nor complete chimeras showed any signs of GVHD [121,123]. The infused donor CD8⁺ T cells do not cause GVHD in either mixed or complete chimeras, because anti-CD3-conditioning also renders the host GVHD-resistant by preventing donor T cell migration into GVHD target tissues as well as up-regulating tissue expression of protective molecules such as B7H1 and indoleamine deoxyginase-2,3 (IDO) that can tolerize infiltrating T cells ([121,123-125] and unpublished data).

Induction of mixed chimerism under anti-CD3/CD8-conditioning regimen reversed autoimmunity and eliminated insulinitis as well as prevented T1D development in prediabetic NOD mice and reversed diabetes in new-onset (3-days after onset) diabetic NOD mice [30,122]. Although induction of mixed chimerism alone failed to reverse late-stage (3-weeks after onset) diabetes in NOD mice [122,126], combination therapy with induction of mixed chimerism and administration of growth factors augmented β cell regeneration and proliferation and reversed late-stage T1D [120]. The induction of mixed chimerism also provides immune tolerance to donor- or host-type islet grafts [126]. Additionally, the native pancreas of the mixed chimeric but not the non-chimeric NOD mice can be used as the site of islet grafts; small amount of donor islets (1/20 of regular dose) in the pancreas of chimeric mice can proliferate to reverse late-stage T1D [126].

Induction of mixed chimerism established thymic deletion of donor-reactive host-type thymocytes in autoimmune NOD mice

Induction of mixed chimerism was able to provide immune tolerance to donor-type organ grafts in both MHC-matched and MHC-mismatched non-autoimmune mouse recipients [127,128] as well as in HLA-matched non-autoimmune patients with kidney transplantation [118,119]. Studies with non-autoimmune mouse models show that presence of donor-type APCs in the thymus of mixed chimeras mediates deletion of donor-reactive host-type thymocytes. For example, B10 mice have a high percentage of V β 11⁺ T cells, and B10A mice express MHC II I-E and mouse mammary tumor virus (MMTV) superantigen that delete V β 11⁺ thymocytes. In the mixed chimeric B10 recipients given

B10A donor bone marrow cells, host-type V β 11⁺ T cells were markedly reduced, but deletion of B10A donor-type cells led to re-emergence of V β 11⁺ T cells in euthymic but not thymectomized B10 recipients [129]. The study demonstrated that donor-type APCs can mediate deletion of donor-reactive host-type T cells in the thymus. Thymic deletion mediated in this manner occurs when donor and host with disparate MMTV superantigens, introducing the superantigen into the host via establishing mixed chimerism leads to loss of particular donor and host V β subsets. Utilizing this method both donor- and host-reactive T cells were shown to be centrally deleted in transplantation models of AKR \rightarrow C57BL/Ka, C57BL/Ka \rightarrow AKR, C57BL/Ka \rightarrow BALB/k [130], AKR \rightarrow C3H [131], BALB/c \rightarrow C57BL/10, C57BL/10.Br \rightarrow C57BL/10 [132]. Similarly, we showed that induction of true mixed chimerism in autoimmune NOD mice resulted in immune tolerance to both donor- and host-type islet and skin grafts, which was associated with thymic deletion of donor- and host-reactive V β subset [121].

Induction of mixed chimerism re-established thymic deletion of autoreactive host-type thymocytes in autoimmune NOD mice

We observed that induction of mixed chimerism with BM transplants from MHC-mismatched (H-2^b) but not MHC-matched (H-2^{g7}) C57BL/6 donors was able to prevent T1D development in NOD mice [30]. Using transgenic BDC2.5 in wild-type NOD background, we showed that induction of mixed chimerism with MHC-mismatched but not matched donor BM mediated thymic deletion of autoreactive BDC2.5 thymocytes at the late development stage of CD4⁺CD8⁺ thymocytes (CD4⁺CD8⁺TCRV β 4⁺) [30], similar to thymic deletion of other autoreactive transgenic T cells [41,133]. This indicates that MHC-mismatched donor-type APC (I-A^b) is able to delete host-type autoreactive thymocytes that recognize (I-A^{g7}). It was reported that the original BDC2.5 clones were not cross-reactive [100]. We found that BDC2.5-tetramer⁺ cells from wild-type but not Rag-1^{-/-} NOD background showed proliferation to H-2^b DC stimulation (Wang, Racine, and Zeng et al: unpublished data), suggesting that BDC2.5 transgenic T cells from wild-type NOD background possess cross-reactivity. This cross-reactivity may mediate their deletion in the mixed chimeras with MHC-mismatched donor APCs (a context which is different from genetic backcrosses where I-A^b is first encountered by developing thymocytes in the cortex). Promiscuous autoreactive T cells are reported to be potent pathogenic T cells in NOD mice [101,134]. It is under investigation whether MHC-mismatched mixed chimerism prevents T1D development by mediating thymic deletion of cross-reactive autoreactive T cells in wild-type NOD mice.

Induction of mixed chimerism re-established peripheral T cell tolerance

Correction of central tolerance by induction of mixed chimerism is one important mechanism for reversal of autoimmunity, re-establishment of peripheral tolerance mechanisms is also needed, as indicated by T1D development in A14-B6.H^{g7} mice, in which thymic negative selection alone cannot be sufficient for diabetes prevention [104]. We observed that induction of MHC-mismatched mixed chimerism through a radiation-free anti-CD3/CD8 conditioning regimen and infusion of donor CD4⁺ T-depleted spleen and bone marrow cells eliminated insulinitis in both prediabetic and late-stage diabetic NOD mice with β cell regeneration [30,122]. This observation strongly suggests that induction of mixed chimerism re-establishes not only central but also peripheral tolerance.

The mechanisms of how induction of mixed chimerism re-establishes peripheral tolerance are not yet clear. We speculate that the first step is to clear memory lymphocyte populations that exist prior to donor BM cell infusion. NOD T cells are less susceptible to irradiation induced cell death [135], as well as cyclophosphamide induced cell death [136], therefore regimens that rely on irradiation or chemotherapeutic agents for memory cell depletion may run into issues in reversing the autoimmunity in NOD mice. In fact, this obstacle has already been observed in mice. Although bone marrow engraftment can occur in NOD mice, it is more difficult than in non-autoimmune strains [112], as onset of overt diabetes increases the difficulty of engraftment due to the large memory T cell population [137].

In our own regimen, infusion of donor CD8⁺ T cells and pre-depletion of host CD8⁺ T cells are required for engraftment in wild-type NOD of either pre- or overt-diabetic states [121]. Others have reported that engraftment of donor hematopoietic cells alone doesn't insure peripheral tolerance induction. While low-levels of donor-chimerism were sufficient for islet tolerance in non-autoimmune strains [138], high-levels of donor chimerism were required for tolerance to occur in NOD mice [138,139]. Even when robust allo-tolerance was achieved in mixed chimeric NOD mice prepared with co-stimulatory blockade, addition of anti-CD4-depleting antibodies were required for reversal of autoimmunity [140]. These observations indicate that donor T cell-mediated graft versus autoimmunity (GVA) that eliminates pre-existing memory autoreactive T cells in chimeric T1D recipients is important for reversal of autoimmunity. Thus, a conditioning regimen that allows for GVA activity without causing any signs of GVHD is required for induction of mixed chimerism in T1D individuals; the radiation-free anti-CD3/CD8 conditioning regimen represents one of these conditioning regimens.

Another important mechanism of peripheral tolerance is the re-establishment of regulatory cell populations that can suppress autoreactive cell activation. Spleen cells from chimeric NOD mice conditioned with co-stimulatory blockade and given B10.BR bone marrow transplants with >80% donor-type T cells in the periphery had the ability to suppress the autoreactive proliferation of naive NOD T cells. This indicated that donor-type cells had sufficient suppressive activity that was not present within non-chimeric NOD [141]. We have observed that, in protected (B6→BDC2.5-NOD) mixed chimeras, a high level of donor-type regulatory T cells were present in the pancreatic lymph nodes (LN), and donor-type T cells and donor-type DCs were both required to suppress diabetes development in secondary NOD-SCID recipients (Racine and Zeng et al, unpublished observations). This active suppression phenotype has been observed by others as mixed chimeric spleens (B6→WT NOD) depleted of donor-type T cells transferred disease into NOD-SCID recipients, while mixed chimeric spleens with donor-type cells intact did not [140]. Thus, donor-type regulatory T cells in the mixed chimeric T1D recipients may actively suppress the residual host-type autoreactive T cells in the periphery.

One more important question for re-establishing peripheral tolerance (as in central tolerance) is whether MHC-mismatched mixed chimerism is a requirement. We observed that induction of true mixed chimerism with MHC-mismatched transplants was able to eliminate insulinitis. However, matched BM transplants were not able to eliminate insulinitis under the radiation-free anti-CD3/CD8 conditioning [30], although the same MHC-matched transplants in lethal TBI-conditioned NOD recipients had previously been shown to prevent T1D development [113]. The latter transplantation actually induced complete rather than mixed chimerism, because a very high

level of donor-type chimerism was reported (>95% donor-type B cells, macrophages, DCs) despite "mixed" status of host-type T cells [113]. In addition, we observed that, although there was a "mixed" state at the T cell level, de novo development of B cells and macrophages in the BM or T cells in the thymus was not present in the TBI-conditioned chimeric NOD recipients [30]. Others have also observed that BM transplants from MHC-matched T1D-free Non-obese resistant (NOR) mice failed to prevent diabetes development in young NOD mice [142]. These indicate that MHC-mismatched mixed chimerism is also required for re-establishing peripheral tolerance. The donor APCs that expresses mismatched MHC and donor Treg cells may work together to tolerize the residual host-type autoreactive T cells with or without cross-reactivity in the periphery.

Induction of mixed chimerism re-established B cell tolerance

Due to the importance of B cells in diabetes development, a question that needs discussion is whether induction of mixed chimerism can correct B cell defects. We observed that induction of mixed chimerism markedly reduced serum levels of anti-insulin autoantibodies (Wang et al., unpublished data), indicating that autoreactive B cells are tolerized in the mixed chimeric NOD mice. However, it is not yet clear whether tolerization of autoreactive B cells is direct, or indirectly results from tolerization of autoreactive T cells and the subsequent lack of T cell help. It is also unclear whether simultaneous tolerization of autoreactive T and B cells are required, and whether leaving one untolerized would prevent the tolerization of the other.

Autoreactive B cells that recognize membrane bound antigen (such as MHC molecules) can be deleted in the bone marrow [143], so alloreactive B cells are likely tolerized in the bone marrow through donor and host cell interacting with the bone marrow niches. Additionally, GalT^{+/+}-donor cells can lead to tolerization of B-1 natural antibody forming B cells in GalT^{-/-} mice after induction of mixed chimerism and this tolerization was dependent on compliment and likely mediated by DC presenting antigen to xeno-reactive B cells [144,145]. However, B cell autoimmunity in T1D is generally considered a B cell intrinsic defect [84,88], and autoreactive B cells develop despite antigen being present. With a decreased B cell turnover, prolonged T2 residency, decreased follicular and expanded MZ B cells seen in NOD mice [84,87,88], one possible mechanism by which mixed chimerism could tolerate autoreactive B cells is providing a larger repertoire of B cells that compete with autoreactive B cells for survival signals. Both B-cell activating factor (BAFF)-dependent [146] and BAFF-independent [147] pathways were shown to rescue and expand autoreactive B cells under lymphopenia situation. One recent report indicates that T cell tolerance as the means by which autoreactive B cells remain tolerized, as autoreactive B cells specific to glucose-6-phosphate isomerase (GPI) could be detected in mice, but only caused antibody mediated arthritis when co-transferred with GPI reactive I-A^{b7} restricted CD4⁺ T cells into T cell deficient secondary recipients [148]. Since mixed chimerism is able to mediate the deletion of a large number of autoreactive T cells, those B cells that manage to escape negative selection in mixed chimeras could remain non-pathogenic. Investigations on how mixed chimerism tolerize autoreactive B cells are ongoing in our lab.

Summary and Future Direction

Many regimens have been reported to be able to induce mixed chimerism in autoimmune NOD mice [32-34,112,113,115, 141]. However, induction of true mixed chimerism should be confirmed with de novo generation of host-type CD4⁺CD8⁺ immature thymocyte in the thymus and immature B220⁺ B cells in the bone marrow, similar

to what we have observed with mixed chimerism in autoimmune NOD mice induced with radiation-free anti-CD3/CD8 conditioning regimen and transplantation of donor bone marrow and CD4⁺ T-depleted spleen cells [30].

Induction of mixed chimerism is a curative therapy for autoimmunity in T1D. MHC-mismatched mixed chimerism eliminated insulinitis and autoantibody production, mediates thymic deletion of autoreactive thymocytes, and maintains peripheral tolerance of residual

autoreactive T cells [30,120], as well as tolerizes autoreactive B cells. The tolerance induction and maintenance in mixed chimeric NOD mice appear to require donor DCs that express mismatched MHC and de novo developed Foxp3⁺ Treg cells. The detailed mechanisms are not yet clear and we propose the following hypotheses.

As depicted in Figure 1, we hypothesize that there are both cross-reactive and non-cross-reactive autoreactive T cells in autoimmune NOD mice, as reported by others [90,94,96]. In the NOD mice with MHC-mismatched mixed chimerism, cross-reactive autoreactive thymocytes encounter donor-type DCs that express mismatched MHC in the thymic medulla and go through apoptosis (Figure 1A). Non-cross-reactive autoreactive T cells and residual cross-reactive autoreactive T cells are exported into the periphery such as lymph nodes and spleen. In the periphery, residual cross-reactive autoreactive T cells interact with donor-type tolerogenic DCs and become apoptotic or anergic (Figure 1B); non-cross-reactive autoreactive T cells interact with host-type tolerogenic DCs and become apoptotic or anergic (Figure 1B). De novo developed donor-type Foxp3⁺ Treg cells that can interact with both donor- and host-type DCs play an important role in maintaining the tolerogenic status of both donor- and host-type DCs in the mixed chimeras; the Treg cells can also directly suppress the expansion of both cross-reactive and non-cross-reactive autoreactive T cells (Figure 1B). Tolerization of autoreactive T cells and production of donor-type B cells in the mixed chimeras can lead to tolerization of autoreactive B cells. Presence of donor-type B cells that take away survival factors such as BAFF and lack of T cell help could result in autoreactive B cell apoptosis or anergy (Figure 1C). In future studies, the role of donor- and host-type DCs, the role of donor-type Treg cells, and the role of DC interaction with donor-type Treg cells in mediating tolerization of cross-reactive and non-cross-reactive autoreactive T cells, as well as tolerization of autoreactive B cells in mixed chimeric NOD mice need to be investigated.

Since induction of mixed chimerism has been demonstrated in animal models and humans not to cause GVHD [118,128], a safe and non-toxic conditioning regimen for induction of stable mixed chimerism would be a curative therapy for established T1D. As reported in animal models, elimination of autoimmunity and insulinitis could allow residual islet β cells to proliferate and reverse new-onset diabetes [122]; combination therapy with induction of mixed chimerism and administration of growth factors can augment β cell neogenesis and proliferation to reverse late-stage T1D [120].

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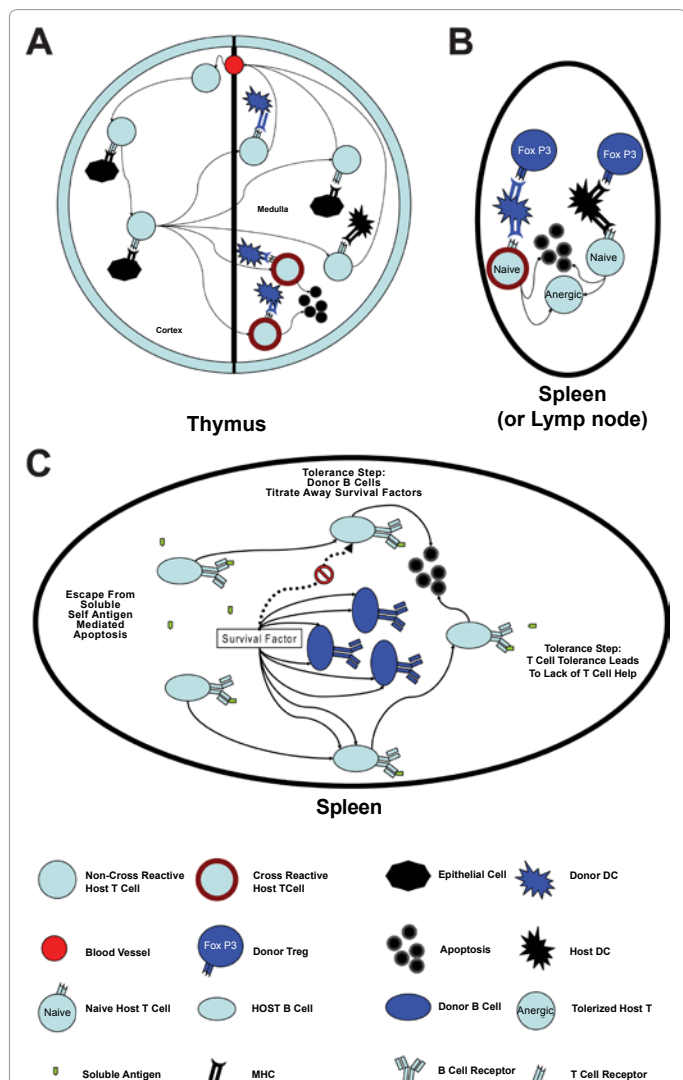


Figure 1: Model for re-establishing central and peripheral tolerance in autoimmune NOD mice after induction of mixed chimerism with bone marrow transplants from MHC-mismatched donors.

A. Developing thymocytes are selected on the cortical epithelial cells with T1D-linked MHC II molecules. In the medulla, cross-reactive autoreactive cells encountering mismatched MHC II molecules undergo negative selection. Non-cross-reactive autoreactive cells are unable to interact with mismatched MHC and fail to undergo negative selection and escape to the periphery. **B.** In the spleen and lymph nodes, donor-type Treg cells interact with both donor- and host-type DCs to confer tolerogenic phenotypes on DCs. Residual cross-reactive autoreactive T cells (which can interact with donor-type DC, as well as host-type DC) and non-cross-reactive T cells (which can only interact with host-type DC) are tolerized. **C.** Autoreactive B cells enter the spleen, and are tolerized through several proposed mechanisms. 1) Autoreactive B cells in competition for survival factors such as BAFF fail to outcompete non-autoreactive donor- and host-type B cells. 2) Autoreactive B cells that manage to survive to maturity fail to find T cell help as the T cells were either deleted in the thymus or spleen, or were rendered anergic after encountering donor-Treg and tolerogenic DCs.

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