Antigen-Encoding Bone Marrow Terminates Islet-Directed Memory CD8+ T-Cell Responses to Alleviate Islet Transplant Rejection

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Islet-specific memory T cells arise early in type 1 diabetes (T1D), persist for long periods, perpetuate disease, and are rapidly reactivated by islet transplantation. As memory T cells are poorly controlled by “conventional” therapies, memory T cell-mediated attack is a substantial challenge in islet transplantation, and this will extend to application of personalized approaches using stem cell-derived replacement β-cells. New approaches are required to limit memory autoimmune attack of transplanted islets or replacement β-cells. Here, we show that transfer of bone marrow encoding cognate antigen directed to dendritic cells, under mild, immune-preserving conditions, inactivates established memory CD8+ T-cell populations and generates a long-lived, antigen-specific tolerogenic environment. Consequently, CD8+ memory T cell-mediated targeting of islet-expressed antigens is prevented and islet graft rejection alleviated. The immunological mechanisms of protection are mediated through deletion and induction of unresponsiveness in targeted memory T-cell populations. The data demonstrate that hematopoietic stem cell-mediated gene therapy effectively terminates antigen-specific memory T-cell responses, and this can alleviate destruction of antigen-expressing islets. This addresses a key challenge facing islet transplantation and, importantly, the clinical application of personalized β-cell replacement therapies using patient-derived stem cells.

It is increasingly recognized that islet-specific memory T cells represent a major barrier to β-cell replacement therapies. After immunogenic antigen encounter, naïve T cells give rise to effectors and a small population of residual memory cells with increased longevity, faster response kinetics, and a reduced dependence on costimulatory signals (1). While this is advantageous for re-encounter with pathogens, formation of T-cell memory can be detrimental under some circumstances. Pathogenic memory T cells are prominent in type 1 diabetes (T1D), where they arise during the early, preclinical phase of disease and are well established by the time of diagnosis (2). In addition to perpetuating disease, islet-specific CD4+ and CD8+ memory T cells are persistent and may remain dormant for extended periods after T1D onset but are rapidly reactivated by islet antigen exposure during islet transplantation and contribute substantially to rejection of transplanted islets (3,4). Thus, immunotherapy for T1D must address memory T cells regardless of disease stage, and this is crucial for success of islet transplantation.

Conventional immunosuppressants such as calcineurin inhibitors and rapamycin control memory much less effectively than naïve T-cell responses (5). Many immunotherapies for T-cell-mediated diseases attempt to reintroduce or expand regulatory T cells (Tregs); however, memory T cells can resist suppression by Tregs (6,7). Therefore, development of new approaches to terminate memory T-cell responses in a controlled, antigen-specific context would be highly beneficial. In the clinical context, this is particularly
pertain for application of islet transplantation and personalized β-cell replacement therapies using patient-derived stem cells where memory autoimmune responses would attack replacement cells.

Dendritic cells (DCs) are increasingly considered clinically appropriate therapeutics for T1D and other autoimmune diseases. Indeed, memory CD4⁺ and CD8⁺ T-cell responses are terminated by DCs or other antigen-presenting cells (APCs) engineered to express cognate antigen (8–10). One strategy to achieve APC-targeted antigen expression to prevent diabetes development therapeutically is to transfer bone marrow (BM) or hematopoietic stem and progenitor cells (HSPCs) genetically modified to encode antigen expression that, once engrafted, continually gives rise to antigen-expressing APCs (11). Autologous hematopoietic stem cell transplantation (HSCT) has been tried for T1D with exciting outcomes but, as currently applied, uses toxic conditioning leading to complete immune ablation to deplete pathogenic immune cells including memory T cells (12,13).

Here, we tested the hypothesis that addition of gene therapy to HSCT, to achieve tolerogenic self-antigen expression in APCs, would terminate established memory T-cell responses, restoring long-lasting tolerance to islet antigens while maintaining protective immunity. We demonstrate that, under immune-preserving conditions, transplantation of BM encoding DC-expressed antigen terminates cognate CD8⁺ memory T-cell responses and alleviates immune destruction of newly transplanted islets.

**RESEARCH DESIGN AND METHODS**

**Mice**

OT-I (14), 11c.OVA (15), and K5.mOVA (16) mice were maintained under specific pathogen-free conditions in the Princess Alexandra Hospital Biological Resource Facility or Translational Research Institute Biological Resource Facility, Brisbane, Australia. C57BL/6 and B6.SJLptprca mice were purchased from the Animal Resources Centre (Perth, Australia). B6.SJL and OT-I mice were crossed to generate CD45.1⁺CD45.2⁺ OT-I mice. 11c.OVA mice were backcrossed to B6.SJLptprca to make CD45.1⁺ OT-I mice. Unless stated otherwise, CD45.1⁺ B6.SJLptprca mice were used as recipients and CD45.2⁺ mice used as BM donors. All animal procedures were approved by the University of Queensland Animal Ethics Committee.

**OT-I T-Cell Transfers**

For the transfer of naive OT-I T cells, suspensions of pooled mesenteric, inguinal, axillary, and brachial lymph nodes (LNs) were prepared and transferred (5 × 10⁶) as previously described (15). Memory OT-I T cells were generated as described previously (8). Briefly, LNs were harvested from OT-I or CD45.1⁺CD45.2⁺ OT-I mice and cultured in complete RPMI with 1% mouse serum, 0.1 μg/mL OVA257-264, and 10 ng/mL rhIL-2. After 3 days, cells were washed and recultured with 10 ng/mL rmIL-15 for 2 days. Where indicated, OT-I T cells were carboxyfluorescein succinimidyl ester (CFSE) labeled before transfer. Unless stated otherwise, 2 × 10⁶ Tmem were transferred (intravenous, lateral tail vein).

**BM Transplantation**

Femurs and tibias were collected into mouse tonicity PBS. BM was flushed with mouse tonicity PBS/2.5% FCS, erythrocytes were lysed (NH₄Cl/TRIS buffer), and BM was injected intravenously within 3 h of irradiation (300cGy, 137Cs source). HSPCs for transfer were prepared by high-speed FACS sorting of lin-negative (lin⁻)/c-kit-positive (c-kit⁺) cells to typically >95% purity from BM. HSPCs were depleted from BM by sorting lin⁺/c-kit⁻ cells from BM.

**In Vitro and In Vivo Assays**

CFSE labeling and flow cytometric analyses including bead-based counting assays were performed as previously described (15,17). OVA/QuilA immunization was as previously described (9). In vivo cytotoxic T lymphocyte (CTL) assays were performed as previously described (15). Monoclonal antibodies were purchased from Biolegend, BD Biosciences, or BioXcell (Lebanon, NH) or grown, purified, and conjugated in-house. ELISpot assays were as previously described (15) using OVA257-264 (0.5 μg/mL) or keyhole limpet hemocyanin (10 μg/mL) stimulation and counted with an ELISpot reader (AID GmbH, Strassburg, Germany).

**Skin and Islet Transplantation**

Skin grafting used double grafting of skin from K5.mOVA and BALB/c donors as previously described (18). For islet transplantation, mice were administered a single dose of streptozotocin (200 mg/kg i.p., pH 4.2 citrate buffer), and blood glucose (BG) was monitored daily using an Accu-Check Advantage (Roche). Hyperglycemic (>16.6 mmol/L) mice were transplanted under the kidney capsule with islets prepared from OVA⁺ve RIP.mOVA or OVA⁻ve littermate donors at a ratio of three donors per recipient. BG was monitored as indicated. Mice in which euglycemia (<16.6 mmol/L) was not initially restored were excluded due to primary graft nonfunction. Rejection was defined as two consecutive BG levels >16.6 mmol/L after initial euglycemia. Mice were nephrectomized ≥100 days postgrafting to determine graft dependence of euglycemia. Kidneys containing islet grafts were formalin fixed and embedded for histological analysis (19).

**Statistical Analysis**

Student t test was used for comparison of means and one-way ANOVA with Newman-Keuls or Tukey posttest for multiple comparisons (GraphPad Prism 5 or Prism 6). Survival data were analyzed using a Log-rank test (Mantel-Cox) (GraphPad Prism 5 or Prism 6).

**RESULTS**

**In Vitro-Generated OT-I Tmem Establish Functional In Vivo Memory Populations**

To study induction of tolerance to islet antigen, we adapted an established system (8,20) to generate and
monitor CD8+ T-cell memory at the individual cell level. LN cells from OVA-specific OT-I T-cell receptor transgenic (Tg) mice were cultured in peptide/interleukin (IL)-2 washed and cultured in IL-15 over a period of 5 days (8,21), which gives rise to predominantly CD44high/CD69−/CD62L+ central memory phenotype cells, referred to here as “Tmem.” These cells establish long-lived memory populations in vivo that readily respond to immunogenic rechallenge (Fig. 1A), which would target OVA-expressing β-cells.

**Low-Dose Irradiation Permits Effective Engraftment of Antigen-Encoding BM While Preserving CD8+ T-Cell Memory**

Low-dose irradiation facilitates engraftment of BM in naive and primed mice (22) and preserves immunity. For testing of whether immunity was preserved in mice with TCR-Tg memory CD8+ T cells, OT-I Tmem were transferred to CD45.1+ recipients. Recipients were left untreated or irradiated 1 week later (300cGy), and BM from CD45.2− non-Tg mice was transferred. No significant loss of responsiveness in OT-I memory populations resulted from low-dose irradiation and transfer of non-Tg BM (Fig. 1B). Antigen-encoding BM engraftment in immune-competent mice is enabled by restricting antigen expression to differentiated CD11c+ DCs (22). For testing of this in mice carrying TCR Tg memory CD8+ T cells, OT-I Tmem were transferred and recipients irradiated (300cGy) 1 week after transfer, and then BM from CD45.2− non-Tg or 11c.OVA donors, which expresses OVA in DCs (15), was transferred. Donor-type (CD45.2+) leukocytes accumulated in blood and spleen, indicating that BM effectively engrafted from OVA-negative and 11c.OVA donors (Fig. 1C and D). Donor-type DCs developed similarly in recipients of 11c.OVA or non-Tg BM (Fig. 1E), indicating that OVA expression was no impediment to DC development in the presence of OVA-specific Tmem.

**Transplantation of Antigen-Encoding BM Inactivates OT-I Memory T Cells**

We next determined whether antigen-encoding BM transfer (BMT) under immune-preserving conditions inactivated preexisting memory CD8+ T-cell responses. OT-I Tmem were transferred to non-Tg mice and 1 week later, mice were irradiated (300cGy) and 11c.OVA or non-Tg BM was transferred. Six weeks later, responsiveness of OT-I cells was tested by immunogenic challenge with OVA/QuilA (8), which substantially expanded OT-I T-cell number in spleens of no bone marrow transfer (no-BMT) controls and non-Tg BM recipients relative to sham-challenged controls (Fig. 2A–C) but not in recipients of 11c.OVA BM (Fig. 2A–C). Intracellular cytokine staining for interferon (IFN)-γ, as a measure of effector function, showed that...
OVA/QuilA challenge elicited a ~200-fold increase in total IFN-γ–producing OT-I T cells in spleens of both no-BMT controls and non-Tg BM recipients (Fig. 2D). The number of splenic IFN-γ–producing OT-I T cells in 11c.OVA BM recipients remained low, however, regardless of challenge (Fig. 2D). Similarly, little CTL activity was elicited in vivo by OVA/QuilA challenge of 11c.OVA BM recipients, but CTL activity was substantial in recipients of non-Tg BM or in no-BMT controls (Fig. 2E). As we have previously reported (22), BMT using low-dose irradiation does lead to some mild, mostly nonsignificant, reduction in responsiveness to OVA challenge (Fig. 2A–D). Together, these data indicate that responsiveness of an established OT-I Tmem population was effectively inhibited by OVA-encoding BM transfer. In additional studies where OT-I Tmem recipients were immunized with KLH/QuilA prior to BM transfer, KLH responses were preserved regardless of whether OVA-encoding or non-Tg BM was transferred (Supplementary Fig. 1), indicating antigen specificity of the tolerogenic effect of OVA-encoding BM transfer.

We observed no change in the proportion of donor-type DCs in either non-Tg or 11c.OVA BM recipients after OVA/QuilA challenge (Fig. 2F), indicating that the lack of OVA257–264–specific CTL activity in 11c.OVA BM recipients (Fig. 2E) is attributable to inactivation rather than redirection of OVA-specific CTL activity to cytotoxic destruction of OVA-expressing DCs.

**BM Transfer Is Effective at High CD8+ Memory T-Cell Frequencies**

For determination of whether the effectiveness of Ag-encoding BM transfer was limited if Ag-specific Tmem were highly abundant, a large number (4 × 10⁷) of OT-I Tmem were transferred and then non-Tg or 11c.OVA BM was transferred. OVA/QuilA challenge, 4 weeks after BM transfer, showed that while OT-I Tmem retained responsiveness and the capacity to proliferate and produce IFN-γ in recipients of non-Tg BM (Fig. 2G and H), this was completely ablated in recipients of OVA-encoding 11c.OVA BM (Fig. 2G and H). This indicates that Ag-encoding BM transfer is an effective strategy to achieve antigen-specific tolerance, particularly for Tmem, even when the frequency of Ag-specific CD8⁺ Tmem is very high, as might occur in T1D.

**Figure 2**—Inactivation of memory OT-I T-cell populations by OVA-encoding BMT. A–F: OT-I Tmem (CD45.1⁺/CD45.2⁺) were transferred to non-Tg mice (CD45.1⁺), and 1 week later mice were irradiated (300cGy) or not and non-Tg or 11c.OVA BM (CD45.2⁺, 10⁷) was transferred. Six weeks after, BMT mice were sham or OVA/QuilA challenged and, 1 week later or as indicated, analyzed by flow cytometry. OT-I T cells (CD45.1⁺/CD45.2⁺/CD8⁺/Vα₂⁺) were serially monitored in blood by cytometry (A) or 1 week after challenge (B). Spleens were analyzed 1 week after OVA/QuilA challenge (C, D, and F). In vivo CTL was performed 1 week after challenge (E). G and H: OT-I Tmem (4 × 10⁷, CD45.1⁺/CD45.2⁺) were transferred to non-Tg mice (CD45.1⁺), and 1 week later mice were irradiated (300cGy) and non-Tg or 11c.OVA BM (CD45.2⁺, 10⁷) was transferred. Four weeks after, BMT mice were sham or OVA/QuilA challenged and, 1 week later, analyzed by flow cytometry. Total number of OT-I per spleen (G) and total IFN-γ–OT-I per spleen (H) were determined. Data are pooled from 3 experiments and represent mean ± SEM (n = 6–11) (A) or individual mice with mean ± SEM (B–F) or pooled from two separate experiments showing individual mice with mean ± SEM (G and H).
Transplantation of Antigen-Encoding BM Prevents Islet Rejection

We sought to determine whether ablation of a cognate preexisting memory CD8<sup>+</sup> T-cell response might prevent memory T-cell-mediated rejection of "replacement" tissues. For islet transplants, this would model the autoimmune component of responses mounted against islet grafts in T1D where, here, OVA substitutes for component of responses mounted against islet grafts in T1D. Memory CD8<sup>+</sup> T cells were established by Tmem transfer, and 1 week later mice were left untreated or irradiated (300 cGy) and non-Tg or 11c.OVA BM was transferred. Four weeks later, mice were rendered diabetic by streptozotocin treatment, islets from syngeneic OVA<sup>ve</sup> RIP.mOVA mice expressing OVA in pancreatic β-cells or OVA<sup>ve</sup> littermate controls were transplanted under the kidney capsule, and glycemia was monitored to determine islet graft survival. OVA<sup>ve</sup> islets transplanted as technical controls survived long-term, stably restoring euglycemia in a graft-dependent manner (Fig. 3A and Supplementary Fig. 2). When grafted into OT-I Tmem recipients to which control non-Tg BM had been transferred, five of eight OVA-expressing islet grafts failed to stably restore euglycemia, and graft failure occurred soon after transplantation in most cases (Fig. 3A and Supplementary Fig. 2). In contrast, in mice carrying memory OT-I T cells that received OVA-encoding 11c.OVA BM, eight of nine islet grafts stably restored euglycemia (Fig. 3A and Supplementary Fig. 2). Insulin-positive cells were prominent in islet grafts of mice that received 11c.OVA BM (Fig. 3B).

To further define the ability of antigen-encoding BM transfer to prevent rejection of transplanted antigen-expressing tissues, we compared survival of allogeneic and OVA-expressing skin grafts. OT-I Tmem were transferred to non-Tg recipients and non-Tg or 11c.OVA BM was transferred after low-dose irradiation. Six weeks later, recipients and no-BMT controls were double grafted with skin from allogeneic BALB/c and syngeneic K5.mOVA donors expressing OVA in skin. Control, non-BM Tmem recipients rejected >80% of OVA-expressing skin grafts rapidly, as did recipients of non-Tg BM. Remaining grafts promptly rejected in response to OVA/QuilA challenge 100 days after placement (Fig. 3C), indicating residual OVA-specific memory. Profoundly, almost two-thirds of OVA-expressing skin grafts survived long-term in the 11c.OVA BM group (Fig. 3C), and none rejected in response to OVA/QuilA challenge, indicating robust graft acceptance in this group. In all mice tested, allogeneic skin rejected with a similar tempo (Fig. 3D), indicating no gross immunosuppression that might contribute to a difference between test groups. These data demonstrate successful induction of tolerance, which alleviates Tmem attack of antigen-expressing islets and skin, addressing a key challenge facing islet transplantation and personalized β-cell replacement therapies.

Tmem Inactivation Requires Engraftment of Antigen-Encoding Hematopoietic Progenitors

As gene-engineered BM transfer could have clinical application, we sought to understand the mechanisms of tolerance induction. To define whether engraftment of HSPCs was required and to negate any contribution from differenti-ated cells cotransferred in BM such as Tregs, sort-purified lin<sup>-ve</sup>/c-kit<sup>ve</sup> HSPCs were transferred to mice carrying OT-I Tmem under engrafting (300cGy) or nonengrafting (no irradiation) conditions and tolerance induction was tested by OVA/QuilA challenge. Donor-type leukocyte accumulation, indicating engraftment, was lower in HSPC than BM recipients (Fig. 4A), as anticipated from previous studies (22). However, 5 weeks after HSPC or BM transfer, total spleen cell number did not differ between groups (Fig. 4B). Whether mice received 11c.OVA HSPCs or BM, OVA/QuilA elicited little expansion of the memory OT-I population as expected (Fig. 4C). Substantial OT-I expansion, similar to that in non-Tg BM or HSPC recipient controls, occurred, however, if mice received HSPC-depleted 11c.OVA BM under engrafting conditions or 11c.OVA HSPCs under nonengrafting conditions. Together, these results indicate that engraftment of antigen-encoding HSPCs is necessary and sufficient for antigen-specific CD8<sup>+</sup> tolerance and nonengrafting components of BM do not contribute. A prerequisite for tolerance induction is steady-state antigen presentation by antigen-expressing DCs (15). Transfer of CFSE-labeled OT-I T cells revealed that 11c.OVA BM transfer, led to long-term presentation of OVA determinants for at least 12 weeks in recipient mice (Fig. 4D). Furthermore, in addition to ablating responsiveness of antigen-specific memory CD8<sup>+</sup> T cells, OVA-encoding BM establishes a long-term tolerogenic environment in recipients (Supplementary Fig. 3) that would prevent reemergence of targeted antigen-specific T-cell populations.

Ablation of Memory OVA-Specific CD8<sup>+</sup> T-Cell Responses by OVA-Encoding BM Does Not Establish OVA-Specific “Regulation”

Using irradiation to facilitate BM engraftment has been associated with generation or expansion of CD4<sup>+</sup> or CD8<sup>+</sup> Treg populations that modulate subsequent immune responses (23,24). To determine whether OVA-encoding BM transfer induced a regulatory response that inhibited subsequent OVA-specific T-cell activation, we adapted a widely used “regulation” assay and compared proliferation of a second adoptively transferred population of OVA-specific CD8<sup>+</sup> T cells in mice where OVA-specific memory CD8<sup>+</sup> T cells had been inactivated or not by BM transfer. Mice were injected with CD45.2<sup>+</sup> OT-I Tmem or not and 1 week later irradiated (300cGy), and non-Tg or 11c.OVA BM was transferred. Six weeks later, a new cohort of congenically distinct (CD45.1<sup>+</sup>) CFSE-labeled naive OT-I T cells was transferred as the "test" population, and CFSE dilution was measured 3 days later. For provision of a consistent source of antigen stimulation between recipients of non-Tg and OVA-encoding BM, some mice were immunized subcutaneously with OVA/QuilA. In non-Tg BM recipients, CFSE dilution indicated that OT-I T cells divided only after immunization (Fig. 5A, left and center.
panels) and proliferation was most extensive in LN draining the site of immunization (Fig. 5A, center panels). In nondraining LN where immunization-derived OVA was not presented, proliferation was most extensive in 11c.OVA BM recipients (Fig. 5A, center and right lower panels). However, after immunization, proliferation of test T cells was similar in draining LN of non-Tg and 11c.OVA BM recipients (Fig. 5A, center and right upper panels), and this was not altered by the presence of the initially transferred, but inactivated, OT-I Tmem (Fig. 5B and C).

Figure 3—Antigen-encoding BM transfer promotes survival of antigen-expressing islet and skin grafts. A and B: OT-I Tmem (4 x 10^7) were transferred to non-Tg mice. One week later, mice were irradiated (300cGy) and non-Tg or 11c.OVA BM was transferred. Four weeks after BM transfer, mice were rendered diabetic with streptozotocin (200 mg/kg) and islets from RIP.mOVA mice transplanted under the kidney capsule. BG was monitored and rejection determined as 2 consecutive readings >16.6 mmol/L. Graft sites were embedded, sectioned, and stained for insulin (B). C and D: OT-I Tmem were transferred to non-Tg mice. One week later, mice were irradiated (300cGy) and non-Tg or 11c.OVA BM was transferred. Six weeks after BMT, mice were double grafted with BALB/c and K5.mOVA skin. Mice retaining skin grafts at 100 days postgrafting were immunized with OVA/QuilA. Data are pooled from 2–3 experiments per group.
Together, these observations show that 11c.OVA BM transfer, whether or not in the presence of OT-I Tmem, had not established OVA-specific regulation. In line with this, no significant differences were observed in the proportion of CD4+CD25+FoxP3+ Treg between groups, and transfer of 11c.OVA did not lead to differentiation of OT-I Tmem to CD8+FoxP3+ Treg (Fig. 5D).

Deletion Contributes to Ablation of CD8+ Memory Responses After Transfer of Antigen-Encoding BM

Serial analyses of peripheral blood (e.g., Fig. 1A) indicated that after an initial expansion phase, the population of OT-I T cells may have been contracting, possibly through deletion. When we serially sampled blood over time, the frequency of OT-I increased similarly in non-Tg and 11c.OVA BM recipients for 3–4 weeks but then appeared to contract in 11c.OVA BM recipients (Fig. 6A). In spleens, where only late time points were analyzed, a similar pattern was apparent and the number of OT-I T cells significantly dropped in 11c.OVA BM recipients between 28 and 49 days after BM transfer (Fig. 6B). Together, these observations were consistent with a pattern of OT-I Tmem expansion followed by deletion in 11c.OVA BM recipients that would be anticipated based on previous studies (22). As host T-cell dynamics after low-dose irradiation might confound this analysis, we tested the contribution of deletion more directly. The proapoptotic bcl-2 family member bim is a crucial mediator of DC-induced T-cell deletion (25). Therefore, we transferred deletion-resistant OT-I.bim<sup>−/−</sup> Tmem or deletion-competent OT-I Tmem, and 1 week later, BM from non-Tg or 11c.OVA mice was also transferred. OT-I and OT-I.bim<sup>−/−</sup> T cells were enumerated in peripheral blood 1 day before and at regular intervals after BM transfer. In recipients of non-Tg BM, few OT-I T cells accumulated in blood, and the number of these diminished slowly with time (Fig. 6C). In 11c.OVA BM recipients, wild-type (WT) OT-I T cells expanded and then began to diminish in number consistent with slow contraction of the population. On the other hand, deletion-impaired bim-deficient OT-I T cells continued to expand and accumulated in many-fold greater numbers than WT equivalents in blood (Fig. 6C) and in spleen (Fig. 6D). Together these results demonstrate that bim-mediated deletion limits OT-I T accumulation after transfer of 11c.OVA BM and enforces contraction of the OVA-specific Tmem population.

Residual, Unresponsive CD8+ Memory Cells Exhibit a CD5high Phenotype Distinct From “Exhaustion”

Although OT-I Tmem undergo deletion after 11c.OVA BM transfer, the number of residual OT-I Tmem in spleen is similar to or higher than that in recipients of non-Tg BM (e.g., Figs. 1C and 2C), but functional studies (Figs. 1 and 2) indicate that these cells are inactivated. Long-term expression of OVA by DC in 11c.OVA BM recipients (Fig. 5) could induce inactivation by “exhaustion” or other mechanisms. For investigation of this, OT-I Tmem were transferred to recipient mice and then 11c.OVA or non-Tg BM (10<sup>7</sup>) was transferred. CFSE-labeled naive OT-I T cells were transferred as indicated and analyzed 3 days later. Proliferation index is indicated (mean ± SD, n = 4). Data are pooled from 4 experiments (A–C) and show individual mice (mean ± SEM) (n = 4 [sham] or 8 [OVA/QuilA] per group) or representative of 4 mice from 2 separate experiments.

Together, these observations show that 11c.OVA BM transfer, whether or not in the presence of OT-I Tmem, had not established OVA-specific regulation. In line with this, no significant differences were observed in the proportion of CD4+CD25+FoxP3+ Treg between groups, and transfer of 11c.OVA did not lead to differentiation of OT-I Tmem to CD8+FoxP3+ Treg (Fig. 5D).
Figure 5—Transfer of OVA-encoding BM does not establish OVA-specific regulation. A–C: OT-I Tmem were or were not transferred to non-Tg recipients. One week later, mice were irradiated (300cGy) and non-Tg (C57BL/6) or 11c.OVA BM was transferred. Six weeks after BM transfer, 5 x 10^6 naive CFSE-labeled OT-I cells were transferred and mice immunized with OVA/QuilA subcutaneously. CFSE dilution in the draining (inguinal) and nondraining (pooled axillary, mesenteric) LN was assessed 3 days later by flow cytometry. D: OT-I Tmem were transferred to non-Tg mice that were irradiated (300cGy) and injected intravenously with BM from non-Tg or 11c.OVA donors (BMT) 1 week later. Controls (no-BMT) were non-Tg or 11c.OVA mice injected with Tm in parallel with BM transfer test mice. Four weeks after BM transfer, spleen cells were examined by flow cytometry. Data are representative of 6 mice in 2 separate experiments (A) or pooled from two separate experiments (B–D). Lines depict mean ± SEM (n = 4–6 per group).
may be present (26). As programmed cell death protein-1 (PD-1) is considered an archetypal marker of exhaustion and has been implicated in induction and maintenance of tolerance, we examined expression of PD-1 and a range of other markers associated with exhaustion. In non-Tg BM recipients and non-Tg controls, PD-1 was expressed at low levels by approximately one-third of residual OT-I cells (Fig. 7A–C). In 11c.OVA BM recipients, PD-1 was expressed by the majority of cells at moderate levels (Fig. 7A–C). In 11c.OVA controls, PD-1 was expressed on almost all residual OT-I Tmem and at high levels relative to the other experimental groups. CD5 negatively regulates intracellular signaling in lymphocytes and dampens T-cell responsiveness by inhibiting TCR signaling (27). CD5 expression has, however, typically been associated not with chronic viral infection–induced exhaustion but, rather, with T-cell “tuning” in response to chronic antigen exposure. Relatively little CD5 was expressed by OT-I in non-Tg BM recipients and non-Tg controls, but a small population expressed moderate levels (Fig. 7A and B). Data were pooled from 2–3 experiments per time point with typically 2–4 mice per group, some of which were sham immunized (PBS/QuilA) at day 42 (A and B), or pooled from 2 experiments (C and D). Data show individual points or mean ± SEM. B: *11c.OVA BM 49 days after BM transfer is significantly lower than 11c.OVA BM 28 days after BM transfer (P < 0.05). C: **OT-I.bim<sup>−/−</sup> 11c.OVA BM is significantly greater than WT OT-I 11c.OVA BM transfer at days 23 and 37 (P < 0.001). **OT-I.bim<sup>−/−</sup> 11c.OVA BM days 23 and 37 significantly greater than days 11 and 18 (P < 0.001).

Figure 6—Deletion is an important contributor to ablation of preexisting memory CD8<sup>+</sup> T-cell responses. A and B: OT-I Tmem (CD45.1<sup>+</sup>/CD45.2<sup>+</sup>) were transferred to non-Tg mice (B6.SJL, CD45.1<sup>+</sup>) and, 1 week later, mice were irradiated (300cGy) and injected intravenously with BM (10<sup>7</sup>) from non-Tg (C57BL/6, CD45.2<sup>+</sup>) or 11c.OVA (CD45.2<sup>+</sup>) donors. At the indicated time points, blood or spleen was examined by flow cytometry. C and D: WT or bim<sup>−/−</sup> OT-I Tmem (CD45.2<sup>+</sup>, 2 × 10<sup>6</sup>) were transferred to non-Tg (B6.SJL, CD45.1<sup>+</sup>) mice. One week later, mice were irradiated (300cGy) and injected intravenously with BM (10<sup>7</sup>) from non-Tg (B6.SJL, CD45.1<sup>+</sup>) or 11c.OVA (CD45.1<sup>+</sup>) donors. OT-I T cells were monitored in blood the day before BM transfer or at the indicated points (C) or in spleen at the end of the experiment (38 days post-BMT) (D). Data are pooled from 2–3 experiments per time point with typically 2–4 mice per group, some of which were sham immunized (PBS/QuilA) at day 42 (A and B), or pooled from 2 experiments (C and D). Data show individual points or mean ± SEM. B: *11c.OVA BM 49 days after BM transfer is significantly lower than 11c.OVA BM 28 days after BM transfer (P < 0.05). C: **OT-I.bim<sup>−/−</sup> 11c.OVA BM is significantly greater than WT OT-I 11c.OVA BM transfer at days 23 and 37 (P < 0.001). **OT-I.bim<sup>−/−</sup> 11c.OVA BM days 23 and 37 significantly greater than days 11 and 18 (P < 0.001).
than those from mice that received 11c.OVA BM transfers (Fig. 7B and C). Of a range of surface markers indicative of T-cell exhaustion, CD160 and to a lesser extent CD244 were expressed by residual OT-I T cells only in non-Tg BM recipients and non-Tg controls, whereas T cell immunoglobulin and mucin domain protein 3 (TIM-3) and lymphocyte-activation gene 3 (LAG-3) were not expressed.

**DISCUSSION**

The autoimmune anti-β-cell response reawakened by islet transplantation is a significant component of recipient immune resistance to islet transplants and remains a significant clinical challenge. Because memory T cells are difficult to control with conventional immunosuppression (3) and are resistant to, for example, cytotoxic drugs and the inhibitory effects of Tregs (6,7,17,28), unwanted, detrimental memory T-cell responses are difficult to control using conventional therapies (5). Thus, current therapeutic approaches are poorly effective or have substantial detrimental side effects, and new approaches are required to control pathogenic memory T-cell responses. Here, using transfer of BM or HSPCs genetically encoding cognate antigen expression under mild, immune-preserving pre-BMT conditioning, we demonstrate a highly effective alternative to current therapies. This approach, which antigen-specifically ablates pathogenic memory T-cell responses, provides an avenue by which autologous HSCT therapies could be modified and optimized to avoid immune suppression while antigen-specifically targeting and preventing the reemergence of detrimental memory T-cell responses.

Memory T cells represent the immunological driving force in many pathological states. As they are programmed...
to rapidly expand and generate large effector populations upon antigen re-encounter, they pose a significant barrier to tissue, cell, and protein replacement therapies in "primed" individuals. Due to their terminal differentiation and costimulation independence, memory T cells have long been considered to be resistant to tolerance induction (29,30). However, pioneering studies including our own, where antigen expression has been enforced in APCs, have shown that memory CD4+ or CD8+ T-cell responses can be silenced (8–10,31–33). We now advance this to show application to the clinical challenge of β-cell–specific memory T-cell responses that represent a strong impediment to islet graft survival. Previous studies showed when using gene-modified BM transfer that restricting antigen expression to differentiated APCs permitted BM engraftment under immune-preserving conditions in naive and primed recipients (22). However, the fate of established memory T cells was not examined. Mechanistic studies here show that with use of this approach, memory CD8+ T-cell populations are inactivated through a combination of deletion and induction of unresponsiveness in residual undeleted cells. Here, we identified deletion by using T-cell donors with a complete functional loss of him. Altered function of some of the bcl family of apoptosis regulators and in deletional pathways has been reported in the NOD mouse (34) and may exist in humans with T1D. While potentially contributing to disease, these alterations are subtle and not comparable to complete gene knockout. In NOD mice, these alterations are unlikely to have a significant impact on tolerance, as our previous studies (11) indicate that enforced expression of the proinsulin islet antigen is sufficient to promote tolerance in this strain. Overall, the data indicate that residual OT-I remaining after deletion, which are rendered unresponsive, exhibit not a classical "exhausted" phenotype but, rather, a phenotype that suggests modulation of responsiveness by mechanisms such as tuning—as indicated by PD-1 and CD5 expression—of TCR responsiveness. In the BM transfer setting, ~30–40% of DCs express OVA and, accordingly, OT-I expression of PD-1 and CD5 is reduced compared with OT-I T cells transferred to 11c.OVA mice, where all DCs express OVA. This is consistent with previous reports that T-cell tuning is dependent on antigen expression levels (35,36). The data also indicate that inactivation is effective even when antigen is expressed by a minority of all DCs. The current study specifically examines the response of CD8+ T cells. We have reported previously that the presence of cognate CD4+ T cells alters the kinetics but does not prevent tolerance induction (15) and that adoptively transferred memory CD4+ T cells are inactivated (10) when antigen is targeted to DCs. In studies where CD4+ T cells have been adoptively transferred to recipients of gene-engineered BM, differentiation of CD4+ Tregs has been reported (37). Such induced CD4+ Tregs are likely to provide bystander suppression and provide additional therapeutic value. Therefore, we propose that this approach would also be effective for control of pathogenic memory CD4+ T-cell responses.

One current approach used clinically to ablate pathogenic T-cell responses in severe autoimmune disease is autologous HSCT. This approach, as currently practiced, while largely effective, relies on the use of cytotoxic agents, typically with or without T-cell depletion (38), to ablate the entire immune repertoire and achieve an immune "reset" in the absence of specific mechanisms leading to protective, antigen-specific tolerance. Some studies have tested the principle of antigen-encoding BM transfer in a primed setting using "nonmyeloablative" approaches, but these have exclusively used T-cell depletion or other immunodepleting strategies (39–41) so that while recipients are ultimately reconstituted with a "tolerant" T-cell repertoire, this is not immune preserving. Our findings represent a significant step forward by providing an immune-preserving procedure and predicate a potential clinically applicable approach to ablating unwanted memory T-cell responses. Additionally, this demonstrates that induction of mixed allogenic chimerism, with its attendant risks, is not required. For instance, the risk of graft-versus-host disease is negated, as the donor cells are syngeneic or autologous to recipients. Similarly, for islet transplantation in T1D there is no risk of disease exacerbation, as replacement islets would be transplanted after T-cell inactivation is complete. If this procedure were to be used to control autoggressive T-cell responses in at-risk individuals, then transient inflammatory exacerbation could potentially be controlled by a short course of immunosuppression. Allogeneic islet or whole pancreas transplantation is currently the only curative therapy for T1D, but its application is limited by several factors including insufficient donor pancreata and the substantial side effects of long-term immunosuppression. With significant advances made recently in generation of stem cell–derived β-cells (42,43) and genetically engineered insulin-secreting cells (44,45), it is conceivable that generation of personalized insulin-secreting cells will be feasible in the future. However, persistent anti-islet memory T-cell responses represent a threat to clinical application of such approaches. Further development of gene-engineered BM transfer protocols may lead to regimens that can be applied with less risk than current organ transplant procedures. Under these circumstances, using gene-engineered BM to ablate memory T-cell responses could reduce the requirements for immunosuppression in allogeneic islet transplantation or, in the case of personalized autologous replacement, avoid the need for immunosuppression completely.

The current study was designed to provide proof-of-principle preclinical data and to define the mechanistic contributions to the therapeutic effects of BMT on autoaggressive memory CD8+ T cells. Nevertheless, before this approach can be translated, a number of challenges remain. An ideal translational application of the single antigen approach demonstrated in these preclinical studies would be to protect insulin-secreting cells engineered
from non-β-cells such as hepatocytes (44,45). However, prevention of T1D progression in at-risk or recent-onset human subjects may require a more complex approach. In an appropriate preclinical model, it would be important to demonstrate simultaneous inactivation of responses to multiple relevant antigens and/or bystander tolerance where regulatory T cells induced toward one antigen regulate immunity toward additional relevant local antigens. Additionally, interpretation based on TCR Tg T cells may be limited, and in future studies endogenous antigen-specific cells would need to be examined using tetramers. Humanized mice grafted with relevant human tissue antigens may represent one preclinical model where such advances could be made. Finally, mild conditioning regimens would need to be developed to support HPSC transfer in young individuals with, or at risk for, T1D. Notwithstanding these challenges, we demonstrate a highly effective method to silence autoaggressive islet-destructive memory T-cell responses that, with further development, has clinical potential.

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References