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Tolerance to MHC class II disparate allografts through genetic modification of bone marrow

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Abstract

Induction of molecular chimerism through genetic modification of bone marrow is a powerful tool for the induction of tolerance. Here we demonstrate for the first time that expression of an allogeneic MHC class II gene in autologous bone marrow cells, resulting in a state of molecular chimerism, induces tolerance to MHC class II mismatched skin grafts, a stringent test of transplant tolerance. Reconstitution of recipients with syngeneic bone marrow transduced with retrovirus encoding $H\text{-}2I\text{-}A^b$ (I-A^b) resulted the long-term expression of the retroviral gene product on the surface of MHC class II-expressing bone marrow derived cell types. Mechanistically, tolerance was maintained by the presence of regulatory T cells, which prevented proliferation and cytokine production by alloreactive host T cells. Thus, the introduction of MHC class II genes into bone marrow derived cells through genetic engineering results in tolerance. These results have the potential to extend the clinical applicability of molecular chimerism for tolerance induction.

Keywords

Tolerance; molecular chimerism; T cells; Treg

INTRODUCTION

Transplantation tolerance, defined as long-term allograft survival without ongoing immunosuppression, remains a major goal in the field of transplantation medicine. Bone marrow derived hematopoietic cells have been shown to be capable of inducing transplantation tolerance (1, 2), and tolerance to allogeneic transplants in adults can be

established by inducing a state of mixed hematopoietic chimerism through allogeneic bone marrow transplantation (2, 3). Mixed chimerism leads to specific tolerance, and permits transplantation of organs matched to the donor bone marrow without immunosuppression (4). However, the use of allogeneic bone marrow transplantation to induce tolerance is associated with serious complications, (5-11). In addition, it has been difficult to reliably establish a stable state of mixed host-donor hematopoietic chimerism in primates (12-15). Further decreasing the clinical applicability of bone marrow chimerism as an approach to tolerance induction, it has been reported that induction of mixed chimerism through bone marrow transplantation is unable to prevent chronic allograft rejection (16), currently the major factor limiting long-term survival of transplants (17-19).

As an alternative to mixed chimerism, gene therapy approaches can be used to express alloantigens in syngeneic bone marrow cells, resulting in molecular rather than cellular chimerism. The induction of molecular chimerism avoids several of the serious complications associated with mixed cellular chimerism. Because genetically engineered bone marrow cells are of host origin, there is no possibility risk of graft vs. host disease in which allogeneic T cells attack the host tissues (20). The possibility of generating full donor chimerism, which results in immuno-incompetence because developing T cells are selected on host thymic epithelium, but must respond to pathogens in a donor-MHC restricted fashion (11), does not exist in molecular chimeras since all engineered bone marrow derived cells express both host and donor MHC molecules. Finally, the difficulty in obtaining a suitable bone marrow donor is avoided since syngeneic bone marrow cells are modified to induce tolerance. These advantages suggest that the induction of molecular chimerism may have clinical applicability in the induction of tolerance.

Tolerance to self is maintained through central mechanisms involving negative selection of self-reactive T cells in the thymus and peripheral mechanisms involving regulatory T cells (Tregs) (21-23). Indeed, it is clear that tolerance to self requires both a central and peripheral component (21). We have previously demonstrated that MHC class I molecular chimerism can induce permanent tolerance to MHC class I mismatched heart and skin allografts (24-26) through both central and peripheral mechanisms (24, 26-29). Notably, chronic rejection of cardiac allografts was not observed in this model. Furthermore, we have demonstrated that tolerance to cytoplasmically expressed antigens can also be induced through molecular chimerism (30). However, to date there have been no reports of successfully inducing tolerance to MHC class II alloantigens through molecular chimerism. To extend the applicability of molecular chimerism for tolerance induction, we examined whether retrovirally mediated expression of MHC class II could induce durable tolerance.

RESULTS

Efficient transduction of bone marrow with retroviruses encoding MHC class II I-A^b

To generate retroviral vectors encoding *H-2I-A^b* (I-A^b), constructs were prepared in which the cytoplasmic tail of the beta chain of I-A^b was fused to enhanced GFP (GFP) to facilitate tracking of MHC class II chains in hematopoietic cells, as previously described (31). I-A^b beta chain–GFP fusion genes were then cloned into the pMMP retroviral vector. Next, the full-length cDNA encoding the alpha chain of I-A^b was cloned into the pMMP-I-A^b vector, downstream of an IRES sequence, to generate a bicistronic vector (Figure 1A). This vector encodes the I-A^b beta chain fused to GFP, and the alpha chain of I-A^b. The pMMP (32) vector encoding GFP alone was used as a control in all experiments (Figure 1A). VSV-G protein enveloped retroviruses were generated in 293T cells by transient transfection of constructs as previously described (24), hereafter referred to as VSV-IA^b, and VSV-GFP respectively.

To validate the ability of VSV-IA^b to confer expression of I-A^b, A20 (*H-2*^{d}) cells were transduced with VSV-IA^b. After 48 hours, cells were stained with a monoclonal antibody specific for I-A^b (AF6-120.1) and analyzed by flow cytometry. Expression of GFP was used as a marker of viral transduction. We observed that GFP$^+$ A20 cells expressed detectable levels of I-A^b α chain on the cell surface (Figure 1B). We next stained A20 cells transduced with VSV-IA^b with a monoclonal antibody that specifically recognizes the I-A^b α/β -peptide complex (KH74 (33)). We observed significant expression of the I-A^b complex on the surface of A20 cells that were GFP$^+$ (Figure 1B). These results indicate that VSVIA^b confers expression of both the alpha and beta chains of I-A^b, and that these retrovirally-encoded proteins are expressed on the cell surface. To determine the ability of VSV-IA^b retrovirus to infect primary murine bone marrow cells, we used GFP as a pseudomarker of I-A^b expression. Bone marrow from 5-FU treated B10.MBR mice was isolated and transduced with either VSV-IA^b or VSVGFP retrovirus. After 72 hours, GFP expression was analyzed by flow cytometry. Mock transduced bone marrow was used as a staining control. Transduction of B10.MBR bone marrow with VSV-IA^b led to expression of GFP in 12.7±3% of cells (Figure 1C). Transduction of bone marrow with VSV-GFP led to expression of GFP in 29.7±14.7% of cells (Figure 1C). GFP was expressed in cells following transduction of bone marrow with either VSV-IA^b or control virus VSV-GFP at a consistently high level suggesting that I-A^b is not inherently unstable in these cells as has been previously reported (34). However, the median fluorescent intensity of GFP in bone marrow cells transduced with VSV-GFP was higher than in VSV-IA^b transduced cells, reflecting lower expression of GFP when expressed as a fusion with I-A^b β chain as observed previously (31).

Mice reconstituted with bone marrow transduced with VSV-IA^b exhibit long-term expression of retrovirally-encoded MHC class II in multiple hematopoietic lineages

We next examined expression of virally encoded MHC class II in the peripheral blood of B10.MBR mice reconstituted with VSV-IAb, or mock transduced syngeneic bone marrow eight weeks after bone marrow transplantation. We observed GFP expression in 20±6% in CD4$^+$ cells, 24±9% of CD8$^+$ cells, 41±4% of Mac-1$^+$ cells, 29±6% of B220$^+$ cells and 43±8% of CD11c$^+$ cells (Figure 2A) in mice that received VSV-IA^b transduced bone marrow. As expected there was no GFP expression in mock transduced controls. We next examined the expression of I-A^b on the cell surface of transduced GFP$^+$ PBMC. 10 weeks after reconstitution, PBMC were harvested and treated with LPS for 48 hours to increase MHC class II expression. Cells were then stained with antibodies specific for B220, I-A^b and endogenous I-A^k. In GFP$^+$ transduced B220+ B cells from mice that received VSV-IA^b transduced bone marrow, detectable levels of I-A^b were observed (Figure 2B), suggesting that the products of the retrovirally encoded MHC class II genes were expressed on the cell surface. GFP negative cells from the same animals did not express I-A^b. Moreover, we did not observe expression of I-A^b on cells from control mice reconstituted with VSV-GFP transduced bone marrow. Thus, VSV-IAb specifically confers I-A^b expression in progeny of transduced progenitors. Expression of retrovirally encoded MHC class II I-A^b did not measurably affect expression of the endogenous I-A^k chain. GFP+ and GFP- cells from the same animal expressed similar levels of I-A^k chain expression regardless of expression of I-A^b (Figure 2B). When CD4 and CD8 T cells were examined, we were unable to detect I-A^b expression on the cell surface, as expected, since in mice T cells do not express MHC class II (Figure 2B). Thus, our retroviral construct conveyed cell surface expression of allogeneic MHC class II only in MHC class II-expressing cell types.

To analyze long-term long term expression of I-A^b in recipients of VSV-IAb transduced bone marrow cells, B10.MBR recipients were reconstituted with 4×10^6 VSV-IA^b or mock transduced bone marrow cells. After 24 weeks animals were sacrificed, and expression of

GFP and I-A^b was analyzed by flow cytometry. Detectable levels of GFP were observed in the spleen of animals reconstituted with bone marrow transduced with VSV-IAb (Figure 2C). Mice reconstituted with VSV-IAb transduced bone marrow also expressed GFP in bone marrow, thymus, blood and lymph nodes (data not shown). As expected, GFP was not expressed in cells from animals reconstituted with mock transduced bone marrow cells. Taken together, these data indicate that long-term, cell surface expression of retrovirally-encoded MHC class II was achieved.

Expression of retrovirally encoded I-A^b induces tolerance to MHC class II mismatched skin allografts

To determine the ability of retrovirally encoded I-A^b to induce immunologic tolerance, B10.MBR (K^b I-A^k, I-E^k D^q) mice were reconstituted with VSV-IAb or VSV-GFP transduced bone marrow. After eight to ten weeks, mice reconstituted with either VSV-IAb or VSV-GFP transduced bone marrow received both a MHC class II mismatched B10.QBR (K^b, I-A^b,I-E^{null}, D^q) and a third-party BALB/c (H-2^d) skin graft. B10.MBR mice reconstituted with VSV-GFP transduced bone marrow rapidly rejected both B10.QBR (Figure 3A, median survival time (MST)= 14 days, n=12) and BALB/c skin grafts (Figure 3B, MST= 16 days, n=12). In contrast mice reconstituted with VSV-IAb transduced bone marrow accepted B10.QBR skin grafts long term (Figure 3A, MST>100 days, n=9, $p <$ 0.006), but rejected third party BALB/c skin grafts rapidly (Figure 3B, MST= 14 days, n=9) indicating that tolerance was specific to grafts expressing I-A^b.

Decreased T cell proliferation in response to allogeneic stimulators in mice expressing retrovirally-encoded allogeneic MHC class II

We next examined the effect of expressing retrovirally encoded I-A^b on proliferation of alloreactive T cells. B10.MBR mice reconstituted with VSV-IAb or control VSV-GFP transduced bone marrow were immunized with 10^7 irradiated B10.QBR splenocytes. After 10 days, splenocytes were harvested, stained with CFSE and cultured with irradiated B10.QBR or BALB/c splenocytes for 72 hours. T cells derived from mice reconstituted with VSV-GFP transduced bone marrow proliferated in response to either B10.QBR or third party BALB/c splenocytes (Figure 4, $p <$0.001 in comparison to syngeneic stimulators). In contrast, T cells derived from mice reconstituted with bone marrow transduced with VSV-IAb did not proliferate when cultured with B10.QBR stimulators, although they did proliferate in response to third party BALB/c splenocytes (Figure 4, $p <$0.05 in comparison to syngeneic stimulators), suggesting that suppression of T cell proliferation is specific. The response of splenocytes from mice reconstituted with bone marrow transduced with VSV-IAb to third party BALB/c splenocytes appeared to be lower than that of mice reconstituted with VSV-GFP transduced bone marrow, although this did not reach statistical significance (Figure 4, $p >$ 0.05). However, mice reconstituted with bone marrow transduced with VSV-IAb were able to rapidly reject skin grafts from BALB/c mice (Figure 3B), suggesting that responses to third party antigens remain intact in these mice.

Induction of molecular chimerism prevents cytokine production by alloreactive T cells

We next determined the ability of T cells from B10.MBR mice reconstituted with VSV-IAb or VSV-GFP transduced bone marrow to produce effector cytokines following stimulation with irradiated MHC class II mismatched B10.QBR splenocytes. B10.MBR mice were reconstituted with VSV-IAb or VSV-GFP transduced bone marrow as described above and then sacrificed eight to ten weeks after reconstitution. Splenocytes were harvested and cultured for 48 hours together with irradiated B10.QBR splenocytes. To measure the alloreactive response, production of IL-2, IL-4, or IFN-γ was analyzed by performing cytokine ELISPOT assays. We observed relatively few IL-2 (13 ± 4 vs. 661 ± 52 per 10^6), IL-4 (28 ± 10 vs. 208 ± 20 per 10^6) and IFN-γ (0.67 ± 0.58 vs. 88 ± 60 per 10^6) producing

cells (Figure 5A) in the spleens of mice reconstituted with VSV-IAb transduced bone marrow when compared to the number observed in the spleens of mice reconstituted with VSV-GFP transduced bone marrow (p <0.05 between groups).

We next analyzed cytokine production using multiplex Luminex assays. B10.MBR mice were reconstituted with VSV-IAb or VSV-GFP transduced bone marrow as described above. Eight to ten weeks after reconstitution, animals were sacrificed, and splenocytes were cultured for 48 hours with irradiated B10.QBR splenocytes. After 48 hours, cell culture supernatants were harvested and production of GM-CSF, IFNγ, IL12 (p70), IL-17, IL-2, IL-4, IL-5, IL-6, IL-10, MIP-1α, RANTES, and TNFα examined by LUMINEX. When supernatants from cells derived from mice reconstituted with VSV-IAb transduced bone marrow were examined, significantly lower levels of GM-CSF, IFNγ, IL-17, IL-2, IL-4, IL-5, IL-6, IL-10, MIP-1α, and TNFα were produced when compared with supernatants from cells derived from mice reconstituted with VSV-GFP transduced bone marrow (p <0.05 between groups for each cytokine pair, Figure 5B). Taken together these data suggest that expression of retrovirally encoded I-A^b in molecular chimeras significantly impairs the production of cytokines in response to I-A^b-expressing allogeneic stimulators.

Regulatory T cells are required for long-term skin graft acceptance

We have previously shown that tolerance induction through molecular chimerism has both a deletional and a regulatory component (24, 25, 27). To test the degree to which regulatory T cells were involved in tolerance to MHC class II mismatched skin grafts, B10.MBR mice were reconstituted with syngeneic bone marrow transduced with VSV-IAb as described. Eight weeks after reconstitution mice were challenged with a B10.QBR skin graft. As previously observed, B10.MBR mice reconstituted with bone marrow transduced with VSV-IAb accepted B10.QBR skin grafts indicating they were tolerant to I-A^b. 55 days after skin grafting, tolerant mice expressing retrovirally encoded I-A^b were then injected with the anti-CD25 antibody PC61. Antibody treatment effectively reduced the frequency of CD4$^+$CD25$^+$ Treg cells in the blood of B10.MBR reconstituted with bone marrow transduced with VSV-IAb (Figure 6A). We also found that FoxP3$^+$ cells were effectively depleted from blood of these animals (11%±1% of CD4 T cells were FoxP3+ prior to administration of PC61, compared with 6%±2% after administration P<0.02). No significant difference was observed in the total frequency of CD4$^+$CD25$^+$ Treg in mice reconstituted with bone marrow transduced with VSV-IAb when compared with bone marrow transduced with VSV-GFP (Figure 6A). We confirmed that in our system >95% of CD4$^+$CD25$^+$ T cells were also positive for FoxP3 by intracellular staining and flow cytometry (data not shown). B10.MBR mice reconstituted with bone marrow transduced with VSV-IAb rejected their skin grafts rapidly following treatment with anti-CD25 antibody (Figure 6B MST= 17 days relative to the initiation of antibody treatment, n=6 p < 0.01 in relation to animals that did not receive PC61). These data suggest that regulatory T cells are involved in maintenance of tolerance to MHC class II mismatched skin grafts. In the absence of anti-CD25 antibody treatment, skin grafts are accepted for the life of the animal, suggesting that MHC class II gene therapy prevents chronic rejection of skin.

DISCUSSION

There has been a great deal of interest in using gene therapy to induce a state of tolerance in the context of restoring normal immune responses in autoimmune models (35-46), in eliminating immune responses to introduced transgenes in the context of replacement gene therapy (47-49), and in inducing tolerance to allergens (50). These approaches focus on the introduction of a single antigen which is then presented to the immune system on host MHC molecules in genetically modified cells. The presentation of these single antigens on bone marrow derived cells, B cells (43, 44, 51, 52), T cells or dendritic cells (53) has been shown

to reproducibly induce tolerance in many models. However, in the transplantation setting, rejection of an allogeneic organ is not mediated by a single antigen difference, but rather through the recognition of a multitude of self-antigens and alloantigens in the context of allogeneic MHC. This can occur directly on donor cells (direct presentation), or through antigen processing and presentation on host cells (indirect presentation). The combination of direct and indirect presentation results in a high precursor frequency of cells capable of responding to the allogeneic organ, thus presenting a particular challenge to the induction of tolerance.

We have previously shown that the induction of molecular chimerism can be used to induce tolerance (24, 31, 54). In the setting of transplantation, we have shown that inducing molecular chimerism can be used to induce tolerance to MHC class I mismatched allografts (24, 25, 27). Mechanistically, tolerance to MHC class I induced through molecular chimerism occurs through both central deletion of alloreactive T cells (27) and through the induction of regulatory T cells (25). These results demonstrate that molecular chimerism is a powerful tool for the induction of tolerance without the need for extensive conditioning (55), and without the possibility of inducing complications such as GVHD since the bone marrow is syngeneic. However, for clinical transplantation it would be highly desirable to induce tolerance to both MHC class I and MHC class II mismatched organs. We therefore set out to determine whether molecular chimerism with donor MHC class II molecules had an equal capacity to induce long-term tolerance in an allogeneic system.

Our bicistronic retroviral construct encoding MHC class II α and β chains confers cell surface expression of I-A^b on transduced A20 cells without reducing cell surface expression of endogenous I-A^k α chain. The levels of endogenous I-A^k detected on transduced cells was higher than the level of I-A^b. While it is possible that retrovirally encoded MHC class II is less efficiently expressed than the endogenous chains, we suggest that it is more likely that the observed effect is due to the presence of k/b heterodimers on the cell surface as has previously been reported when α/β chain expression is driven by a strong exogenous promoter (56). Our I-A^b specific antibody recognizes I-A^b when complexed with peptide, while the antibody used to detect I-A^k α recognizes both b/k and k/k dimers present on the cell surface. Thus we suggest that increased levels of I-A^k observed may be due to increased detection.

Unlike MHC class I which is expressed on all nucleated cells, MHC class II expression is restricted to a subset of cells including antigen presenting cells such as B cells, macrophages, and dendritic cells (reviewed in (57)). Cell surface expression of MHC class II in these cells is tightly regulated (reviewed in (58)). When bone marrow transduced with VSV-IAb was used to reconstitute conditioned recipients, we observed expression of GFP in all cell lineages examined. However, cell surface expression of I-A^b was observed only on MHC class II expressing cell types. We suggest that the restriction of I-A^b expression to antigen presenting cells is due to the requirement for the cellular machinery that allows for MHC class II expression. Furthermore, we found that expression of the retrovirally encoded MHC class II was inducible. When cells from mice reconstituted with bone marrow transduced with VSV-IAb were examined directly ex vivo, only low levels of retrovirally encoded MHC class II were detected on the surface. However, when cells were stimulated with LPS, expression of MHC class II was up-regulated in fashion similar to endogenous MHC class II. This suggests that the induction of MHC class II in primary cells is controlled by cellular mechanisms unrelated to prevalence of mRNA encoding MHC class II. Taken together, these data suggest that retrovirally encoded MHC class II genes are expressed on the cell surface in a physiologically relevant manner.

Our data demonstrate that reconstitution of mice with VSV-IAb transduced bone marrow results in long-term multi-lineage expression of retrovirally-encoded genes. This point is of particular importance, since we have previously demonstrated that long-term expression of antigen is critical for tolerance induction in molecular chimeras (59). Indeed, long-term multilineage expression of retrovirally encoded I-A^b resulted in long-term acceptance of I-A^b expressing B10.QBR skin allografts. Acceptance of skin grafts is a robust measurement of tolerance induction, and therefore this finding indicates that inducing expression of MHC class II molecules through genetic engineering of autologous bone marrow results is an effective and robust approach that can result in tolerance to allografts.

In contrast to our results, LeGuern et al. have recently reported that expression of MHC class II on bone marrow derived cells is not sufficient to induce tolerance (34). These authors found that the primary T cell response to MHC class II was unchanged in mice receiving MHC class II transduced bone syngeneic marrow. However, they were able to observe prolongation of heart allografts following depletion of CD8 T cells in mice reconstituted with bone marrow transduced with virus encoding I-A, similar to what had previously been observed following CD4 T cell depletion(60). In contrast, we were able to observe long-term survival of skin grafts without the requirement for T cell depletion, a much more rigorous test of tolerance. Thus while we observed tolerance induction, the LeGuern et al. posited only an increase in the generation of regulatory T cells. Notably LeGuern et al. failed to obtain cell surface expression of MHC class II, and expression of MHC class II intracellularly (as measured by GFP as a pseudomarker) was rapidly extinguished. We therefore suggest that, since long-term antigen expression is required for the induction of tolerance (61), the failure to achieve tolerance in this study is related to a failure to achieve substantial long-term MHC class II expression. Our study shows that it would be incorrect to interpret the results of LeGuern et al. (34), and previous work by the same group in large animal models (62) to demonstrate that molecular chimerism with MHC class II fails to induce tolerance, but only that as we have demonstrated, long-term expression of retrovirally encoded products is required.

We have previously shown that induction of tolerance to MHC class I antigens through molecular chimerism results in deletion of alloreactive T cells in the thymus, and the generation of regulatory T cells (25). Depletion of regulatory T cells did not disrupt skin graft tolerance, indicating that central deletion is dominant in the case of mice rendered tolerant to MHC class I following induction of molecular chimerism. Here we have shown induced skin graft tolerance to MHC class II can be reversed through the depletion of CD25$^+$ cells, suggesting that in an MHC class II model of molecular chimerism, regulatory T cells are required for tolerance. In contrast to the assertion by LeGuern et al. that donor specific transfusion is required to "activate" Treg in vivo in mice receiving MHC class II transduced bone marrow (34), our data show that in molecular chimeras, Tregs develop which are directly involved in controlling alloreactive T cell responses. We note that the level of MHC surface expression we were able to achieve may facilitate more efficient Treg generation.

CD4$^+$ regulatory T cells can be either Foxp3$^+$ regulatory T-cells (Tregs) and IL-10-producing regulatory type I (Tr1) cells. Activated Tr1 cells can express CD25, and are characterized by a unique pattern of cytokine expression consisting primarily of IL-10, TGF-β and IL-5 (63-65). While no specific markers allow us to unambiguously distinguish Tr1 cells, we would suggest that Tr1 cells are probably not involved in this model, as we do not detect significant amounts of IL-10.

Our results demonstrate that genetic modification of syngeneic hematopoietic stem cells to express allogeneic MHC genes can be used to facilitate tolerance to allografts, essentially

resulting in matching at MHC class I and II. Given that MHC matching results in profound improvements in long-term survival of allogeneic transplants we suggest that the use of gene therapy may represent one way to improve organ allograft survival.

MATERIALS AND METHODS

Mice

B10.MBR (K^b, I-A^k, I-E^k, D^q), and BALB/c (H-2^d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B10.QBR (K^b, I-A^b, I-E^{null}, D^q) mice were bred in our facility. Mice were housed under microisolator conditions in autoclaved cages and maintained on irradiated feed and autoclaved acidified drinking water. Four to six week old female mice were used in all experiments. All experiments were performed in accordance with the humane use and care policies of our Institution.

Monoclonal antibodies and Flow Cytometry

All cell surface staining and flow cytometry was performed as described previously (66, 67). Monoclonal antibodies (mAbs) specific for CD4 (L3T4), CD8 (52-6.7), I-A^b (KH74 or AF6-120.1), I-A^k (11.5-2), CD25 (7D4), CD11b (Mac-1, M1/70), CD11c (HL3) and B220 (RA3-6B2) were obtained from BD PharMingen (San Diego, CA). FoxP3 staining kit was obtained from eBioscience (San Diego, CA). Depleting/inactivating anti-CD25 (PC61) antibody was the kind gift of Dr. Mohamed Sayegh (Brigham and Women's Hospital, Boston).

Retroviruses

The full-length cDNA encoding *H-2* I-A^b alpha chain was cloned into the MMP retroviral vector containing the I-A^b beta chain fused to enhanced green fluorescent protein (eGFP) previously described (31) to generate pMMP-I-A^b. VSV-G envelope protein pseudotyped viruses were prepared by packaging the pMMP-I-A^b vector in 293T cells by transient transfection, and titered as described (31).

Retroviral transduction of bone marrow cells

Bone marrow cells were harvested from B10.MBR mice and transduced as described previously (67). Briefly, bone marrow cells from mice treated 7 days before with 150 mg/kg 5-Fluorouracil were cultured in tissue culture plates coated with Retronectin (Takara Biomedicals, Shiga, Japan) and transduced in Dulbecco minimum essential medium containing 15% lot-tested fetal calf serum and cytokines to achieve a final concentration of 100 ng/ml human interleukin 6 (IL-6; R&D Systems, Minneapolis, MN), 100 ng/ml recombinant mouse stem cell factor (R&D Systems), 50 ng/ml recombinant mouse thrombopoietin (R&D Systems), and 50 ng/ml recombinant mouse Flt-3 ligand (R&D Systems). All transductions were performed at 37°C with 5% CO_2 for 96 h. Viral supernatants and transduction media were replaced after 72 h. After 96 hours, the cells were harvested, washed twice in Hanks' balanced salt solution, and counted.

Bone marrow transplantation

Mice were conditioned with 11Gy whole-body irradiation 1 day prior to bone marrow transplantation. On the day of reconstitution, 4×10^6 transduced B10.MBR bone marrow cells were injected into the tail vein of recipient mice.

Mixed Lymphocyte Reaction

B10.MBR mice reconstituted with transduced bone marrow as above were immunized intraperitoneally (i.p.) with an injection of 10^7 irradiated (25 Gy) B10.QBR splenocytes. Ten

days after immunization, animals were sacrificed and splenocytes were harvested. Splenocytes were stained with 0.1μM CFSE (Invitrogen, Carlsbad CA) for 8 min at room temperature and cultured in the presence of irradiated (25 Gy) B10.QBR splenocytes or third party BALB/c splenocytes for 96 hrs. Cell proliferation of CD4 and CD8 T cells was assessed by flow cytometry. Percent proliferation was calculated using FloJo software proliferation platform.

ELISPOT Assays

Eight to 12 weeks after B10.MBR mice were reconstituted with transduced bone marrow, recipients were immunized i.p. with 10^7 irradiated (25 Gy) B10.QBR splenocytes. Ten days later animals were sacrificed and single-cell suspensions of splenocytes were prepared and used in a standard cytokine ELISPOT assay as described (66). ELISPOT data are presented as the number of cytokine producing cells per 10^6 T cells.

Luminex

Luminex assays were performed using the Millipore (Billerica, MA) Milliplex Mouse 12 Cytokine/Chemokine Panel kit according to the manufacturer's instructions. Cytokine profile was analyzed on Luminex 100™ (Luminex Corporation, Austin, TX) based on manufacturer's instructions. Duplicate wells were used for each sample.

Skin grafting

Tail skin grafting was performed by a modification of a method previously described (67). Full-thickness tail skin grafts were harvested from the tails of B10.QBR or BALB/c mice. Recipients were anesthetized with 250 mg/kg Avertin and grafted without suturing onto prepared sites on the flanks. Skin grafts were dressed with Vaseline-impregnated gauze and an adhesive bandage for the first 7 days after surgery. Thereafter, skin grafts were assessed three times weekly, and rejection was defined as the first day on which the entire graft surface appeared necrotic.

Statistical Analysis

All statistical calculations were preformed using GraphPad Prism 4.0a software (Graphpad Software, San Diego, CA). The Kaplan and Meier method with a 95% CI was used for the calculation of survival curves. Survival curves were compared using the log rank test. P values <0.05 using Student's t test were considered statistically significant.

Acknowledgments

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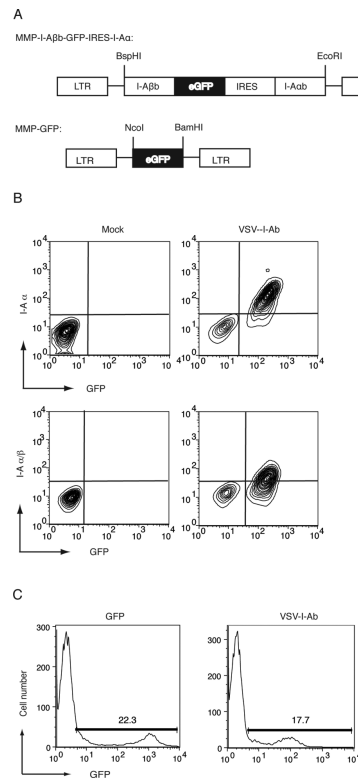
Figure 1. Bicistronic retroviral vector encoding MHC class II confers cell surface expression of I-A^b, and transduces murine bone marrow

(A) Schematic of the bicistronic retroviral construct containing the I-A^b β gene fused to GFP, an internal ribosomal entry site and I-A^b α. Control virus encodes GFP alone (B) A20 cells were transduced with VSV-I-A^b (right panels) or mock transduced (left panels) and cell surface expression of the I-A^b α chain (upper panels) or the I-A^b/ peptide complex (lower panels) was measured by cell surface staining and flow cytometry. Mock transduced A20 cells were used as staining controls. (C) Bone marrow cells were harvested from mice treated 7 days prior with 5-FU, and transduced with VSV-GFP control virus (left panel) or VSV-I-A^b(right panel). 72 hours later, transduction was monitored via GFP fluorescence and flow cytometry. Shown is one representative of three independent experiments.
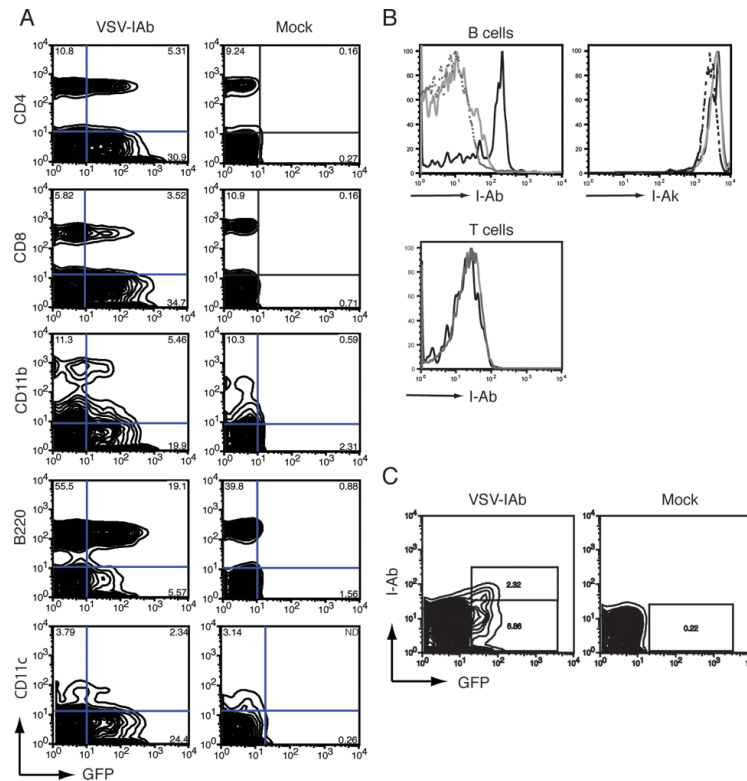
Figure 2. Long-term multi-lineage expression of retrovirally encoded proteins
(A) 4 weeks after reconstitution with bone marrow transduced with VSV-I-A^b (left column) or mock transduced (right column), B10.MBR mice were bled, and peripheral blood mononuclear cells (PBMC) were examined by cell surface staining with the lineage markers CD4, CD8, B220, CD11b and CD11c. Cells were then analyzed by flow cytometry. Shown is one representative of three independent experiments. (B) 11 weeks mice were reconstituted with bone marrow transduced with VSV-I-A^b, PBMC were harvested and stimulated with LPS. After 72 hours, cells were stained with antibodies specific for IA^b, IA^k, B220 and CD3 prior to analysis by flow cytometry. Shown are B220+ cells (upper panels) gated on transduced GFP+ cells (solid black line) or GFP- cells (solid gray line. Cells from naïve B10.MBR stimulated with LPS were used as a control (dashed line). Also shown are GFP+ (black line) and GFP- (gray line) CD3+ T cells (lower panel). (C) 24 weeks after bone marrow reconstitution, recipients of VSV-I-A^b (left panel) or mock (right panel) transduced bone marrow were bled, and GFP and IA^b expression in lymphocytes was analyzed by flow cytometry. Shown is one representative of three independent experiments.
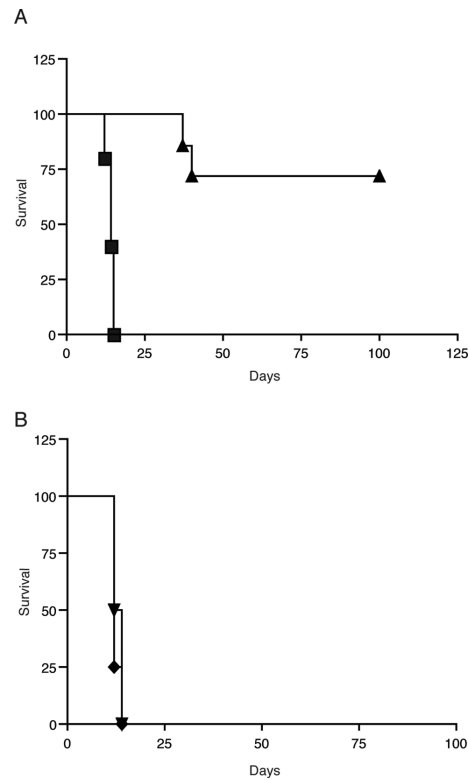
A

B

Figure 3. Expression of retrovirally encoded MHC class II proteins induces specific tolerance to skin grafts

(A) 8-10 weeks after bone marrow reconstitution, B10.MBR recipients of VSV-I-A[b] (triangles) or VSV-GFP (squares) transduced bone marrow received MHC class II mismatched B10.QBR skin grafts. (B) B10.MBR recipients of VSV-I-A[b] (triangles) or VSV-GFP (diamonds) transduced bone marrow also received BALB/c skin grafts. Shown are the combined results of 3 independent experiments.
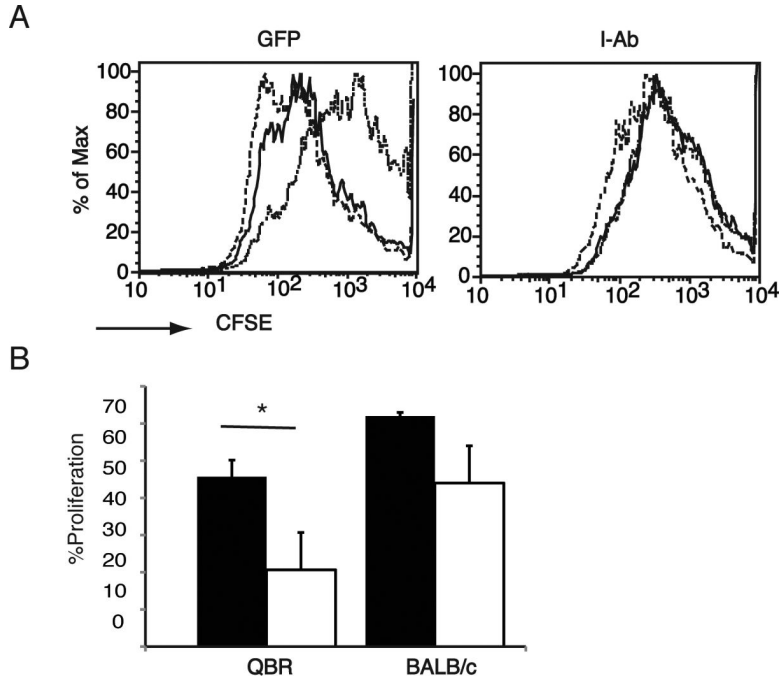
Figure 4. Expression of retrovirally encoded MHC class II prevents T cell proliferation in response to MHC class II mismatched splenocytes
B10.MBR mice were reconstituted with VSV-I-A^b (I-Ab) or control VSV-GFP (GFP) transduced bone marrow. Recipients were immunized with 10^7 irradiated B10.QBR splenocytes and then sacrificed 10 days later. (A) Splenocytes were CFSE labeled and stimulated with irradiated syngeneic B10.MBR (dotted line), or allogeneic B10.QBR (solid line)or BALB/c dashed line. Proliferation was monitored by flow cytometry after 72 hours. (B). The results of the experiment in panel A expressed percent proliferation. VSV-I-A^b (white bars) or VSV-GFP (black bars). Shown is one representative experiment of two. Each experiment assayed 2-3 individual mice per group.
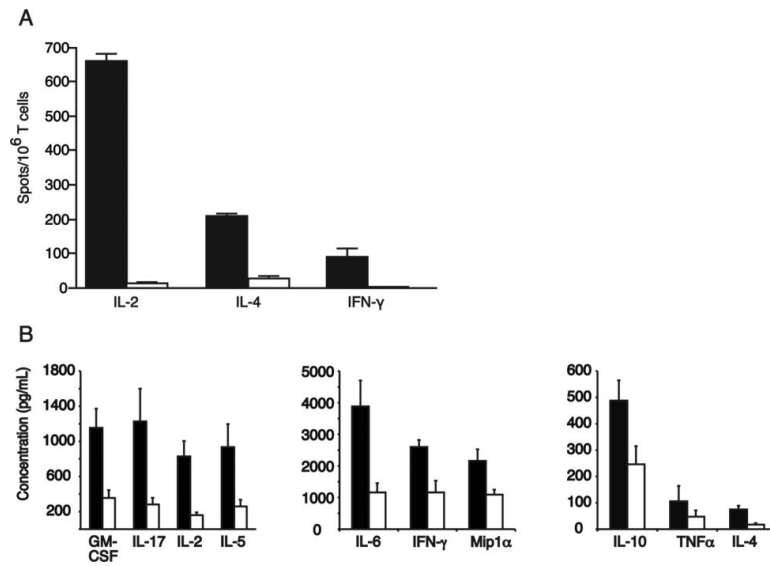
Figure 5. Expression of retrovirally encoded MHC class II prevents T cell cytokine production in response to MHC class II mismatched splenocytes
B10.MBR mice were reconstituted with VSV-I-A^b (white bars) or control VSV-GFP (black bars) transduced bone marrow. 8-10 weeks after reconstitution, mice were immunized with 10^7 irradiated B10.QBR splenocytes and sacrificed 10 days later. (A) Splenocytes were re-stimulated with irradiated B10.QBR splenocytes and analyzed by ELISPOT for IL-2, IL-4 or IFN-γ. Data are presented as spots per 10^6 T cells for each cytokine examined. Shown are the cumulative mean values and standard deviations obtained from three separate experiments. (B) Splenocytes were re-stimulated with B10.QBR or syngeneic B10.MBR splenocytes, and after 48 hours, supernatants were collected and analyzed for cytokine expression by Luminex assay. Background response to B10.MBR has been subtracted from the results shown. Shown are the cumulative mean values and standard deviations obtained from three separate experiments.
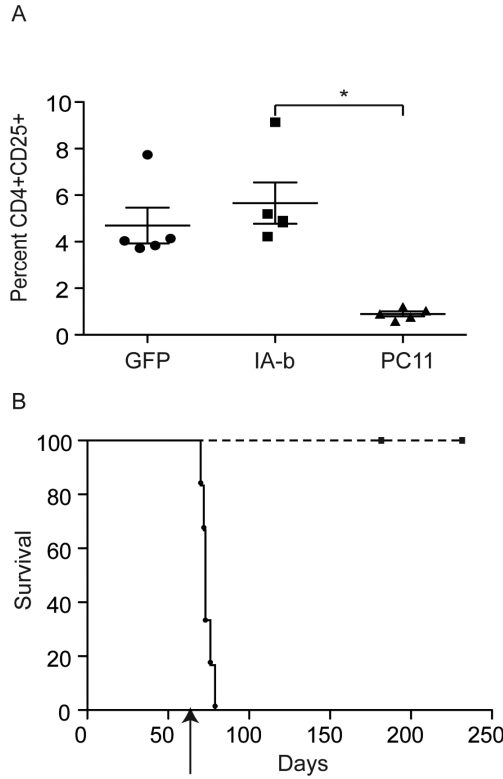
A

B

Figure 6. Tolerance induced by retrovirally encoded MHC class II is dependent on regulatory T cells

A. Frequency of CD4+CD25+ T cells in mice that received VSV-GFP (GFP) or VSV-I-A[b] before and after administration of PC61. Immediately prior to injection with PC61, recipients of bone marrow transduced with VSV-GFP (GFP) or VSV-I-A[b] were bled, and PBMC were examined by cell surface staining and flow cytometry for expression of CD4 and CD25. N=5 for all groups B. 4 weeks after bone marrow reconstitution, B10.MBR recipients of VSV-I-A[b] transduced bone marrow received MHC class II mismatched B10.QBR skin grafts. 56 days after skin grafting, mice were treated with anti-CD25 antibody PC61 (0.125 mg/mouse administered every other day), circles. N=6 Control mice that did not receive antibody are represented by squares. N=8. Shown are combined results of three independent experiments.