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Induction of Central Deletional T Cell Tolerance by Gene Therapy

Eun-Suk Kang and John Iacomini

Transgenic mice expressing an alloreactive TCR specific for the MHC class I Ag K\(^b\) were used to examine the mechanism by which genetic engineering of bone marrow induces T cell tolerance. Reconstitution of lethally irradiated mice with bone marrow infected with retroviruses carrying the MHC class I gene H-2K\(^b\) resulted in lifelong expression of K\(^b\) on bone marrow-derived cells. While CD8 T cells expressing the transgenic TCR developed in control mice reconstituted with mock-transduced bone marrow, CD8 T cells expressing the transgenic TCR failed to develop in mice reconstituted with H-2K\(^b\) transduced bone marrow. Analysis of transgene-expressing CD8 T cells in the thymus and periphery of reconstituted mice revealed that CD8 T cells expressing the transgenic TCR underwent negative selection in the thymus of mice reconstituted with K\(^b\) transduced bone marrow. Negative selection induced by gene therapy resulted in tolerance to K\(^b\). Thus, genetic engineering of bone marrow can be used to alter T cell education in the thymus by inducing negative selection. The Journal of Immunology, 2002, 169: 1930–1935.

The induction of tolerance to transplantation Ags is a major goal in transplantation because of its potential to allow for replacement of host organs without the need for lifelong immunosuppression. One method to establish stable lifelong tolerance to allogeneic transplantation Ags is through the induction of hematopoietic chimerism following allogeneic bone marrow transplantation (1). The establishment of mixed, host and donor, hematopoietic cellular chimerism through bone marrow transplantation leads to specific tolerance in an otherwise fully immunocompetent host (reviewed in Ref. 2). However, the use of allogeneic bone marrow transplantation to induce tolerance has several potential drawbacks, including the severity of the preparative regimen required to achieve bone marrow engraftment, the potential of inducing graft-vs-host disease, and the possibility of engraftment failure. We have recently shown that tolerance can be achieved using a gene therapy-based approach to induce molecular rather than cellular chimerism (3–5). Molecular chimerism is induced by modifying autologous bone marrow cells to express retrovirally transduced Ags. Expression of allogeneic MHC Ags on otherwise autologous bone marrow-derived cells eliminates many of the complications associated with allogeneic bone marrow transplantation used to establish mixed cellular chimerism. Reconstitution of mice with syngeneic bone marrow cells infected with retroviruses carrying the allogeneic MHC class I gene H-2K\(^b\) resulted in lifelong expression of K\(^b\) on bone marrow-derived cells. T cells from mice receiving H-2K\(^b\) transduced bone marrow were unable to kill cells expressing K\(^b\), but were able to kill third party cells. In addition, mice reconstituted with H-2K\(^b\) transduced bone marrow exhibited long-term acceptance of H-2K\(^b\) mismatched skin grafts, although third party control grafts on the same mice were rapidly rejected. Thus, efficient expression of K\(^b\) on bone marrow-derived cells was sufficient to induce tolerance to the product of the transduced MHC gene rather than hyporesponsiveness, which was observed previously in mice expressing low levels of the transduced MHC gene (6–8).

Our results demonstrate that induction of molecular chimerism by gene therapy results in T cell tolerance; however, the mechanism of tolerance induction remains to be elucidated. We hypothesized that, following reconstitution of mice with H-2K\(^b\) transduced bone marrow, tolerance to K\(^b\) may be established by a central deletional mechanism resulting from negative selection of alloreactive T cells in the thymus. Alternatively, functional tolerance could be established by peripheral mechanisms involving induction of T cell anergy (9) or the generation of regulatory T cells (10–12). To address these issues, we made use of BM3.3 TCR-transgenic mice which express an alloreactive TCR on CD8 T cells specific for K\(^b\) that can be detected using the anticonalotypic mAb T98 (13, 14). Our results suggest that induction of molecular chimerism leads to negative selection of alloreactive CD8 T cells in the thymus. Thus, genetic engineering of bone marrow can be used to fundamentally reshape the T cell repertoire by influencing thymic education.

Materials and Methods

Mice

Female B10.AKM/SnJ (H-2K\(^b\), I\(^d\), D\(^o\)), B10.MBR (H-2K\(^b\), I\(^d\), D\(^o\)), and BALB/c (H-2\(^d\)) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The B10.MBR strain was derived from a recombination event that occurred during the back-crossing of B10.AKM to C57BL/10 and differs from the B10.AKM strain in the MHC class I H-2K region (15). BM3.3 TCR-transgenic mice (H-2k, CBA/Ca background (13)) were kindly provided to us by Dr. A. L. Mellor (Medical College of Georgia, Augusta, GA) and bred in our facility. Mice were housed using microisolator conditions in autoclaved cages and maintained on irradiated feed and autoclaved acidified drinking water. All sentinel mice housed in the same colony were viral Ab free. Six- to 12-wk-old female mice were used in all experiments.

Retroviruses

VSV-Kb virus has been described previously (3). Briefly, to generate VSV-Kh, the cDNA encoding H-2K\(^b\) (K\(^b\)) was cloned into the MMP retroviral vector kindly provided by Dr. R. C. Mulligan (Children’s Hospital, Boston, MA) to generate pMMP-K\(^b\). The MMP retroviral vector is a derivative of

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VSV-Kb used in this report was obtained from the Gene Therapy Initiative. The viral titer obtained for the preparation of VSV-Kb virus was determined by analyzing expression of K\textsuperscript{b} on NIH-3T3 cells by cell surface staining and flow cytometry following infection. All virus preparations were made in affiliation with the Harvard Institute for Human Genetics Gene Therapy Initiative. The viral titer obtained for the preparation of VSV-Kb used in this report was \(2 \times 10^6\) infectious particles per milliliter.

**Retroviral transduction of bone marrow cells**

Bone marrow cells were harvested from B10.AKM mice and transduced as described previously (3, 4). Briefly, bone marrow cells from mice treated 7 days prior with 5-fluorouracil (150 mg/kg) were cultured in Retronectin (Takara Biomedicals, Shiga, Japan)-coated tissue culture plates in transduction medium (DMEM containing 15% lot tested FCS and cytokines to achieve a final concentration of 100 ng/ml human IL-6 (R&D Systems, Minneapolis, MN), 100 ng/ml recombinant mouse stem cell factor (BioSource International, Camarillo, CA), 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\textsubscript{2} for 96 h. Retronectin-coated plates were prepared according to the manufacturer’s instructions. Bone marrow cells were cultured at a density of 6 \(\times\) 10\textsuperscript{6} cells per milliliter together with VSV-Kb virus to achieve a multiplicity of infection of \( \sim 1\). Viral supernatants and transduction medium were placed at 72 h after the start of the transduction. Mock transductions were performed in the same manner, except viral supernatants were replaced with transduction medium. Twenty-four hours later, the cells were harvested, washed twice in HBSS, and counted.

**Bone marrow transplantation**

BM3.3 bone marrow was harvested from mice treated 7 days prior with 5-fluorouracil as described above. All BM3.3 bone marrow donors also received a depleting dose of anti-CD8 (116-13.1 (19)) and CD4 (GK1.5 (20)) mAbs 4 days before bone marrow harvest to eliminate alloreactive T cells in vivo. Transduced B10.AKM and freshly isolated BM3.3 bone marrow cells were then used to reconstitute B10.AKM mice, which received a lethal dose of irradiation (10.25 Gy), a depleting dose of the anti-CD8 (116-13.1 (19)) and anti-CD4 (GK1.5 (20)) before bone marrow harvest led to complete depletion of T cells from bone marrow (data not shown).Recipient B10.AKM mice were then conditioned with lethal irradiation (10.25 Gy) and a depleting dose of anti-CD8 and anti-CD4 mAbs, and reconstituted with the following dose varying with ratios of cultured B10.AKM and BM3.3 bone marrow. Mice received 2–3 \(\times\) 10\textsuperscript{6} B10.AKM bone marrow cells and decreasing doses of BM3.3 bone marrow cells.

By \(~9\) wk following bone marrow transplantation, mice receiving a ratio of 5:6:1 B10.AKM:BM3.3 bone marrow cells consistently contained a population of CD8 T cells in their peripheral blood that was detectable by cell surface staining and flow cytometry with the anti-BM3.3 clonotypic Ab T98. Approximately 2.9 \(\pm\) 1.2% \((n = 5)\) of CD8 T cells in blood expressed the BM3.3 TCR, detectable using T98. At 19 wk after bone marrow transplantation, as high as 10% of CD8 T cells expressed the BM3.3 TCR (Fig. 1). Increasing the proportion of BM3.3 cells used for reconstitution led to a dominance of BM3.3 TCR-positive cells (data not shown). Clonotype-positive CD8 T cells were not detectable in the blood of either naive B10.AKM controls or against K\textsuperscript{b}, we first set out to reduce the frequency of BM3.3 CD8 T cells by generating bone marrow irradiation chimeras. This was done to obtain mice containing a population of alloreactive T cells which exists at physiologically relevant frequencies and could be tracked using the anti-BM3.3 clonotypic Ab T98.

B10.AKM (H-2\textsuperscript{k}, R, D\textsuperscript{k}) bone marrow was harvested from mice treated 7 days prior with 5-fluorouracil and cultured in vitro using mock transduction conditions. This approach was used to ensure that the reconstitution potential of the bone marrow would be similar to that observed after retroviral transduction in later experiments. BM3.3 (H-2\textsuperscript{k}, CBA/Ca background) bone marrow was similarly harvested from 5-fluorouracil-treated mice; however, BM3.3 bone marrow cells were not cultured in vitro in any experiments. In addition, BM3.3 mice also received a depleting dose of anti-CD8 and anti-CD4 mAbs before bone marrow harvest to eliminate alloreactive T cells in the bone marrow. Treatment of BM3.3 mice with anti-CD8 (116-13.1 (19)) and anti-CD4 (GK1.5 (20)) before bone marrow harvest led to complete depletion of T cells from bone marrow (data not shown).

**Skin grafting**

Tail skin grafting was performed and evaluated as previously described (23).

**Results**

**Development of a TCR-transgenic model to examine mechanisms of tolerance induced by gene therapy**

To create a model in which the fate of alloreactive T cells could be monitored in mice reconstituted with H-2\textsuperscript{k} transduced bone marrow, BM3.3 transgenic mice that express an alloreactive TCR on CD8 T cells were used (13). However, because essentially 100% of CD8 T cells in BM3.3 mice are alloreactive...
B10.AKM mice reconstituted with B10.AKM bone marrow cells alone (Fig. 1).

Generation of B10.AKM-BM3.3 chimeras following retroviral transduction of bone marrow with retrovirus carrying the gene encoding Kβ

To examine how expression of a retrovirally transduced MHC class I gene in bone marrow-derived cells affected the development of alloreactive T cells, B10.AKM bone marrow was harvested from 5-fluorouracil-treated mice and either transduced with vesicular stomatitis virus G protein-enveloped retroviruses carrying the gene encoding H-2Kβ, hereafter referred to as VSV-Kb as described (3), or mock transduced. Immediately following transduction with VSV-Kb, ~10–20% of B10.AKM bone marrow cells expressed Kβ on their cell surface at levels readily detectable by cell surface staining and flow cytometry (Fig. 2). As expected, Kβ was not detected on the surface of mock transduced cells (Fig. 2). Recipient B10.AKM mice were then conditioned with lethal irradiation (10.25 Gy) and a depleting dose of anti-CD8 and anti-CD4 mAbs. The mice were reconstituted the following day with a 5:1 ratio of either Kβ or mock transduced B10.AKM (2 × 10^6 total cells per mouse) and BM3.3 bone marrow cells (0.4 × 10^6 total cells per mouse) harvested from 5-fluorouracil-treated BM3.3 mice depleted of T cells in vivo as described above.

Following reconstitution, bone marrow-derived cells expressing Kβ on their surface were detectable by flow cytometry in the blood of mice receiving VSV-Kb transduced marrow at all time points analyzed over the 29-wk follow-up period (Figs. 2 and 3). The percentage of cells in the blood expressing Kβ appeared to remain stable (Fig. 3), suggesting that early progenitor cells had been transduced, as we have observed previously (3). Furthermore, expression of Kβ was detectable on T cells (CD4+ and CD8+), B cells (CD19+), macrophages (Mac-1, CD11b+), dendritic cells (CD11c+), granulocytes (Ly-6G, Gr-1+), and NK cells (DX5+) in peripheral lymphoid tissues (data not shown). In control experiments, the frequency of Kβ-positive cells in mice receiving a mixture of VSV-Kb transduced B10.AKM bone marrow and BM3.3 cells was similar to that observed in control mice receiving VSV-Kb transduced B10.AKM bone marrow alone (data not shown).

FIGURE 2. Analysis of bone marrow transduction efficiency and expression of Kβ on bone marrow-derived cells in the periphery of reconstituted mice. A. Expression of Kβ on the surface of VSV-Kb (solid line) or mock transduced (dashed line) B10.AKM bone marrow before bone marrow transplantation. B. Expression of Kβ on the surface of bone marrow-derived cells in the blood of B10.AKM mice reconstituted with either a mixture of VSV-Kb transduced B10.AKM and BM3.3 bone marrow (solid line) or mock transduced B10.AKM and BM3.3 bone marrow (dashed line) 13 wk after bone marrow transplantation. Shown are representative data.

Expression of retrovirally transduced Kβ on bone marrow-derived cells prevents the development of CD8 T cells expressing the BM3.3 TCR

Starting at 3 wk after bone marrow transplantation, mice reconstituted with either VSV-Kb or mock transduced B10.AKM and BM3.3 bone marrow cells were bled every other week to examine hematopoietic reconstitution and development of CD8 T cells expressing the BM3.3 TCR. CD8 T cell recovery was complete by ~8 wk after bone marrow transplantation. CD8 T cells expressing the BM3.3 TCR were readily detectable in the blood of B10.AKM mice reconstituted with a mixture of mock transduced B10.AKM and BM3.3 bone marrow cells by cell surface staining with Ti98 (Fig. 4). In contrast, we were unable to detect the presence of CD8 T cells expressing the BM3.3 TCR in the blood of B10.AKM mice reconstituted with a mixture of VSV-Kb transduced B10.AKM and BM3.3 bone marrow cells (Fig. 4). The failure to detect CD8 T cells expressing the BM3.3 TCR was not simply due to a delay in reconstitution, because BM3.3 clonotype-positive CD8 T cells remained undetectable in the blood on mice expressing Kβ on bone marrow-derived cells for up to 29 wk after transplantation, at which time the experiment was terminated (data not shown).

At 19 wk after reconstitution, groups of mice were sacrificed and CD8 T cells from spleen and lymph nodes were analyzed for expression of the BM3.3 TCR by cell surface staining and flow cytometry with Ti98. CD8 T cells expressing the BM3.3 clonotype were readily detected in lymphoid tissues from mice reconstituted with a mixture of mock transduced B10.AKM and BM3.3 bone marrow cells (Fig. 5). In contrast, we were unable to detect BM3.3 clonotype-positive CD8 T cells in peripheral lymphoid organs of mice reconstituted with a mixture of VSV-Kb B10.AKM and BM3.3 bone marrow cells (Fig. 5). Similar results were observed at earlier time points (data not shown).

To determine whether the inability to detect BM3.3 clonotype-positive T cells in mice reconstituted with a mixture of VSV-Kb transduced B10.AKM and BM3.3 bone marrow cells resulted from a failure of BM3.3 bone marrow to engraft, blood cells were harvested from mice reconstituted with either Kβ or mock transduced B10.AKM and BM3.3 bone marrow cells and DNA was isolated. Because BM3.3 transgenic mice carry the genes encoding the transgenic TCR in all tissues, the presence of transgene encoded Vβ2 gene segments in reconstituted mice would be indicative of engraftment, even if CD8 T cells expressing the BM3.3 clonotype
failed to develop. Therefore, PCR primers complementary to the framework region of V<sub>β</sub>2/H9252 and the complementarity-determining region 3 of the BM3.3/H9252-β-chain were synthesized and used to amplify DNA isolated from chimeric mice in each group. Amplification of DNA isolated from eight of eight mice reconstituted with VSV-Kb transduced B10.AKM and BM3.3 bone marrow cells, and seven of eight control mice receiving a mixture of mock transduced B10.AKM and BM3.3 bone marrow produced a PCR product of the correct size using BM3.3 TCR-specific primers. The amount of PCR product generated was similar in samples from both groups of mice (data not shown). Thus, BM3.3 bone marrow was able to engraft in conditioned hosts, suggesting that the failure to detect T cells expressing the BM3.3 TCR in mice expressing K<sub>b</sub> was not due to engraftment failure.

Expression of retrovirally transduced K<sub>b</sub> on bone marrow-derived cells induces negative selection of BM3.3 clonotype-positive T cells in the thymus

To examine whether CD8 T cells expressing the BM3.3 TCR underwent negative selection in mice receiving VSV-Kb transduced bone marrow, we analyzed development of BM3.3 clonotype-positive CD8 T cells in the thymus of mice reconstituted with either K<sub>b</sub> or mock transduced B10.AKM and BM3.3 bone marrow cells using the mAb Ti98. CD4/CD8 double positive as well as CD8 single positive T cells expressing the BM3.3 clonotype were readily detectable in the thymus of B10.AKM mice reconstituted with a mixture of mock transduced B10.AKM and BM3.3 bone marrow cells (Fig. 6). In contrast, B10.AKM mice reconstituted with a mixture of VSV-Kb transduced B10.AKM and BM3.3 bone marrow cells contained in their thymus relatively few clonotype-positive CD4/CD8 double positive T cells (Fig. 6). CD8 single positive T cells expressing the BM3.3 receptor were not detected in the thymus of mice reconstituted with VSV-Kb transduced B10.AKM and BM3.3 bone marrow cells (Fig. 6). These data strongly suggest that expression of a retrovirally transduced allogeneic MHC class I gene in bone marrow-derived cells results in negative selection of alloreactive CD8 T cells in the thymus.

Mice reconstituted with a mixture of VSV-Kb-transduced B10.AKM and BM3.3 bone marrow are tolerant to K<sub>b</sub>

To examine the immunological responsiveness to K<sub>b</sub> in mice reconstituted with a mixture of VSV-Kb transduced B10.AKM and BM3.3 bone marrow, mice were grafted with B10.MBR skin. Control mice reconstituted with a mixture of mock transduced B10.AKM and BM3.3 bone marrow cells rejected B10.MBR skin grafts rapidly (Fig. 7). In contrast, mice reconstituted with a mixture of VSV-Kb transduced B10.AKM and BM3.3 bone marrow cells accepted their B10.MBR skin grafts for >95 days (p < 0.0003 between groups, Fig. 7). In control experiments, mice in both groups were able to rejected third party BALB/c skin graft within 15 days, demonstrating that both groups of mice were immunocompetent.
Discussion

Inducing tolerance to transplantation Ags has the potential to overcome rejection without the use of lifelong immunosuppression and therefore remains a major goal in the field. Building on the concept of hematopoietic chimerism, we have developed a gene therapy-based approach designed to induce tolerance following the induction of molecular chimerism. We have shown previously that establishing molecular chimerism results in functional tolerance (3). However, it is important to note that tolerance can be induced by a variety of mechanisms, including negative selection in the thymus, induction of T cell anergy in the periphery, or the generation of regulatory T cells. Central deletional tolerance which occurs in the thymus during T cell development as a result of negative selection is perhaps the most robust form of tolerance, because potentially alloreactive T cells are eliminated from the repertoire (25). In contrast, functional tolerance established by inducing T cell anergy or generating regulatory T cells has the potential of being broken because potentially alloreactive T cells remain, albeit in an unresponsive state. Understanding which mechanisms lead to functional tolerance following the establishment of molecular chimerism is critical in terms of predicting the stability of tolerance induced.

To examine the mechanism by which molecular chimerism induces tolerance, we used BM3.3 mice that express an alloreactive TCR on essentially 100% of CD8 T cells specific for K\(^b\). By generating irradiation bone marrow chimeras using a mixture of B10.AKM and BM3.3 bone marrow, we were able to reduce the frequency of CD8 T cells expressing the BM3.3 receptor to more physiologically relevant levels and generate mice containing a population of alloreactive CD8 T cells that could be reliably tracked using the anticonnotypic Ab Ti98. Expression of K\(^b\) on the surface of bone marrow-derived cells prevented the development of clonotype-positive CD8 T cells in mice reconstituted with a mixture of clonotype-positive CD8 T cells expressing K\(^b\) on bone marrow-derived hematopoietic cells led to negative selection of CD8 T cells expressing the BM3.3 TCR in the thymus. While relatively few CD4/CD8 double positive thymocytes expressing the BM3.3 clonotype were present in mice expressing K\(^b\), CD8 single positive T cells expressing the BM3.3 receptor in the thymus were not detected. These data are consistent with negative selection occurring at the double positive stage of T cell development (26, 27).

In control mice reconstituted with a mixture of mock transduced B10.AKM and BM3.3 bone marrow cells, typically 2–4% of CD8 T cells expressed the BM3.3 receptor. Therefore, the frequency of T cells expressing the alloreactive BM3.3 TCR was significantly greater than the frequency of alloreactive T cell observed naturally, which has been estimated to be in the order of ~1 in 1000 (28). Thus, expression of retrovirally transduced K\(^b\) on bone marrow-derived cells was able to induce negative selection of a relatively large alloreactive T cell precursor population. In mice reconstituted with VSV-Kb transduced B10.AKM and BM3.3 bone marrow, K\(^b\) was expressed long term on multiple hematopoietic cell lineages. Long-term chimeras failed to develop CD8 T cells expressing the BM3.3 TCR (data not shown). We suggest lifelong expression of K\(^b\) on bone marrow-derived cells maintains negative selection of K\(^b\)-reactive CD8 T cells for the life of the animal. Furthermore, based on our skin graft data, mice receiving VSV-Kb transduced bone marrow exhibited long-term survival of B10.MBR skin grafts. These data suggest that, in addition to BM3.3 clonotype-positive cells, endogenous K\(^b\)-reactive T cells derived from B10.AKM bone marrow were also negatively selected in these mice.

In previous studies, inefficient retroviral transduction and expression of retrovirally transduced H-2K\(^b\) induced hyporesponsiveness to K\(^b\) via peripheral mechanisms that could be broken upon Ag challenge (6, 8). Recent work from our laboratory demonstrated that tolerance, rather than hyporesponsiveness, could be induced by gene therapy following efficient transduction and expression of K\(^b\) on bone marrow-derived cells, which was achieved using improved transduction conditions and retroviral vectors (3). T cells from mice receiving H-2K\(^b\) transduced bone marrow were unable to kill targets expressing K\(^b\) even after rigorous Ag challenge and accepted skin grafts expressing K\(^b\) indefinitely (3). We suggest that improved expression of K\(^b\) on the surface of bone marrow-derived cells induced stable T cell tolerance, rather than hyporesponsiveness, by inducing negative selection of alloreactive CD8 T cells in the thymus.

Deletion of alloreactive T cells in the thymus as a result of genetic engineering of bone marrow suggests that tolerance induced by this approach is relatively robust and not subject to being broken by the presence of helper determinants on tissues expressing K\(^b\) (29, 30). Using genetically modified autologous bone marrow to establish molecular chimerism eliminates many of the complications.
associated with allogeneic bone marrow transplantation to establish mixed cellular chimerism, but retains the same ability to induce stable and specific tolerance induced by mixed cellular chimerism, including negative selection in the thymus (2). To our knowledge, this is the first example in which gene therapy has been shown to induce negative selection of alloreactive T cells. The observation that efficient expression of retrovirally transduced allogeneic antigen on bone marrow–derived cells is sufficient to induce T cell negative selection in the thymus supports the notion that specific immunological tolerance induce by gene therapy can be used to fundamentally reshape the immunological repertoire. Similar approaches may eventually be applicable for the induction of tolerance in immunological disorders such as autoimmune disease.

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