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Induction of transplantation tolerance by combining non-myeloablative conditioning with delivery of alloantigen by T cells

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Abstract

The observation that bone marrow derived hematopoietic cells are potent inducers of tolerance has generated interest in trying to establish transplantation tolerance by inducing a state of hematopoietic chimerism through allogeneic bone marrow transplantation. However, this approach is associated with serious complications that limit its utility for tolerance induction. Here we describe the development of a novel approach that allows for tolerance induction without the need for an allogeneic bone marrow transplant by combining non-myeloablative host conditioning with delivery of donor alloantigen by adoptively transferred T cells. CBA/Ca mice were administered 2.5Gy whole body irradiation (WBI). The following day the mice received K^b disparate T cells from MHC class I transgenic CBK donor mice, as well as rapamycin on days 0–13 and anti-CD40L monoclonal antibody on days 0–5, 8, 11 and 14 relative to T cell transfer. Mice treated using this approach were rendered specifically tolerant to CBK skin allografts through a mechanism involving central and peripheral deletion of alloreactive T cells. These data suggest robust tolerance can be established without the need for bone marrow transplantation using clinically relevant non-myeloablative conditioning combined with antigen delivery by T cells.

Keywords

Tolerance; T cells; Transplantation; Negative Selection; Non-myeloablative conditioning

INTRODUCTION

It has been known for some time that bone marrow derived hematopoietic cells are potent inducers of transplantation tolerance [1,2]. One of the most reliable ways to establish life-long tolerance to allogeneic transplants in adults is to induce a state of mixed hematopoietic chimerism through allogeneic bone marrow transplantation [3,4]. Mixed chimerism leads to specific tolerance, and permits transplantation of organs matched to the donor bone marrow without immunosuppression [5]. However, the use of allogeneic bone marrow transplantation to induce tolerance is associated with serious complications, such as graft-vs-host disease

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(GvHD), the inability to recover complete immunocompetence, engraftment failure, and infectious complications [6–11].

Recently, we have shown that in the context of hematopoietic chimerism induced by bone marrow transplantation expression of donor major histocompatibility complex (MHC) on bone marrow derived lymphocytes is required to induce tolerance to allogeneic skin grafts [12]. We have also shown that adoptive transfer of alloantigen expressing mature T cells into lethally irradiated mice undergoing a syngeneic bone marrow transplant can be used to induce tolerance to skin grafts matched to the T cell donor strain [13]. Induction of tolerance appears to be related, at least in part, to the ability of adoptively transferred alloantigen expressing T cells to re-enter the thymus [14] and mediate negative selection of developing alloreactive T cells [13]. Importantly, these data suggest that delivery of alloantigen by mature T cells can reshape the immune repertoire.

While the use of T cells to deliver alloantigen in mice receiving lethal whole body irradiation (WBI) and syngeneic BMT represents a novel approach to tolerance induction, avoidance of lethal WBI and its inherent toxicity and the need for harvesting autologous hematopoietic stem and progenitor cells is desirable for clinical translation. We therefore set out to examine whether expression of alloantigen on adoptively transferred T cells is sufficient to allow for tolerance induction in hosts prepared using a non-myeloablative conditioning regimen consisting of low dose WBI, rapamycin and CD40-CD154 blockade. Our data indicate that adoptive transfer of MHC class I disparate T cells alone into hosts receiving low dose WBI, rapamycin and anti-CD154 allows for the induction of long-term tolerance to MHC class I disparate skin allografts without the need for bone marrow transplantation. Mechanistically, tolerance induced by this approach involved negative selection of alloreactive T cells.

MATERIALS AND METHODS

Mice

CBA/CaJ (*H-2^k*) and BALB/cByJ mice were purchased from The Jackson Laboratory, Bar Harbor, ME. CBK (*H-2K^b* transgenic mice on the CBA/Ca background [15]) and BM3.3 T cell receptor (TCR) transgenic mice (*H-2^k*, CBA/Ca background [16]) were kindly provided by Dr. Andrew L. Mellor, Medical College of Georgia, Augusta, GA. Mice were housed using micro-isolator conditions in autoclaved cages and maintained on irradiated feed and autoclaved drinking water. All sentinel mice housed in the same colony were viral antibody-free. Six to eight week old female mice were used in all experiments. All experiments involving were conducted in accordance with institutional guidelines.

Antibodies

Monoclonal antibodies specific for *H-2K^b* (AF6-88.5), CD3 (2C11), CD4 (RM4-5), CD8 (53-6.7), B220 (RA3-6B2), CD11b (Mac-1, M1/70), NK cells (DX5) and CD11c (HL3), Ly-6G (Gr-1, RB6-8C5) were obtained from BD PharMingen (San Diego, CA). The hybridoma secreting the BM3.3 anti-clonotypic antibody Ti98 was kindly provided by Dr. A. L. Mellor [17]. Ti98 Ab was purified in our laboratory and labeled with FITC using standard methods.

Development of BM3.3-CBA chimeras

Bone marrow cells were harvested from femurs of CBA/CaJ and BM3.3 mice as described [13]. CBA/CaJ recipients were irradiated (10.25Gy) using a ¹³⁷Cs source. Radiation was given at a dose rate of 1Gy per minute. Mice were reconstituted the following day with a mixture of 12×10^6 CBA/CaJ and 2×10^6 BM3.3 freshly isolated bone marrow cells.

T cell purification, host conditioning and adoptive transfer

T cells were prepared as described [13]. Briefly, spleens from donor mice were harvested and single cell suspension was prepared. CD4⁺ and CD8⁺ T cells were positively selected using magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The resulting cells were then further purified to 99% purity by cell sorting based on expression of CD4 and CD8 as described [12]. Prior to adoptive cell transfer, recipients received 2.5 Gy WBI. The following day, day 0, mice were injected i.v. with 10⁷ CBK T cells or syngeneic CBA/CaJ T cells as described [13]. Recipient mice were also treated with 1.5mg/kg/day rapamycin (Wyeth, Madison, NJ) diluted in carboxymethylcellulose and polysorbate solution by i.p. injection on days 0–13 relative to T cell transfer and 0.2mg/day anti-CD154 [18] in PBS on days 0–5, 8, 11 and 14.

Flow cytometry

Cell surface staining and flow cytometry was performed as described previously [19]. Expression of K^b was evaluated as described in [12]. Antibodies specific for *H-2K^b* (AF6-88.5), CD4 (RM4-5), CD8 (53-6.7), B220 (RA3-6B2), CD11b (Mac-1, M1/70), and CD11c (HL3) were obtained from BD Biosciences Pharmingen (San Diego, CA). Peripheral blood was processed and stained in FACS lysing solution (Becton Dickinson, San Jose, CA) according to manufacturer's instructions.

Skin grafting

Eight weeks after adoptive T cell transfer, mice received both a CBK and a third-party BALB/cByJ (*H-2^d*) skin graft. Tail skin grafting was performed and evaluated as previously described [20].

Histology

Tissue sections were prepared and stained as described previously [21]. Histology was performed one hundred days after skin transplantation.

RESULTS

Long-term persistence of adoptively transferred alloantigen expressing T cells in hosts receiving non-myeloablative conditioning

In order to examine whether adoptive transfer of alloantigen expressing T cells could be used to induce tolerance in hosts conditioned with non-myeloablative conditioning we first set out to examine whether a conditioning regimen consisting of low doses of WBI together with rapamycin and costimulatory blockade would permit long-term survival of alloantigen expressing T cells (Fig. 1A). To this end, CBA/Ca (*H-2^k*) mice were given 2.5Gy WBI. The following day the mice received 10⁷ purified T cells from either CBK transgenic mice or syngeneic CBA/Ca controls. In all cases, the purity of transferred T cells was greater than 99% (not shown). CBK mice express the *H-2K^b* gene under control of its autologous promoter on the CBA/Ca background [15], but are otherwise syngeneic with CBA/Ca mice. Therefore, CBK T cells are unable to induce a GVH response in CBA/Ca mice. On the day of T cell transfer, the mice also received rapamycin (1.5mg/kg) and anti-CD154 (0.2mg). We continued to give rapamycin daily from days 0–13 relative to T cell transfer and anti-CD154 on days 0–5, 8, 11 and 14.

Starting at 2 weeks, mice were bled on a weekly basis to examine persistence of CBK T cells based on expression of the allogeneic MHC class I antigen K^b. Adoptively transferred CD4 and CD8 T cells expressing K^b were detectable in the blood of conditioned mice receiving CBK T cells for at least 24 weeks by cell surface staining and flow cytometry (Fig. 1B). The

frequency of K^b expressing T cells was statistically the same from 6 to 24 weeks ($P>0.05$). Adoptively transferred T cells expressing K^b were also detected in peripheral lymphoid tissue and the thymus (Figs. 1C and 1D). We were unable to detect B220⁺, CD11b or CD11c⁺ cells that expressed K^b in the lymph nodes, thymus or blood of adoptive cell transfer recipients (not shown). CD4 and CD8 T cells expressing K^b in mice receiving CBK T cells were either CD4 or CD8 single positive (Fig. 1C and 1D). As expected, we were unable to detect K^b expressing cells in control mice receiving syngeneic CBA/Ca T cells (Fig. 1B).

Adoptive transfer of alloantigen expressing T cells into hosts conditioned using low dose WBI, rapamycin and anti-CD154 results in tolerance

Our previous work demonstrated that long-term persistence of alloantigen expressing T cells adoptively transferred into lethally irradiated mice simultaneously undergoing a syngeneic bone marrow transplant resulted in tolerance to skin grafts matched to the T cell donor strain [13]. We therefore examined whether presence of adoptively transferred CBK T cells in mice receiving non-myeloablative conditioning resulted in tolerance. To test this possibility, CBA/Ca mice were conditioned using 2.5 Gy WBI, rapamycin and anti-CD154 as described above and received either K^b disparate CBK or syngeneic CBA/Ca T cells. Eight weeks later, the mice received both CBK and BALB/c third party skin grafts. Mice receiving CBK T cells accepted their CBK skin allografts long-term (>100 days), but rapidly rejected third party BALB/c grafts (Fig. 2A). CBK skin grafts on these mice appeared normal upon visual inspection (Fig. 2B). Moreover, CBK skin grafts in these animals appeared normal upon histological analysis (Fig. 2C). In contrast, mice that received syngeneic CBA/CaJ T cells rapidly rejected both their CBK and third party skin grafts (Fig. 2A. $P<0.001$ between groups).

Tolerance induced by adoptively transferred T cells involves a deletional mechanism

We next sought to examine the mechanism of tolerance induced by adoptive transfer of alloantigen expressing T cells in hosts prepared using non-myeloablative conditioning. To determine possible mechanisms by which allogeneic T cells are able to induce tolerance we generated bone marrow irradiation chimeras in which CBA/Ca mice were lethally irradiated and reconstituted with a mixture of bone marrow from CBA/Ca mice and BM3.3 T cell receptor (TCR) transgenic mice to generate BM3.3-CBA mice. BM3.3 mice (CBA/Ca background) express a transgenic TCR on CD8 T cells that recognizes the alloantigen K^b [16]. The BM3.3 TCR can be detected by cell surface staining and flow cytometry using the anti-clonotypic antibody Ti98 [17]. Using this approach, it is possible to generate mice in which there exists a physiologically relevant frequency of T cells expressing the BM3.3 receptor that can be tracked by cell surface staining with Ti98 followed by flow cytometry [13].

Eight weeks after generation of BM3.3-CBA chimeras, these mice were conditioned using 2.5 Gy WBI, rapamycin and anti-CD154 as described above and received either K^b disparate CBK or syngeneic CBA/Ca T cells. Blood cells were then harvested and development of clonotype positive CD8 T cells was analyzed by cell surface staining and flow cytometry. Prior to conditioning and adoptive transfer approximately 7.1±2.9 and 6.2±2.4% of blood cells in mice receiving CBK or syngeneic CBA T cells respectively were clonotype positive. At 3 weeks after the infusion of control CBA/Ca T cells, CD8 T cells expressing the BM3.3 TCR were readily detectable in blood (Fig. 3A and 3B), comprising approximately 3.8±1.4% of CD8 T cells. In contrast, we were unable to detect clonotype positive T cells in the blood of mice receiving CBK T cells by 3 weeks after adoptive T cell transfer (Fig. 3A and 3B). Similarly, while clonotype positive CD8 T cells were detectable in the spleen and lymph nodes of mice receiving syngeneic CBA/Ca T cells, we were unable to detect clonotype positive T cells in the spleen or lymph node of mice receiving CBK T cells (Fig. 3C). These data suggest that adoptive transfer of CBK T cells into hosts prepared using the non-myeloablative conditioning

regimen described resulted in peripheral deletion of K^b-specific BM3.3 expressing CD8 T cells.

Adoptively transferred T cells mediate negative selection of newly developing alloreactive T cells in the thymus

We next sought to examine whether the inability to detect clonotype positive T cells in the periphery of BM3.3-CBA mice receiving CBK T cells was related to an effect on development of alloreactive T cells in the thymus. We therefore analyzed the development of K^b reactive BM3.3 derived CD8 T cells in the thymus of BM3.3-CBA mice receiving K^b disparate CBK or syngeneic CBA/Ca T cells. Clonotype positive CD4 and CD8 double positive T cells as well as CD8 single positive T cells were readily detectable in the thymus of conditioned mice that received syngeneic CBA/Ca T cells (Fig. 4). The thymi of mice that received CBK T cells contained a reduced number of clonotype positive CD4 and CD8 double positive T cells when compared with controls (Fig. 4). Moreover, we were unable to detect clonotype positive CD8 single positive T cells in the thymi of BM3.3-CBA mice that received CBK T cells (Fig. 4). These data suggest that the presence of CBK T cells in the thymus was sufficient to induce negative selection of developing alloreactive T cells.

DISCUSSION

Studies in irradiation chimeras have clearly shown that bone marrow derived hematopoietic cells are potent inducers of tolerance [2–5]. While the use of bone marrow to induce tolerance leads to robust tolerance, the use of allogeneic bone marrow transplantation is associated with serious complications, such as graft-vs-host disease (GvHD, [10]), the inability to recover complete immunocompetence [8,9] and infectious complications [6]. Chronic GvHD occurs in approximately 25–45% of HLA-matched and 40–70% of HLA-unmatched allogeneic stem cell transplants [22]. Furthermore, it has been difficult to establish a stable state of mixed host-donor hematopoietic chimerism in primates and humans [23–26].

Building on the concept that bone marrow derived hematopoietic cells are potent inducers of tolerance, we set out to identify a long-lived hematopoietic cell lineage capable of inducing tolerance upon adoptive transfer. We hypothesized that identifying such a cell type could allow for delivery of donor transplantation antigens on genetically modified autologous cells to induce tolerance without the need for allogeneic bone marrow transplantation thereby avoiding problems associated with transplantation of allogeneic bone marrow. To this end, we have previously shown that expression of alloantigens on bone marrow derived mature lymphocytes is critical to induce tolerance in lethally irradiated hosts reconstituted with autologous bone marrow genetically modified to express an alloantigen [12]. In addition, using an adoptive cell transfer system, we have shown that mature MHC class I disparate T cells are able to survive long-term when adoptively transferred into lethally irradiated conditioned hosts undergoing a syngeneic bone marrow transplant and induce tolerance to skin grafts matched to the T cell donor strain through a mechanism that involved negative selection of newly developing alloreactive T cells [13]. Similarly, T cells have been shown to induce tolerance across minor antigen disparities [27]. Together, these data suggested that the delivery of alloantigen mature T cells can reshape the immune repertoire.

In so far as our previous results showed that T cells are able to induce transplantation tolerance when administered to lethally irradiated mice undergoing a simultaneous syngeneic bone marrow transplant, we set out to address the question of whether similar results could be achieved in hosts prepared without lethal irradiation and without the need for bone marrow transplantation. To address this issue, we first set out to define a non-myeloablative conditioning regimen that would allow for survival of adoptively transferred alloantigen expressing T cells. We used a conditioning regimen that was based on previous studies showing

that in hosts receiving minimal WBI, rapamycin and anti-CD154 act synergistically to permit the induction of mixed chimerism and long-term tolerance [28]. Based on our data, 2.5Gy WBI combined with short-term treatment with rapamycin and anti-CD154 allowed for long-term survival of adoptively transferred K^b disparate CBK T cells. Moreover, using this conditioning regimen, adoptive transfer of CBK T cells resulted in tolerance to K^b, permitting long-term survival of K^b disparate CBK skin grafts. Given that mice receiving the same conditioning regimen together with syngeneic T cells rejected K^b disparate skin grafts, conditioning alone was not sufficient to allow for long-term skin graft acceptance. Rather induction of tolerance also required delivery of alloantigen by the adoptively transferred T cells.

Using BM3.3-CBA mixed chimeras as hosts we examined the mechanism of tolerance induced. We first observed that BM3.3 clonotype positive T cells were undetectable in the blood of mice receiving CBK T cells by three weeks after adoptive cell transfer. Similarly, clonotype positive alloreactive CD8 T cells were not detected in the spleen or lymph nodes of mice receiving CBK T cells. We suggest that the disappearance of pre-existing clonotype positive T cells in the periphery involves a mechanism in which peripheral deletion of alloreactive CD8 T cells is achieved. Analysis of T cell development in the thymus revealed that in mice receiving CBK T cells there is a reduction in the absolute number of immature CD4 and CD8 double positive thymocytes expressing the BM3.3 clonotype and a lack of CD8 single positive T cells expressing the BM3.3 clonotype. Therefore, developing T cells expressing the BM3.3 TCR undergo negative selection in the thymus of mice that received CBK T cells. The notion that peripheral and central deletion mechanisms play an important role in the tolerance induced is supported by the observation that CBK skin grafts on tolerance mice were free of lymphocytic infiltration. While we cannot rule out the possibility that regulatory T cells may be induced in this setting that control CD4 T cells that recognize alloantigen via the indirect pathway as we have seen in other settings [29], the mechanism of alloreactive CD8 T cell tolerance appears to be largely deletional.

We have recently reported that the ability of T cells to induce tolerance when adoptively transferred into mice undergoing a syngeneic bone marrow transplant is mechanistically linked to their ability to re-enter the thymus and that T cells become licensed for thymic re-entry after they have undergone homeostatic proliferation in the periphery and acquire a memory-like phenotype [14]. The preparative regimen we have used does induce lymphopenia [28]. We suggest that the lymphopenia induced by the preparative regimen employed may facilitate acquisition of a phenotype that allows for thymic re-entry of adoptively transferred T cells and negative selection of newly developing alloreactive CD8 T cells in the thymus.

Our data clearly suggest that alloreactive CD8 T cells tolerance involves at least in part a deletional mechanism. However, it has been shown that CD4 T cells are able to mediate rejection of K^b disparate skin allograft through the indirect pathway [20,30]. Therefore, in so far as permanent survival of K^b disparate skin is achieved using our approach, CD4 T cells that recognize K^b through the indirect pathway are also tolerized using our approach. We are currently attempting to elucidate the mechanism of tolerance that is induced in alloreactive CD4 T cells that mediate allograft rejection through the indirect pathway. Nevertheless, we wish to point out that acceptance of skin allografts is the most stringent test of tolerance [31], suggesting that the ultimate mechanism of tolerance induced by our approach in the alloreactive CD8 and CD4 compartment is robust.

Together, our data provide a proof of principle that robust transplantation tolerance can be achieved by adoptive transfer of alloantigen expressing T cells into hosts undergoing non-myeloablative conditioning without the need for bone marrow transplantation. To our knowledge, this is the first demonstration that one can combine non-myeloablative conditioning regimen together with adoptive transfer of alloantigen expressing T cells to induce

tolerance to skin allografts. While other approaches such as donor specific transfusion combined with costimulatory blockade are able to prolong skin allograft survival, they are unable to induce tolerance to skin allografts unless they are used in combination with an allogeneic bone marrow transplant [32,33]. The observation that T cells can deliver alloantigen in a tolerogenic fashion opens up the possibility of using autologous T cells genetically modified ex vivo to express donor MHC to induce tolerance. We suggest that induction of tolerance using this approach may have significant clinical application because it avoids the potential of inducing GvHD and avoids complications associated with bone marrow transplantation. A major issue is how many alloantigens could one simultaneously induce tolerance by using this approach. Interestingly, human T cells express, in addition to MHC class I, MHC class II antigens [34–36], suggesting that tolerance to MHC class II could also be induced using this approach. While we are currently investigating this issue, we wish to point out even if it is only possible to induce tolerance to donor MHC class I using this approach the clinical relevance would be significant because it is clear that in solid organ transplantation, HLA matching even at a limited number of loci, or even a single loci has a profound impact on long-term allograft survival [37,38].

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Figure 1. Long-term survival of adoptively transferred MHC class I mismatched mature T cells in mice receiving the non-myeloablative conditioning

(A). Schema of the conditioning regimen to permit long-term survival of alloantigen expressing T cells and induce tolerance to K^b . (B) Conditioned CBA/CaJ mice were adoptively transferred with either 10^7 CBK T cells (CBK T, closed squares) or CBA/CaJ T cells (CBA T, open squares). At 2, 6, 12, 20, and 24 weeks after transfer, peripheral blood from these animals was examined for K^b expression by flow cytometry following cell surface staining. Shown are the percentages of K^b expressing cells. Seven to eight mice in each group were analyzed at each time point. Percentage of K^b expressing cells in the peripheral blood showed no significant difference after 6 weeks following adoptive T cell transfer and was therefore stable ($n=8$, $P>0.01$). Shown are representative data from one experiment of two. Twenty-four weeks after adoptive transfer, mice receiving CBK T cells were sacrificed. Lymph nodes (C) and thymi (D) were then harvested and stained with antibodies specific for K^b and CD4, CD8, B220, CD11b, CD11c prior to analysis by flow cytometry. In each panel, the frequencies of K^b cells are shown. Quadrants in which the percentage of K^b cells could not be detected ($<0.1\%$) are marked "U". Seven to eight mice were analyzed in each group. Shown are representative data from one experiment of two.

Figure 2. Mature allogeneic T cells induce tolerance to skin allografts under the non-myeloablative regimen

(A) Eight weeks after adoptive T cell transfer, CBA/CaJ mice that received either 10^7 purified CBK T cells (closed squares, $n=8$) or syngeneic CBA/CaJ T cells (closed triangles, $n=7$) were transplanted with both a CBK (solid symbols) and third party BALB/c skin graft (open squares, $n=15$). Survival of third party BALB/c skin graft was identical between groups and therefore the results were combined for both groups. In all cases the combined results of skin graft survival from two independent experiments is shown. (B) One hundred days after skin transplantation, the skin grafts were harvested from CBA/CaJ mice that received 10^7 purified CBK T cells and then fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (right panel). Shown is the boundary of donor skin graft and recipient skin. Gross picture of a CBK skin graft skin from the same mouse is shown in the left panel. Shown are representative data from one mouse of eight.

Figure 3. BM3.3 clonotype-positive CD8 T cells are not present in the periphery of mice adoptively transferred with CBK mature T cells under the non-myeloablative conditioning

Eight weeks after development of BM3.3-CBA chimeras as described in the text, the mice received either 10^7 purified CBK T cells or syngeneic CBA/CaJ T cells using the nonmyeloablative regimen described. **(A)** Three weeks after adoptive transfer, CD8 T cells expressing the BM3.3 TCR were analyzed by staining with clonotypic antibody Ti98 in the blood of mice that received CBK T cells (CBK T, right panel) or syngeneic CBA/CaJ T cells (CBA T, left panel). The percentage of CD8 T cells expressing Ti98 is shown. The flow cytometry data are representative of 8 mice per group. **(B)** Kinetic analysis of BM3.3 TCR clonotype positive T cells in blood. CD8 T cells expressing the BM3.3 TCR were analyzed by

staining with clonotypic antibody Ti98 in the blood of either mice that received either CBK (diamonds, $n=8$) or syngeneic CBA/CaJ T cells (squares, $n=8$) at 3 and 8 weeks after adoptive transfer of T cells. The percentage of clonotype positive cells pre-transfer is shown for comparison. The mean percentage of CD8 T cells expressing Ti98 at the indicated time points is shown. Data are representative from one of two experiments. (C) Eight weeks after adoptive transfer, T cells expressing the BM3.3 TCR were analyzed by staining with Ti98 in the spleens (top panels), and lymph nodes (bottom panels) of mice that received CBK T cells (CBK T, right panels, $n=8$) or syngeneic CBA/CaJ T cells (CBA T, left panels, $n=8$). The percentage of cells expressing the Ti98 clonotype is shown. Shown are representative data from one experiment of two.

Figure 4. Expression of K^b on mature T cells induces negative selection of T cells expressing the BM3.3 clonotype under non-myeloablative conditioning

Four weeks after development of CBA/CaJ-BM3.3 chimeras as described in the text, mice received either 10⁷ purified CBK T cells (CBK T, right panel, *n*=8) or syngeneic CBA/CaJ T cells (CBA T, left panel, *n*=8) after non-myeloablative conditioning. Eight weeks after adoptive transfer, mice were sacrificed and thymi were removed. The thymi were then triple stained with antibodies specific for CD4, CD8, and the anti-clonotypic antibody Ti98. Shown is expression of CD4 and CD8 on thymocytes from representative mice following gating on Ti98-positive cells. The absolute numbers of cells in each quadrant are indicated. The number of CD8 single positive T cells staining with Ti98 in the right panel is similar to background observed in controls (data not shown). Shown are representative data from one experiment of two.