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Tolerance Induction in Presensitized Bone Marrow Recipients by Veto CTLs: Effective Deletion of Host Anti-Donor Memory Effector Cells1,2

Shlomit Reich-Zeliger, Esther Bachar-Lustig, Avichai Bar-Ilan, and Yair Reisner3

Veto cells have been defined as cells capable of inducing apoptosis of effector CD8 cells recognizing their disparate MHC Ags. Tolerance induced by donor-type veto cells is desirable, because it is restricted to depletion of anti-donor clones without depletion of other immune specificities. It has been shown that anti-third party CTLs exhibit marked veto activity with reduced capacity to induce graft-vs-host disease, when tested on naive effector cells. However, presensitized T cells could play an important role in graft rejection, and therefore, their sensitivity to veto cells could be critical to the implementation of the latter cells in bone marrow transplantation. To address this question, we compared naive and presensitized TCR transgenic effector CD8 T cells, bearing a TCR against H-2d. Both cell types exhibited similar predisposition to killing by veto CTLs in vitro, and this killing was dependent on large doses of bone marrow (BM)4 cells, adequately depleted of T cells as the latter cells are activated faster, with a lower threshold for activation and with a reduced requirement for costimulatory signals. Thus, Valujskikh et al. (36) have shown that the presence of presensitized cells prevents the beneficial effects of anti-CD40L in prolonging allograft survival. An early study addressing the potential of veto CTLs to suppress presensitized T cells, suggested that in the context of presensitization to minor histocompatibility Ags, a cytotoxic clone with a marked veto activity against naive T cells exhibited significantly less effective...
veto reactivity against presensitized cells (17). However this suggestion was not tested in vivo. Moreover, the clone tested expressed a CD8+ CD4- phenotype which is unusual for CTLs.

In the past, it was difficult to track memory cells directed against a particular Ag in vivo, but with the advent of TCR transgenic mice it has become possible to retrieve such memory cells and examine their sensitivity to veto cells. Thus, in the present study, we initially addressed this question by comparing in vitro the interaction of veto CTLs with naive and presensitized T cells, using effectors from TCR transgenic and Fas knockout mice. Furthermore, the capacity of the veto cells to delete presensitized T cells, was investigated in vivo, using a stringent model for T cell mediated BM allotraft rejection.

Materials and Methods

Animals

Mice used were 6- to 12-wk-old females. BALB/c (H-2d), FVB (H-2j), SJL (H-2a), and C57BL/6 (H-2b) were obtained from the Weizmann Institute Animal Center (Rehovot, Israel). DBA/2 (H-2b) and C3H/HeJ (H-2e) mice were obtained from the Roscoe B. Jackson Memorial Laboratory. A breeding pair of BALB/c (H-2b) mice expressing the TCR from the transgenic clone 2c with specificity for H-2Ld was provided by Janko Nikolic-Zugic (Sloan-Kettering, New York). Progeny of these Tg mice were bred at the Weizmann Institute Animal Breeding Center. 2c-Ipr Tg mice were produced by breeding 2c Tg mice with C3H.MRL-Fas/-mice as previously described (37). The F1 2c/2c mice were bred again with C3H.MRL-Fas/-mice. All mice were kept in small cages (five animals in each cage) and fed sterile food and water.

Preparation of host nonreactive anti-third party CTLs from donor-type spleen cells

Anti-third party CTLs were prepared as described by Bachar et al. (22). In brief, spleen cells from BALB/c or DBA/2 origin were harvested, lysed in 0.15 M cold ammonium chloride-potassium (ACK) buffer (0.5 ml spleen) to remove RBC, and brought to a concentration of 2×10^6 cells/ml in RPMI 1640 supplemented with 25 mM HEPES (pH 7.4), 10% heat-inactivated FCS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM l-glutamine, 5×10^{-3} M-2-ME, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. This splenic single-cell suspension was cocultured for 6 days at 37°C in a 5% CO2/air incubator without exogenous IL-2. This splenic single-cell suspension was cocultured for 6 days at 37°C in a 5% CO2/air incubator without exogenous IL-2. The IL-2 deprivation period was established based on the maximal time that mouse spleen T cells can be maintained in MLR culture without IL-2 (data not shown). However, attempts to grow large numbers of cells for the investigation of these veto cells in vivo revealed irregularities in the cell composition of the harvested cells due to difficulties in controlling outgrowth of lymphokine-activated killer (LAK) cells and/or CD4+ T cells. This problem was addressed by removal of CD4+ and NK cells at the end of the IL-2 deprivation, before the addition of IL-2. Thus, cells were fractionated on Ficoll and the lymphoid fraction was then subjected to positive selection of CD8+ cells using magnetically labeled anti-CD8 Abs and a MACS system (Miltenyi Biotec). The isolated cells (2×10^6/ml) were then re-stimulated with irradiated (20 Gy) splenocytes from the original third party donors (FVB, 2×10^6/ml), and human rIL-2 (50 U/ml; European collaborative) was added from that day every second day to the MLR culture (day 6, 8 and 10). At day 10 the MLR cultures were harvested, fractionated on Ficoll-Paque plus (Amerham Pharmacia Biotech), analyzed by FACS for their CD8 level and tested for their veto activity at different cell ratios, as described in Results.

Generation of presensitized T cells

Irradiated (20 Gy) BALB/c splenocytes (5×10^6) were injected i.p. into naive 2c Tg hosts at day 0 and day 21. In experiments described in Figs. 1–4 the whole population of presensitized spleen cells was harvested at 45–60 days after the end of sensitization and tested for its responsiveness to the veto activity of anti-third party CTLs. In the experiment shown in Fig. 2c presensitized wild-type spleen cells were obtained by immunization of C3H/HeJ mice as described above. In the experiment shown in Fig. 5, C3H/HeJ mice were immunized as described above and CD44+ T cells were isolated from their splenocytes as described below.

Assay for veto activity in the 2c TCR Tg mouse model rashu

Spleen cells of 2c Tg H-2b mice, expressing a TCR-αβ with specificity for H-2Ld mice were used throughout as effectors cells in the veto assay as previously described (38), except that, in addition to naive 2c mice, we also used presensitized 2c mice as described above. Briefly, spleen cells were harvested, lysed in cold ACK buffer to remove RBC, and brought to a concentration of 2×10^6 cells/ml in RPMI complete tissue culture medium (CTCM). The cells (2×10^6/ml) were then stimulated with irradiated (20 Gy) BALB/c splenocytes (2×10^6/ml) in the presence or absence of relevant (BALB/c origin) and nonrelevant (SJL origin) veto CTLs at two concentrations (5, 10%). Cultures were incubated for 72 h in 24-well plates. The deletion of specific effector T cells was monitored by cytofluorometric analysis, measuring the level of 2c Tg cells, specifically stained by the 1B2 Ab, directed against the clonotypic anti-H-2Ld TCR.

Cytofluorometric analysis

FACS analysis was performed using a modified FACScan (BD Biosciences, Mountain View, CA). Fluorescence data were collected using 3-decade logarithmic amplification on 25–50×10^4 viable cells, as determined by forward light scatter intensity. Cells were stained with anti-CD8a (Ly-2)-FITC, anti-CD8α (Ly-2)-CyChrome, anti-CD8b (Ly-2)-allophycocyanin, anti-CD222-PE (TM-61), anti-CD95 (Fas)-FITC, anti-CD45-FITC, and anti-CD4-PE (BD Pharmingen, San Diego, CA). Biotinylated 1B2 Abs (provided by J. Nikolic-Zugic, Sloan-Kettering Institute) were stained with R-PE streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA).

Detection of apoptotic cells

Annexin V-Cy5 was used to detect apoptotic cells. Cells were incubated in annexin V binding buffer (1.4M NaCl, 25 mM CaCl2, 0.13M HEPES Ph 7.4) and supplemented with 5 μl of annexin V-Cy5. The cells were incubated at room temperature for 5 min in the dark, and then washed in binding buffer. Positive cells were monitored by flow cytometry.

The mouse model for T cell mediated BM allotraft rejection

C3H/HeJ female mice were exposed to a single dose of 10 Gy (supralethal conditioning) TBI on day 0. The following day, the mice received i.v. 1×10^6 purified host T cells. Transplantation of 2×10^6 allogeneic T-depleted BALB/c BM cells was performed on day 2 in conjunction with either rapamycin alone, veto CTLs alone or both. The survival of the mice was monitored daily. Due to changes of the radiation source from Cobalt-60 to Cesium at the Weizmann Institute of Science, we experienced some subtle differences in our graft rejection mouse model which required minor adjustments. Thus, to reproduce our previous data on the synergistic role of veto CTLs and Rapamycin in this model (22), we introduced the following changes: 10 Gy instead of 11 Gy TBI was used for conditioning, 1×10^6 host T cells instead of 1.5×10^6 T cells were used for induction of rejection, and half dose of rapamycin as μg/mouse/day for 5 days) was used for immune suppression. As in our previous study, under these conditions rapamycin alone, or veto cells alone, could only marginally overcome graft rejection mediated by naive host T cells, but the two agents together led to marked engraftment and survival (data not shown). Once this was reproduced we could begin comparing the effect of veto CTLs on naive vs presensitized host T cells.

Purified naive and presensitized host T cells were prepared by selection with magnetic beads. Briefly, host (C3H/HeJ) splenocytes were obtained from naive or from mice presensitized with irradiated BALB/c spleen cells, as described above. The cells were fractionated on Ficoll/Paque and the isolated mononuclear cells were subjected to a negative selection of T cells, removing myeloid (CD11b), NK (DX5 (CD49b)) and B cells (CD45R B220)) and sparing CD4+ and CD8+ T cells. As in our previous study, under these conditions rapamycin alone, or veto cells alone, could only marginally overcome graft rejection mediated by naive host T cells, but the two agents together led to marked engraftment and survival (data not shown). Once this was reproduced we could begin comparing the effect of veto CTLs on naive vs presensitized host T cells.

Results

Inhibition of presensitized CD8 effector T cells by veto CTLs in vitro

The 2c TCR transgenic mouse model. The 2c TCR transgenic mouse, in which the CD8+ T cells express a TCR transgene
against H2d (stained by the clonotypic Ab 1B2) enables monitoring the fate of anti-H2d clones upon interaction with relevant veto cells of H2d origin, as opposed to nonrelevant veto cells of a different H2 origin. Thus, by using this model we were able previously to demonstrate that CTLs of H2d origin induce deletion of primary effector cells via a Fas-FasL mechanism (37, 39). In the present study, to define the role of veto CTLs in the context of presensitized effectors, the same 2c mice were used except that the mice were presensitized by infusion of irradiated BALB/c spleen cells, as previously described (40).

Following presensitization, the lymphoid compartment in the spleens of presensitized mice was compared by FACS analysis to spleens obtained from un-sensitized mice. As can be seen in Fig. 1, 1B2+ cells in immunized mice exhibit a memory phenotype, namely a high level of CD44 (68%) or CD122 (60%) in contrast to primary 1B2+ cells which exhibit reduced expression of these markers (18% and 6%, respectively).

As explained above, the 2c model enables FACS monitoring of veto activity, either by measuring the inhibition of proliferation or the induction of apoptosis (Annexin staining) in the effector cells. In the present study we used both assays to test whether veto CTLs suppress the allo-immune response mediated by memory CD8+ T cells. Thus, when measuring the proliferation and expansion of 2c cells by their cognate MHC stimulators, the same 2c mice were used except that the mice were presensitized by infusion of irradiated BALB/c spleen cells, as previously described (40).

In the presence or absence of 10% nonrelevant veto cells (origin of H2s, black bars), or relevant veto cells (origin of H-2d, white bar) (p < 0.001). The results represent average ± SD of three different experiments. To address the inhibitory activity of veto CTLs on naive (A) and presensitized (B) wild-type C3H/HeJ responders, relevant BALB/c (white bar) or nonrelevant SJL (black bar) veto CTLs were added to MLR in which C3H/HeJ splenocytes were stimulated against irradiated BALB/c stimulators and then tested for their cytotoxicity of BALB/c targets by the 51Cr release assay (c).

FIGURE 2. Veto activity of anti-third party CTLs on naive and presensitized 2c mice. The inhibitory effect of 10% relevant CTLs (origin of H-2d) on naive (A) and presensitized 2c T cells (B) was measured after 72 h of culture. The percentage (a) or the total cell number (b) of 1B2+CD8+ cells is shown in the presence of 10% nonrelevant veto cells (origin of H2d black bars), or relevant veto cells (origin of H-2d, white bar) (p < 0.001). The results represent average ± SD of three different experiments. To address the inhibitory activity of veto CTLs on naive (A) and presensitized (B) wild-type C3H/HeJ responders, relevant BALB/c (white bar) or nonrelevant SJL (black bar) veto CTLs were added to MLR in which C3H/HeJ splenocytes were stimulated against irradiated BALB/c stimulators and then tested for their cytotoxicity of BALB/c targets by the 51Cr release assay (c).

The role of Fas-FasL apoptosis in the deletion of presensitized effectors by veto CTLs

Previous studies demonstrated that the activation of the 2c effectors is associated with up-regulation of the cell surface Fas receptor (CD95), leading to apoptosis by interaction with FasL on the effector T cells. In vivo against minor histocompatibility Ags (17). To address potential discrepancy between TCR transgenic and wild type effectors, we also tested the inhibitory activity of anti-third party veto CTLs upon addition to MLR in which in vivo presensitized wild type C3H/HeJ splenocytes were re-stimulated in vitro against irradiated BALB/c stimulators. As can be seen in Fig. 2c, the killing of BALB/c targets by CTLs generated from both naive and presensitized wild type C3H splenocytes, as measured by the Cr51 release assay (used by Fink et al.), was effectively inhibited by the veto CTLs. Thus, there is no significant difference (p > 0.15) in the sensitivity of wild type naive and presensitized effectors to inhibition by anti-third party veto CTLs.

Considering that veto CTLs were previously shown to kill naive effector T cells by apoptosis, it was of interest to test the induction of annexin V on presensitized effectors upon their interaction with veto CTLs. As shown in Fig. 3 presensitized CD8 cells (Fig. 3, c-d) similarly to naive CD8 cells (Fig. 3, a-b), undergo deletion in the presence of 10% veto CTLs. The annexin V level on naive or presensitized 2c cells, in the presence of veto CTLs was significantly reduced upon the addition of relevant anti-third party CTLs from the DBA (H2b) background (3310 ± 381 and 1573 ± 360, respectively). A similar trend was also found by recording the percentages of surviving 1B2+CD8+ cells at the end of the 72 h MLR in the presence of nonrelevant or relevant veto CTLs.

It could be argued that the veto sensitivity exhibited by presensitized 2c TCR transgenic T cells, might not be relevant to wild type T cells, due to the artificial expression of TCR on the transgenic cells. In particular, this is important in light of the early study of Fink et al. suggesting that a veto CTL clone which was effective against naive effectors was not effective in blocking presensitized
CTLs that were described in cells in the MLR cultures with (pressed by the stimulated naive (relevant veto CTLs. Bb sitized 2c-lprated splenocytes from BALB/c mice. Thus, Fas negative presen-
a, c), Histograms showing the level of annexin V expressed by the stimulated naive (a and b) and presensitized (c and d) 2c T cells in the MLR cultures with (b and d) and without (a and c) relevant veto CTLs that were described in A.

veto CTLs (37, 39). To further establish the role of Fas in the apoptosis of presensitized CD8 T cells, 2c cells were harvested from 2c-lpr mice following presensitization with 1 × 10⁶ irradiated splenocytes from BALB/c mice. Thus, Fas negative presensitized 2c-lpr cells were tested for their sensitivity to veto CTLs in comparison to Fas positive presensitized 2c splenocytes. As can be seen in Fig. 4, similarly to previous results with naive effector 2c cells, (37) deletion by veto cells was markedly reduced when testing the Fas deficient 2c-lpr presensitized cells compared with Fas positive 2c presensitized cells. Thus, presensitized CD8 T cells, similarly to naive cells, are likely deleted by veto CTLs via a Fas-FasL mediated mechanism.

Enhancement of engraftment of T cell-depleted BM in presensitized mice by veto CTLs

It has been shown in a stringent mouse model for T cell mediated BM allograft rejection, that optimal enhancement of BM allografting by veto CTLs is attained in combination with rapamycin based conditioning (22). In this model, recipient mice are conditioned by supralethal TBI and radio protected with T cell-depleted BM. Rejection is induced by adoptively transferred purified host T cells (HTC). To evaluate the potential of veto CTLs to overcome rejection in presensitized mice, we used the same model except that host type CD44⁺ T cells, harvested from presensitized mice, were used instead of naive T cells. The CD44⁺ T cells were isolated by a two step procedure, comprising an initial negative selection of HTC followed by positive selection of CD44⁺ cells. As can be expected, the rejection mediated by 5 × 10⁶ adoptively transferred presensitized HTC is more vigorous compared with that mediated by naive HTC. Thus, in 4 independent experiments average survival time in recipients infused with presensitized HTC was 19.2 ± 1.2 days compared with 30 ± 3 days in recipients of naive HTC. Upon infusion of 1 × 10⁶ HTC, the difference in the rate of survival between recipients of presensitized or naive HTC is less pronounced (21 ± 2 days and 23 ± 1 respectively) presumably due to the strong antigenic stimulus mediated by fully allogeneic BM cells. Considering that we have previously found that veto cells are markedly enhanced by short treatment with rapamycin in this in vivo model, we have now compared their efficacy to neutralize naive and presensitized HTC, in the presence or absence of rapamycin. As can be seen in Fig. 5, while rejection mediated both by naive and presensitized host type T cells (Log-Rank and Wilcoxon test). Thus, in accordance with the in vitro studies, the presensitized HTC are as prone as the naive HTC (p > 0.05 Log-Rank and Wilcoxon test) to the inhibitory reactivity of veto CTLs in vivo.

Interestingly, when veto CTLs were infused alone, enhancement of engraftment was more effective in mice adoptively transferred with presensitized HTC compared with naive HTC (35% and 54%
survival, respectively.) However, this trend suggesting potential higher sensitivity of presensitized HTC was not significant ($p > 0.05$ Log-Rank and Wilcoxon test).

As can be expected for BM recipients conditioned with supralethal TBI, full donor-type chimerism (96–100%) was exhibited at 60 days post transplant in both naive and presensitized groups treated with veto CTLs and rapamycin. Splenocytes recovered from the chimeric mice of both groups exhibited significant capacity to generate alloreactive CTLs against nonrelevant SJL stimulators (30% and 66% cytotoxic index, respectively) while diminished responses were recorded against host (2.3% and 6.5%, respectively) or donor-type stimulators (0.6% and 3%, respectively).

**Discussion**

Immune memory is an indispensable component of the immune response protecting the host from future challenges of the same pathogen or even in certain settings of unrelated pathogens. As a consequence, anti-viral effector T cells capable of directly recognizing foreign MHC might be generated, inducing long-lived alloreactive memory cells (41). The use of immunosuppressive drug treatment predisposes graft recipients to viral infections which, in turn, sensitize alloreactive immune cells to induce graft rejection. In addition to such alloreactive memory cells, many patients undergoing BM transplantation are presensitized to a broad range of donor MHC Ags from multiple blood transfusions. Consequently, many presensitized patients are either precluded from receiving a transplant or may experience an increased rate of early rejection episodes that are irreversible or difficult to treat with current immunosuppressive agents. This has led to the emerging concept that environmental exposures and large memory pools may constitute a more formidable barrier to tolerance induction. Furthermore, it was shown that memory T cells which exhibit selective resistance to therapeutic depletion, are resistant to several immunosuppressive agents and are less dependent on costimulatory signals. Thus, although in vivo depletion decreases the allosppecific precursor frequency, the remaining cells are less susceptible to therapies that successfully interrupt naive cell activation.

Our previous data clearly have shown that, anti-third-party CTLs generated under IL-2 deprivation, afford a suitable source for effective veto cells that can enhance BM allografting without GVHD in lethally irradiated mice, adoptively transferred with graduated numbers of purified naive host-type T cells. This model, which has been designed to distinguish between T cell mediated rejection as opposed to rejection mediated by non-T cell mechanisms, including failure to engraft due to stem cell competition, has also been used recently to evaluate the role of Treg cells (42).

An early study has suggested that veto CTLs might not be capable of effectively inhibiting in MLR, effector T cells presensitized in vivo (17).

Considering that with the advent of TCR transgenic models it has become possible to follow and isolate memory T cells after presensitization in vivo, we reinvestigated this important question in the context of BM allograft rejection, and we demonstrate both in vitro and in vivo that presensitized effectors are as sensitive as their primary counterparts to the inhibition of veto CTLs. Furthermore, by using Fas knockout 2c mice, we were able to show that similarly to naive effector cells, deletion of presensitized effectors is mediated through Fas-FasL apoptosis.

It could be argued that the conclusion raised by our in vitro studies differ from the early study of Fink et al. (17), due in part to the use in the present study of artificial TCR transgenic effectors. However, the same efficacy of the veto CTLs was also demonstrated against wild type effectors presensitized in vivo, using the same chromium release cytotoxic assay used in the earlier study. Clearly, such assays measuring functional inhibition of CTLp frequency, do not allow to monitor directly the fate of the specific effector cells, in contrast to the 2c model in which effectors can be decorated with an appropriate clonotypic Ab.

The discrepancy between the earlier study and our present results might be attributed to the clone used in the former, which bears an unusual phenotype and is likely not representative of the anti-third party CTL preparation from which CD4+ cells are removed at the end of the culture.

Our results seem to be also in line with tolerance induction by veto CTLs out of the context of BM allografting, namely for CTLs presensitized against viral Ags which were found to be as susceptible in vitro to veto cell inhibition as their naive counterparts (43). However, this veto activity on presensitized effectors directed against viral Ags has to be confirmed in vivo.

The sensitivity of presensitized effectors to veto CTLs is clearly important for BM transplantation, however an obstacle remaining is their reduced reactivity in vivo compared with their remarkable ability to delete anti-donor clones in vitro. The enhancement mediated by short term treatment with rapamycin is compatible with the recent demonstration that veto CTLs operate via Fas-FasL triggering, upstream of rapamycin inhibition of IL-2R signaling. Thus, effector T cells that might have escaped deletion by the veto cells could still be eliminated by rapamycin. Moreover, Anderson et al. (43) suggested that rapamycin prevents the responding cells from differentiating into fully activated CTLs and that the point of inhibition precedes the loss of a veto sensitive phenotype.

One attractive explanation could be offered by the suggestion that up-regulation of L-selectin on veto CTLs by rapamycin, (44–46) might direct them more effectively to lymph nodes, where the deletion and inhibition of host T cells by veto cells likely occurs. However, considering the broad spectrum of rapamycin cellular reactivities, other pathways and mechanisms must be considered.

Recently, it has been suggested that anti-CD70 treatment could prevent CD8 T cell-mediated rejection (47), and it was further demonstrated that it could inhibit the proliferation and activation of effector CD8 T cells, and diminish the expansion of effector and memory CD8 T cells in vivo (48). Thus, the C27-CD70 pathway seems critical for CD28-independent effector/memory CD8+ alloreactive T cell activation in vivo. Considering that Anderson et al. (43) showed recently that addition of rapamycin extends the window during which responding cells are susceptible to deletion by veto cells, it would be interesting to test the possibility that blockade of the CD27–CD70 costimulatory pathway might be synergistic with veto CTLs, so as to potentially substitute the need for treatment with rapamycin.

In conclusion, our results suggest that allograft rejection mediated by naive or presensitized T cells can be equally overcome by combining rapamycin and veto CTLs, and that the deletion of the presensitized effector CD8 cells is dependent on Fas/FasL interaction, as shown for naive T cells. This conclusion is highly relevant to the prospect of clinical application of anti-third party veto cells in leukemic recipients of BM transplantation, in whom rejection is often mediated by residual presensitized T cells. A protocol for effective large scale production of human veto CTLs has been described recently (49) and clinical trials in leukemia patients are in the final stages of preparation. The present data showing the effective deletion of presensitized T cells, suggest that if successful in leukemic BM recipients, this approach could be further extended to induction of tolerance for organ transplantation or in autoimmunity, in which presensitized T cells present a major obstacle.
Disclosures

The authors have no financial conflict of interest.

References


