Tolerance Induction by Veto CTLs in the TCR Transgenic 2C Mouse Model. I. Relative Reactivity of Different Veto Cells

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Tolerance Induction by Veto CTLs in the TCR Transgenic 2C Mouse Model. I. Relative Reactivity of Different Veto Cells

Shlomit Reich-Zeliger, Esther Bachar-Lustig, Judith Gan, and Yair Reisner

Several bone marrow cells and lymphocyte subpopulations, known as veto cells, were shown to induce transplantation tolerance across major histocompatibility Ags. Due to the low frequency of the effector T cells against which the veto cells inhibitory activity is aimed, the fate of the effector cells was traditionally followed indirectly by functional limiting dilution assays, which are cumbersome and depend on numerous parameters. In the present study the fate of the effector T cells was monitored directly by FACS, using TCR transgenic mouse CD8+ T cells in which the transgene is directed against H-2d (the 2C model). This assay is validated by demonstrating the potency, selectivity, radiation sensitivity, and contact dependency of anti-third-party CTLs previously demonstrated by the limiting dilution assay. In contrast to veto CTLs, nonactivated CD8+ T cells lack veto activity. Comparison by FACS in the 2C model revealed a hierarchy of veto cells, in the order of veto CTLs, activated CD4+ T cells, and activated CD8+ T cells, and activated B cells. The latter cells as well as nonactivated CD4+ or NK cells were shown to be completely devoid of veto activity. The Journal of Immunology, 2004, 173: 6654–6659.

B one marrow transplantation after supralethal radiochemotherapy is associated with dangerous infections due to the slow immune reconstitution during the first year posttransplant (1–3). Thus, the use of reduced intensity conditioning, associated with less severe immune ablation, could have a remarkable potential in the treatment of a variety of nonmalignant diseases or for the induction of mixed chimerism as a prelude for cell therapy in cancer or organ transplantation (4–7). However, preparatory regimens represent a difficult barrier for the engraftment of donor cells.

In patients with advanced hematological malignancies who cannot withstand myeloablative conditioning because of age and/or performance status, recent attempts were made to develop low toxicity conditioning protocols in conjunction with HLA-matched transplants. Potent post-transplant immunosuppression and the presence of large numbers of alloreactive T cells in the graft enabled a high rate of engraftment. However, graft-vs-host disease (GVHD), particularly chronic GVHD, remains a major obstacle (7). Although in high risk leukemia, such transplant-related mortality is acceptable, it would be totally intolerable if applied to patients with long life expectancy. Thus, the use of purified allogeneic stem cells, which do not pose any risk for GVHD and which can continuously present donor type Ags in the host thymus, thereby inducing durable tolerance to donor cells or tissues, represents one of the most desirable goals in transplantation biology.

One approach to address this challenge is based on the use of donor veto cells depleted of host reactive T cells.

Veto activity was defined in 1980 by Miller as the capacity to specifically suppress cytotoxic T cell precursors directed against Ags of the veto cells themselves, but not against third-party Ags (8–11). Interestingly, it has been shown that some of the most potent veto cells are of T cell origin; in particular, a very strong veto activity was documented for CD8+ CTL lines or clones (12–17). The specificity of the veto effect mediated by CTL clones was shown by several studies to be unrelated to their TCR specificity (18–20). The suppression of effector CTL-precursor directed against the veto cells is both Ag-specific and MHC-restricted, resulting from the unidirectional recognition of the veto cell by the responding CTLs, but not vice versa (18). Furthermore, it has been shown that this suppression is mediated by apoptosis (18, 21).

Although CD8+ CTLs are endowed with very potent veto activity, their use in allogeneic stem cell transplantation is limited due to their marked graft-vs-host reactivity. To address this problem we developed a new approach for the generation of host-nonreactive CTLs based on stimulation of donor CD8+ T cells against third-party stimulators under IL-2 deprivation. This approach is based on the observation that only those CTLs activated are capable of surviving IL-2 starvation in the primary culture. Other studies used T cytotoxic 2 CTLs, which were shown to be associated with a reduced GVH reactivity (22), or NK cells (23), which are not associated with any risk for GVHD. One obstacle in the study of basic questions related to the mechanism of action of veto cells is presented by the assay for these cells. Due to the low frequency of the effector T cells against which the veto cell inhibitory activity is directed, the fate of the effector cells was traditionally followed indirectly by functional limiting dilution assays, which are cumbersome and depend on numerous parameters. However, the availability of new TCR transgenic (Tg) strains of mice, in which the transgene is directed against H-2 determinants, as well as the development of tetramer technology have made it possible to directly monitor in such MLR cultures the fate of the effector T cells by FACS analysis. Thus, the 2C Tg mouse model, the T cells of which bear a transgene directed against H-2d, was used to document the veto reactivity of activated bone marrow.
cells (21, 23, 24). In the present study we used this Tg mouse model to study the relative veto reactivities of various veto cells, including host nonreactive CD8– T cells, activated CD4+ T cells, B cells, and NK cells.

Materials and Methods

Animals

The mice used were 6- to 12-wk-old females. BALB/c (H-2b), FVB (H-2d), SJL (H-2d), and C57BL/6 (H-2d) were obtained from the Weizmann Institute Animal Center (Rehovot, Israel). DBA/2 (H-2b) and C3H/HeJ (H-2b) mice were obtained from the Roscoe B. Jackson Memorial Laboratory (Bar Harbor, ME). A breeding pair of Tg H-2b mice expressing the TCR from the CTL clone C with specificity for H-2Ld was provided by J. Nikolic-Zugic (Sloan-Kettering Institute, New York, NY). Progeny of these Tg mice were bred at the Weizmann Institute Animal Breeding Center. All mice were kept in small cages (five animals in each cage) and were fed sterile food and acid water.

Preparation of host-nonreactive, donor anti-third-party CTLs

Anti-third-party CTLs were prepared as described by Bachar-Lustig et al. (25). Briefly, splenocytes of BALB/c or DBA/2 origin were cultured with antigen-primed (20 Gy) FVB, C3H/HeJ, or SJL (third-party stimulators) splenocytes. Responders (2 × 10^5/ml) and stimulators (2 × 10^5/ml) were cultured for 6 days in RPMI 1640 complete tissue culture medium at 37°C in a 5% CO2/air incubator. Six days after culture initiation, cells were fractionated on Ficoll, and the lymphoid fraction was subjected to positive selection of CD8+ cells using magnetically labeled anti-CD8 Abs and a MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany). The isolated cells (2 × 10^5/ml) were restimulated with irradiated (20 Gy) splenocytes from the original third-party donors (FVB, C3H/HeJ, or SJL; 2 × 10^5/ml), and human rIL-2 (40 U/ml; Eurocetus, Milan, Italy) was added, beginning that day, every second day to the MLR culture (days 6, 8, and 10). On day 10, the MLR cultures were harvested, fractionated on Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden), analyzed by FACS for CD8 level, and tested for veto activity at different cell ratios, as described in Results.

Preparation of host-nonreactive, anti-third-party CD4+ cells

Splenocytes of BALB/c or FVB mice (nonspecific H-2 control) were harvested, and single cell suspensions were prepared. The cell suspensions were treated with Tris-buffered ammonium chloride to remove RBC, and the isolated mononuclear cells (2 × 10^5/ml) were stimulated with irradiated (20 Gy) C3H/HeJ or SJL (third-party stimulators) splenocytes (2 × 10^5/ml). Responders and stimulators were cultured for 10 days in a complete tissue culture medium at 37°C in a 5% CO2/air incubator. Six days after culture initiation, cells were fractionated on Ficoll, and the lymphoid fraction was subjected to positive selection of CD4+ cells using magnetically labeled anti-CD4 Abs and a MACS system (Miltenyi Biotec). Human rIL-2 (40 U/ml; Eurocetus) was added every second day to the MLR culture. On day 10, the MLR cultures were harvested, fractionated on Ficoll-Paque Plus (Miltenyi Biotec). The purified cells were then cultured in the presence of LPS (20 µg/10^6 cells; Difco, Detroit, MI). On day 4, the cultures were harvested, fractionated on Ficoll-Paque Plus (Amersham Biosciences), and analyzed by FACS for their veto activity at different cell ratios, as described in Results.

Assay for veto activity in the 2C TCR Tg mouse model

Spleen cells of 2C Tg H-2b mice, expressing the TCR-αβ with specificity for H-2Ld mice (provided by J. Nikolic-Zugic, Sloan-Kettering Institute), were collected as described above. The cells (2 × 10^6/ml) were then stimulated with irradiated (20 Gy) BALB/c splenocytes (2 × 10^6/ml) in the presence or the absence of 20, 10, or 2% cells of specific (BALB/c origin) and nonspecific (FVB origin) veto CTLs. 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 FACSscan (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-CD8α (Ly-2)-FITC, anti-CD8α (Ly-2)-CyChrome, anti-CD8α (Ly-2)-allophycocyanin, anti-CD3e-PE, anti-CD95 (Fas)-FITC (BD Pharmingen, San Diego, CA), and anti-CD4-Quantum Red (r) (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 (26–28) and supplemented with 5 µl of annexin V-Cy5. The cells were incubated at room temperature for 5 min in the dark, then washed in binding buffer. Positive cells were monitored by flow cytometry.

Results

Veto activity of anti-third-party CTLs in the 2C TCR Tg mouse model

Direct FACS analysis of veto activity. The TCR Tg anti-H-2d CD8+ T cells of the 2C mouse can be identified by immunostaining with a clonotypic Ab directed against the transgene TCR. Thus, it is possible to monitor by FACS the induction of apoptosis of the effector cells by specific veto cells.

To adapt this model for the study of veto cells, anti-third-party veto CTLs, which were previously characterized by functional inhibition of specific target killing (limiting dilution assay of the Cr release assay), were initially used. In these experiments, anti-third-party CTLs generated from the BALB/c (H-2d) or FVB/N (H-2b) mice served as veto cells.

Preparation of activated NK cells

Splenocytes of BALB/c mice or FVB mice were harvested, and single cell suspensions were prepared. The cell suspensions were treated with Tris-buffered ammonium chloride to remove RBC, and the isolated mononuclear cells (2 × 10^6/ml) were subjected to positive selection of NK cells using magnetically labeled anti-NK Abs (DEX-5) and a MACS system (Miltenyi Biotec). The purified cells were then cultured in the presence of LPS (20 µg/10^6 cells; Difco, Detroit, MI). On day 4, the cultures were harvested, fractionated on Ficoll-Paque Plus (Amersham Biosciences), and analyzed by FACS for their veto activity at different cell ratios, as described in Results.

Preparation of activated B cells

Splenocytes of BALB/c mice or FVB mice were harvested, and single cell suspensions were prepared. The cell suspensions were treated with Tris-buffered ammonium chloride to remove RBC, and the isolated mononuclear cells (2 × 10^6/ml) were subjected to positive selection of B cells using magnetically labeled anti-B-220 Abs and a MACS system (Miltenyi Biotec). The purified cells were then cultured in the presence of LPS (20 µg/10^6 cells; Difco, Detroit, MI). On day 4, the cultures were harvested, fractionated on Ficoll-Paque Plus (Amersham Biosciences), and analyzed by FACS for their veto activity at different cell ratios, as described in Results.
Table I. Veto activity of anti-third party CTLs

<table>
<thead>
<tr>
<th>Veto:Effector Ratio</th>
<th>Without veto cells</th>
<th>With veto cells</th>
<th>% Inhibition</th>
<th>t test</th>
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<tr>
<td>0.0025</td>
<td>53</td>
<td>59</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.005</td>
<td>49</td>
<td>46</td>
<td>6</td>
<td>0.7</td>
</tr>
<tr>
<td>0.01</td>
<td>48</td>
<td>48</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.02</td>
<td>40</td>
<td>43</td>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>0.1</td>
<td>43</td>
<td>36</td>
<td>16</td>
<td>0.03</td>
</tr>
<tr>
<td>0.2</td>
<td>46</td>
<td>22</td>
<td>51</td>
<td>0.014</td>
</tr>
</tbody>
</table>

*Results expressed as a percentage of inhibition are calculated as follows: % inhibition = 1 – (frequency of 1B2+ CD8+ cells in the presence of veto cells)/(frequency 1B2+ CD8+ cells in the absence of veto cells) × 100. The SD of replicate values was consistently <10% of the mean.

T cell activation is required for veto reactivity. To evaluate the role of cell activation in veto activity, we compared CD8+ veto CTLs of F1 origin to fresh CD8+ spleen T cells. As shown in Table II, the latter cells exhibit only marginal veto activity. Likewise, fresh CD8+ T cells purified from alloreactive mice (sharing the H-2 determinants of the stimulator cells) did not exhibit veto activity, similar to the level exhibited in the control group by fresh CD8+ T cells purified from alloreactive mice not sharing the H-2 determinants of the stimulator cells.

Role of cell contact and veto cell irradiation

As previously suggested by other indirect assays, the veto activity of the CTLs is dependent on cell contact. Thus, upon separating veto CTLs from effector cells in Transwell plates, the veto activity was completely lost (Table II). Likewise, irradiation of the veto cells abrogated their reactivity (Table II).

Relative potencies of different veto cells

Several lymphocyte subpopulations and bone marrow cells were shown to exhibit veto activity (29–41). The establishment of a quick and effective direct assay using 2C TCR Tg murine cells enabled us to compare the relative potencies of different veto cells using the same scale. Thus, the veto activity of anti-third-party CTLs in the 2C model, reflected by expansion inhibition of CD8+ 1B2+ responder cells, was compared with that exhibited by the following lymphocyte subpopulations: 1) resting CD4+ T cells, 2) resting CD8+ T cells, 3) resting NK cells, 4) activated CD4+ T cells, 5) LPS-activated B cells, and 6) H-2-activated NK cells. As
addition of anti-third-party CTLs of the H-2q background did not
lead to any appreciable inhibition up to a veto:effector ratio of 0.2
(p = 0.005; Table III).

By this analysis, activated NK cells exhibited lower veto activity,
attaining significance at a veto:effector ratio of 0.1 (p = 0.004;
Table III). However, considering that at a 0.05 ratio the inhibition
was close to being significant (p = 0.06), it could be that effective
inhibition might have been attained between a veto:effector ratio of
0.05 and 0.1, suggesting 10- to 20-fold reduction in veto activity
compared with veto CTLs.

Activated CD4 cells exhibited inhibition only at a veto:effector
ratio of 0.2, suggesting reduction of veto activity compared with
NK cells by 2- to 5-fold, whereas activated B cells are completely
devoid of veto activity.

Finally, all cell types tested failed to exhibit veto activity if
not activated before addition to the 2C culture (Fig. 2).

Discussion

In the present study we characterized several attributes of veto

cells as reflected by direct FACS measurement of H-2a-specific
effecter T cells in the 2C TCR Tg mouse model. These attributes
include definition of the effective range of veto:effector ratios,
the role of cell contact and irradiation, and the role of cell activation.
Furthermore, by establishing the optimal conditions for this direct
assay it was possible to compare the veto reactivities of different
lymphocyte subpopulations on the same scale. Clearly, this direct
assay is not only more simple and straightforward, but it is also
less likely to be associated with artifacts typical of the indirect
lengthy functional assays. One major observation in the 2C model
is the lack of any appreciable veto activity exhibited by nonacti-
vated lymphocytes. Thus, CD8+ or CD4+ T cells as well as NK
cells uniformly require prior activation to be endowed with veto
activity. In contrast, B cells do not exhibit veto activity even if
activated before testing. The importance of cell activation was
previously indicated by an in vivo study in which CD8 T cells of male
origin were infused into female TCR Tg mice bearing a transgene
against the HY male Ag. Thus, although the infused veto cells
were able to induce reduction of the specific anti-male clones, this
activity was lost when using donor cells lacking the intracellular
domain of the CD8 molecule, important for the activation of CD8
T cells (16). Taken together, these indications pointing to the
importance of cellular activation for the veto activity are intriguing
considering that freshly isolated (donor × host)F1 CD8+ T cells
were shown to facilitate bone marrow (35, 42, 43) or skin al-
lografting (44). This discrepancy could be explained if these non-
alloreactive F1 T cells were activated in vivo by a mechanism
similar to homeostasis, driven by the conditioning of the recipients
(45–47). Alternatively, it is possible that freshly isolated CD8+ T
cells might induce tolerance to donor cells by a mechanism inde-
pendent of the veto mechanism (48).

A second major finding of the present study is the establishment
of the relative hierarchy of veto activity, comparing anti-third-
party CTLs, activated NK cells, and activated CD4+ T cells.

The role of NK cells in this context could be complex, because
it has been shown that alloreactive NK cells could potentially re-
duce host immune cells without GVHD, thereby enhancing en-
graftment of stem cell allografts (49–51). Such ablation of host-
type immune cells is possibly also associated with undesirable
infections. However, the donor-specific veto reactivity measured
in the present study at different veto:effector ratios in the absence

![Table III. The relative veto activity of activated lymphocyte subpopulations](image)

<table>
<thead>
<tr>
<th>Cells</th>
<th>Veto:Effector ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>CD8 T cells</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>% 1B2 CD8+ cells</td>
</tr>
<tr>
<td></td>
<td>% Inhibition</td>
</tr>
<tr>
<td></td>
<td>Statistical significance</td>
</tr>
<tr>
<td>B</td>
<td>% 1B2 CD8+ cells</td>
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<tr>
<td></td>
<td>% Inhibition</td>
</tr>
<tr>
<td></td>
<td>Statistical significance</td>
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<tr>
<td>NK A</td>
<td>% 1B2 CD8+ cells</td>
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<td></td>
<td>% Inhibition</td>
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<td></td>
<td>Statistical significance</td>
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<tr>
<td>B</td>
<td>% 1B2 CD8+ cells</td>
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<tr>
<td></td>
<td>% Inhibition</td>
</tr>
<tr>
<td></td>
<td>Statistical significance</td>
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<tr>
<td>CD4 T cells</td>
<td>% 1B2 CD8+ cells</td>
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<td></td>
<td>% Inhibition</td>
</tr>
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<tr>
<td>NK B</td>
<td>% 1B2 CD8+ cells</td>
</tr>
<tr>
<td></td>
<td>% Inhibition</td>
</tr>
<tr>
<td></td>
<td>Statistical significance</td>
</tr>
</tbody>
</table>

Results expressed as a percentage of inhibition, calculated as follows: % inhibi-
tion = 1 – (frequency of 1B2 CD8+ cells in the presence of veto cells)/(frequency
of 1B2 CD8+ cells in the absence of veto cells) × 100. For each cell subpopu-
lation, inhibition was tested in presence of specific cells of H-2a origin (A) or non-
specific H-2a origin (B). Statistical significance was evaluated by Student’s t test.

![Figure 2. The relative veto activities of different lymphocyte subpopulations. The inhibitory effects of CD8 cells (■), NK cells (⧫), CD4 cells (□), and B cells (●) were compared with that of a control MLR without veto cells (□). The figure shows the veto effect of specific (origin H-2a; A) and nonspecific (origin H-2a; B) freshly isolated cells. The results represent the average ± SD of three different experiments.](image)
of alloreactivity (as evidenced by the control group in which NK cells of a genetic background not recognized by the 2C effector cells did not lead to deletion of the effector cells) strongly support previous studies (23) suggesting that nonalloreactive NK cells could induce tolerance with minimal ablation of host immunity.

It is well established that the level of immune debulking is inversely proportional to the number of vetto cells required to overcome graft rejection. Thus, although megadose stem cell transplants could suffice to allow engraftment in heavily conditioned patients, large numbers of other veto cells might be required to establish stem cell engraftment in patients exposed to reduced intensity conditioning. Considering that new methodologies have been developed to expand ex vivo NK cells (52–55) as well as host nonreactive CD4+ T cells (56, 57), it might be possible in the future to use all these different sources of vetto cells to facilitate engraftment of hemopoietic allografts without the unwanted complications of GVHD-producing T cells.

References


