Anti-Third Party Veto CTLs Overcome Rejection Of Hematopoietic Allografts: Synergism with Rapamycin and BM cell dose

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Abstract

Several bone marrow cells and lymphocyte subpopulations, known as ‘veto cells’, were shown to induce transplantation tolerance across major histocompatibility antigens. Some of the most potent veto cells are of T cell origin, and in particular a very strong veto activity was documented for CTL lines or clones. However, these cells also possess marked GVH reactivity. In the present study we evaluated a new approach to deplete CTLs of anti-host clones by stimulating the donor T cells against third party stimulators in the absence of exogenous IL-2. We demonstrate that such CTLs are depleted of GVH reactivity while maintaining marked veto activity in vitro. Furthermore, marked synergism was exhibited between the veto CTLs and rapamycin when tested in a murine model, which measures T cell, mediated bone marrow allograft rejection, or in sublethally irradiated allogeneic hosts.

Our results suggest that engraftment of early progenitors could be enhanced by using host non-reactive anti-third party CTLs, in conjunction with non-myeloablative rapamycin based conditioning protocols, thereby significantly reducing the toxicity of allogeneic transplantation.

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Introduction

Bone marrow transplantation following supralethal radiochemotherapy is associated with dangerous infections due to the slow immune reconstitution during the first year post transplant\textsuperscript{1-6}. 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 non-malignant diseases, or for the induction of ‘mixed chimerism’ as a prelude for cell therapy in cancer or in organ transplantation. However, the marked level of host hematopoietic and immune cells surviving mild preparatory regimens represents 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 \textsuperscript{7-10}. Potent post-transplant immunosuppression and the presence of large numbers of alloreactive T cells in the graft enabled a high rate of engraftment. However, GvHD, particularly lethal chronic GvHD, remains a major obstacle\textsuperscript{9,11-13}. While 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 antigens in the host thymus, thereby induce durable tolerance to donor cells or tissues, represents one of the most desirable goals in transplantation biology.

One approach to overcoming immune rejection of incompatible stem cells rigorously depleted of T cells, made use initially of increased doses of T cell depleted bone marrow in mice\textsuperscript{14-17} and rats\textsuperscript{18}. Subsequently the cell–dose escalation concept was also shown with purified stem cells\textsuperscript{19-22}. However, although this modality has become a clinical reality in the treatment of patients with leukemia, conditioned by intensive chemotherapy, it has been suggested in studies in mice\textsuperscript{21} and non-human primates (X. Yao, unpublished data, July 2001) that the number of hematopoietic precursors required to overcome the immune barrier in hosts pretreated with sublethal regimens cannot be attained with the state-of-the-
art technology for stem cell mobilization. Rachamim et al.\textsuperscript{23} demonstrated that when purified CD34\textsuperscript{+} cells were added to bulk mixed lymphocyte reaction (MLR), they suppressed CTLs against matched stimulators but not against stimulators from a third party\textsuperscript{23}. These results strongly indicated that cells within the human CD34\textsuperscript{+} population are endowed with potent veto activity. Veto activity was defined in 1980 by Miller\textsuperscript{24} as the capacity to specifically suppress cytotoxic T cell precursors directed against antigens of the veto cells themselves, but not against third party antigens.

Interestingly, it has been shown that some of the most potent veto cells are of T cell origin and, in particular, a very strong veto activity was documented for CD8\textsuperscript{+} CTL lines or clones\textsuperscript{25-31}. The specificity of the veto effect mediated by CTL clones was shown by several studies to be unrelated to their T cell receptor specificity\textsuperscript{32-34}. The suppression of effector CTL-p directed against the veto cells is both antigen-specific and MHC-restricted, resulting from the unidirectional recognition of the veto cell by the responding cytotoxic T-lymphocytes, but not vice versa\textsuperscript{33}. Furthermore, it has been shown that this suppression is mediated by apoptosis\textsuperscript{33,35} and that co-expression of both CD8 and FasL is a prerequisite for the veto reactivity of the CTLs\textsuperscript{36} (Fig 1).
Fig 1: Veto CTLs induce apoptosis in the effector T cells by a Fas-FasL mediated mechanism. Upon engagement between the TCR of the effector cell and class I of the veto cell, the effector cell is activated and Fas is upregulated allowing for the FasL on the veto CTL to induce apoptosis. However, inhibitors such as FLIP protect the activated effector T cell. The high affinity afforded by the interaction between CD8 on the veto cell and class I on the effector cell enables prolonged association until Flip is down regulated and apoptosis can take place\textsuperscript{36}.

Clearly, the limitations of CD34\textsuperscript{+} collection in humans might be overcome if it was possible to supplement these cells with other veto cells provided the latter also lack GVH reactivity. Despite their remarkable potent veto activity, CD8 CTLs could not be used for tolerance induction in allogeneic stem cell transplantation due to their marked GVH reactivity. To address this problem we developed a new approach for the generation of host-non-reactive 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 the activated
CTLs are capable of surviving the IL-2 starvation in the primary culture. In the present study we optimized the conditions that afford large numbers of anti-third party CTLs under IL-2 deprivation, and we tested the efficacy of such cells to induce specific transplantation tolerance without GVHD. Furthermore, considering that previous studies documented the role of the immunosuppressive drug rapamycin in GVHD prevention and in overcoming rejection, the potential synergy between rapamycin and veto CTLs was evaluated.
Methods

Experimental Procedures

Animals. Mice used were female 6-12 weeks old. Balb/c, Balb/c-Nude, FVB, SJL, F1(C3HxBalb) and C57BL/6 were obtained from the Weizmann Institute Animal Center (Rehovot, Israel). C3H/HeJ mice were obtained from the Roscoe B. Jackson Memorial Laboratory (Bar Harbor, ME). A breeding pair of Transgenic (Tg) H-2\textsuperscript{b} mice expressing the TCR from the CTL clone 2C with specificity for H-2L\textsuperscript{d}, was kindly provided by Janko Nikolic-Zugic (Sloan-Kettering, NY). Progeny of these Tg mice was bred at the Weizmann Institute Animal Breeding Center (Rehovot, Israel). All mice were kept in small cages (five animals in each cage) and fed sterile food and acid water containing ciprofloxacin (20 µg/ml).

Preparation of donor non-host reactive CTLs. Spleen cells from Balb/c mice (donor origin), 6 to 16 weeks of age were harvested, lysed in (0.5 ml/spleen) cold ACK buffer (0.15M Potassium-ammonium chloride buffer) to remove red blood cells and brought to a concentration of 2x10\textsuperscript{6} cells/ml in RPMI 1640 supplemented with 15 mm HEPES (PH 7.4), 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 5x10\textsuperscript{-5} 2-ME and 100 U/ml penicillin, 0.1 mg/ml streptomycin. This splenic single cell suspension was co-cultured at a ratio of 1:2 with irradiated (2000 cGy), ACK treated spleen cells from C57BL/6 or FVB mice (third party origin) and cultured at 37°C in a humidified 5% CO\textsubscript{2} 95% O\textsubscript{2} atmosphere. Six days later the co-cultures were fractionated on Ficoll and the lymphoid fraction was enriched for CD8\textsuperscript{+} cells by positive selection using magnetically labeled anti-CD8 antibodies and a magnetic cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach Germany). The CD8 enriched fraction was then re-suspended at 0.5x10\textsuperscript{6} cells/ml and co-cultured at a ratio of 1:4 with the same irradiated (20 Gy) third party stimulators, in the presence of 40 IU/ml recombinant human IL-2 (rhIL-2). The CTL cultures were re-stimulated weekly and rhIL-2 was added every 3 days for 2 more weeks.

In Vitro assay of anti-third party veto CTLs: Deletion of 2C effector CD8 T cells. To determine whether non-alloreactive donor anti-third party CTLs possess veto activity, spleen cells of 2C Transgenenic H-2\textsuperscript{b} mice expressing the TCR-αβ with specificity for H-2L\textsuperscript{d}...
mice (kindly provided by Janko Nikolic-Zugic, Sloan-Kettering, NY), were collected as described above. The cells (2x10^6/ml) were then stimulated with irradiated (20 Gy) Balb/c splenocytes (2x10^6/ml) in the presence of 10% cells of the veto anti-third-party CTLs from Balb/c origin while anti-third-party CTLs of C57BL/6 and/or SJL background serve as non-specific controls. Cultures containing 10% veto CTLs were cultured for 72 in 6-well plates. The deletion of the transgenic T cells was monitored by cytofluorimetric analysis, measuring the level of 2C transgenic cells specifically stained by the 1B2 antibody directed against the clonotypic anti-H-2L^d TCR.

**Evaluation of apoptosis by flow cytometry using Cy 5-annexin V.**
Cells (1-2x10^5) were stained with Cy 5-annexin V (MBL Medical & Biological Laboratories Co., Naka-ku Nagoya, Japan) according to manufacturer’s protocol. Briefly, the cells were suspended in 100 µl of binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2) and incubated for 10 min. The cells were then washed with binding buffer and analyzed by flow cytometry.

**Bone marrow transplantation.** For GVH reactivity determination, C3H/HeJ mice were exposed to a single dose of 11 Gy (lethal conditioning) TBI from a Gamma beam 150-A 60Co source (produced by the Atomic Energy of Canada, Kanata, Ontario) with focal skin distance of 75 cm, at a 0.65 Gy/min dose rate. The following day the mice received intravenously, optimal dose (2x10^6) of Balb/c–Nude BM supplemented with 2x10^6 anti third party CTLs of specific (Balb/c) and non-specific (SJL) origin and unmanipulated donor splenocytes.

In the experiments studying the synergistic effect between veto CTLs and rapamycin under reduced conditioning, C3H/HeJ mice were sublethally irradiated (7 Gy) and 24 hours later inoculated with Balb/c-Nude BM (4-5x10^6) with or without veto CTLs (10x10^6). Chimerism was determined 30 days post transplant.

**Chimerism analysis.** Chimerism was determined by cytofluorimetry. Peripheral blood cells were fractionated on Ficoll-Paque plus, and the isolated mononuclear cells of each mouse were double stained by direct immuno-fluorescence with FITC anti-H2^d monoclonal
antibody specific for the donor and PE anti-H2K for specific host. The antibodies were obtained from Pharmingen: FITC- H-2Dd (clone 34-2-12) and PE- H-2Kk (clone 36-7-5).

**T cell mediated allograft rejection model.** C3H/HeJ female mice (6-8 weeks of age) were exposed to a single dose of 11Gy (supra-lethal conditioning) TBI on day –2. The following day the mice received, intravenously, 1x10^4 unseparated host T cells. Transplantation of 2x10^6 allogeneic Balb/c-Nu BM cells was performed on day 0 in conjunction with the veto cells to be evaluated. The weight of the mice and the survival rate are monitored twice a week.

**Host T cell preparation.** Splenocytes of host C3H/HeJ mice were fractionated on Ficoll/Paque and the isolated mononuclear cells were subjected to a positive selection of T cells (CD4 plus CD8) by magnetic cell sorting (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany). Cytofluorimetric analysis of the fractionated cells is carried out by triple immunofluorescent staining, using the following directly labeled antibodies (obtained from Pharmingen San Diego, CA): fluorescein isothiocyanate (FITC)- CD4/L3T4 (clone H129.19), R-phycoerythrin (PE) –CD3e (clone 145-2C11) and Cy-Chrome - CD8a/ Ly-2 (clone 53-6.7).

**Cytofluorimetric analysis.** FACS analysis was performed using a modified Becton Dickinson FACScan. Fluorescence data were collected using 3-decade logarithmic amplification on 25-50 x 10^3 viable cells as determined by forward-light-scatter intensity. Cells were stained with a CD8a (Ly-2)-FITC, CD4 (L3T4)-CyCherome, CD8a (Ly-2)-APC, CD3ε-PE, (Pharmingen) CD4-Qantum Red (sigma), 1B2 biotinated (kindly provided by Janko Nikolic-Zugic, Sloan-Kettering, NY), R-PE streptavidin (Jackson Immuno. Research Lab. Inc.)
Results

Optimization of an IL2 deprivation protocol for generating anti-third party veto CTLs devoid of GVH reactivity.

Cell recovery and purity

We have shown in-vitro that host non-reactive anti-third party CTLs generated under IL-2 deprivation for 5 days are endowed with marked veto activity\textsuperscript{36}. The IL-2 deprivation period was established based on the maximal time that mouse spleen T cells can be maintained in MLR culture without IL2 (data not shown). However, attempts to grow large cell numbers 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 LAK cells and/or CD4 T cells (data not shown). Thus, this problem was addressed by removal of CD4\textsuperscript{+} and NK cells at the end of the IL-2 deprivation, prior to the addition of IL2.

![Diagram](image)

**Fig 2:** Preparation of non-alloreactive veto CTLs.
As can be seen in Fig.2 the preparation of anti-third party CTLs comprises three major steps: i) Anti-third party stimulation under IL2 deprivation for 6 days. ii) Positive selection of CD8+ T cells (at day 6). iii) CTL expansion under re-stimulation in the presence of IL2 (day 6 to 25).

The cell recoveries obtained in five experiments from Balb/c donors and in one experiment from FVB mice are shown in Table1. The average number of the splenocytes in the initial cell preparation was 2023 x10^6 ± 579 x10^6. Following 6 days of incubation with irradiated (20 Gy) third party splenocytes at an effector/stimulator ratio of 1:2, live mononuclear cells were harvested by Ficoll density separation (316 x10^6 ± 213 x10^6) and the number of CD8+ T cells recovered after positive selection with magnetic beads was further reduced to 69 x10^6 ± 50 x10^6 cells. These anti-third party CTLs were then re-stimulated and cultured in the presence of IL-2 (40 IU/ml), affording 437x10^6±116x10^6 cells within 18-25 days.
Table 1. Generation of host non-reactive anti-third party CTLs: cell recovery following different stages of the protocol

<table>
<thead>
<tr>
<th>Responder/third party stimulators 1:2</th>
<th>Culture initiation cells (x10^6)</th>
<th>Before CD8 selection cells (x10^-6)</th>
<th>After CD8 selection cells (x10^-6)</th>
<th>End of culture cells (x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/c --- &gt; FVB</td>
<td>1875</td>
<td>183</td>
<td>36</td>
<td>492</td>
</tr>
<tr>
<td>Balb/c --- &gt; FVB</td>
<td>2500</td>
<td>305</td>
<td>67</td>
<td>480</td>
</tr>
<tr>
<td>FVB ---&gt; C57BL</td>
<td>1750</td>
<td>315</td>
<td>72</td>
<td>470</td>
</tr>
<tr>
<td>Balb/c --- &gt; FVB</td>
<td>1950</td>
<td>190</td>
<td>42</td>
<td>350</td>
</tr>
<tr>
<td>Balb/c --- &gt; FVB</td>
<td>2950</td>
<td>770</td>
<td>174</td>
<td>590</td>
</tr>
<tr>
<td>Balb/c --- &gt; FVB</td>
<td>1115</td>
<td>135</td>
<td>23</td>
<td>230</td>
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<td></td>
<td>2023±579</td>
<td>316±213</td>
<td>69±50</td>
<td>437±116</td>
</tr>
</tbody>
</table>
FACS analysis of the cells that were positively selected for CD8 at day 6, as well as the cells harvested at the end of the culture period, showed that more than 90% of the cells are CD8^+CD3^+ T cells (Fig 3).

Fig 3: Purification profile of anti-third party CTLs detected by FACS analysis at day 6 (a-c) and at the end of the culture period (d). Unseparated cells (a) CD8 negative fraction (b) CD8 positive fraction (c, d).

**Depletion of GVH reactivity by IL2 deprivation of anti-third party CTLs.**

The CTLs recovered by the above protocol were routinely tested for GVH reactivity in fully mismatched hosts, lethally irradiated and radio-protected with BM from Balb/c-'nude' donors. As can be seen in Fig 4 showing a typical experiment, when C3H/HeJ hosts were conditioned by 11 Gy TBI all the mice died within 10 days while more than 80% of the mice receiving 2x10^6 BM cells from Balb/c-'nude' donors survived. Infusion of 2x10^6 veto CTLs originating from Balb/c, F1(C3HxBalb) or SJL donors, together with the BM, was associated with 0%, 0% and 15% mortality respectively, in contrast to transplantation of 2x10^6 Balb/c splenocytes, together with the BM cells that led to more than 80% mortality within the first month. Thus, the IL2 deprivation procedure for the preparation of anti-third party CTL culture is associated with marked depletion of GVHD reactivity.
Donor (Balb/c) anti-third party (C57BL/6) CTLs are depleted of GVH reactivity against the host (C3H/HeJ). Host mice were conditioned with 11 Gy TBI and radioprotected with 2x10^6 Balb/c-nude BM cells. The GVH reactivity of the CTLs or the unseparated spleen cells is reflected by the percent survival following infusion of 1x10^6 cells.

**In-vitro determination of veto activity of anti-third party CTLs in the 2C TCR transgenic mouse model.**

Considering that the LDA assay used to monitor inhibition of CTLp by veto cells is lengthy and cumbersome, we tested whether effector T cells from a TCR transgenic mouse model could afford a more convenient assay for veto activity, allowing the use of FACS for monitoring deletion of effector T cells by the veto CTLs. For this purpose, spleen cells of the 2C TCR transgenic mouse, in which the CD8 T cells express a TCR transgene directed against H-2^d^ class I^40^, were used as effector cells. The transgene can be stained by a clonotypic monoclonal antibody (1B2 kindly provided by Janko Nikolic-Zugic, Sloan-Kettering, NY), allowing to monitor expansion or deletion of the effector cells upon stimulation in the presence or absence of veto cells.

As can be seen in Fig 5, following a short incubation for 72 hr with veto CTLs the percentage of CD8^+^1B2^+^ cells is reduced from 80% to 11%, if the CTLs are of H-2^d^ origin, compared to reduction from 80% to 71% upon incubation with CTLs generated from...
spleen cells of H-2k background. Furthermore, annexin staining of the CD8+1B2+ showed that marked apoptosis is induced by the veto cells between 48-72 hr of incubation with the veto cells, in agreement with previous reports suggesting that veto CTLs exert their inhibitory effect by a deletion based mechanism36. Along with the depletion of CD8+1B2+ cells by the specific veto cells, the CD81B2- and CD8+1B2- cell subpopulations were notably increased from 0.5% and 1% in Fig 5Aa, to 19% and 22% respectively in Fig 5Ac.

**Fig. 5:** A) Specific deletion of 2C effector CD8 T cells by veto CTLs following 72 hr incubation. (a) 2C levels in the absence of veto CTLs. (b) 2C levels in the presence of non-specific veto CTLs of C57BL/6 background. (c) 2C levels in the presence of veto CTLs of Balb/c background (recognized by the 2C TCR transgene).

B) Annexin staining demonstrating the induction of apoptosis by the specific veto CTLs.
By using this assay the optimal effector: veto cell ratio was found to be between 1:20 to 1:50 (Fig 6). At this ratio the non-specific killing by CTLs generated from mice of SJL origin (H-2^s) is minimal. Likewise, as expected from studies based on the LDA of CTLp, resting CD8 T cells of H-2^d background, which can be recognized by the effector T cells but which have not been activated, lack veto activity and do not delete the 2C effector cells (data not shown.)

**Fig. 6**: A dose response curve comparing the inhibitory effect of specific anti-third party CTLs recognized by the 2C effectors (origin of H-2^d) and non-specific CTLs (origin of H-2^k). Average ± standard deviation of 5 different experiments.

Taken together, these results show that upon generation of host non-reactive CTLs as described above, it is possible to assess by a short and simple FACS assay the veto activity of the generated line prior to further in-vivo experiments.

**In vivo determination of tolerance induction by veto CTLs: Synergy between veto CTLs, bone marrow cell dose and rapamycin.**

The veto activity of the CTLs was tested initially in sublethally irradiated (7Gy) mice. As expected, following infusion of 5x10^6 Balb/c-'nude' BM cells the level of chimerism was very low (2/7). However, it was markedly enhanced when veto CTLs were added (4/4) (Fig 7).
Fig 7: Donor type chimerism in sublethally (7Gy) irradiated hosts (C3H/HeJ) of 5x10^6 allogeneic BM(Balb/c-nude): enhancement by veto CTL.

The importance of stem cell dose in this equation was emphasized again in these experiments. Thus, upon reducing the BM cell dose to 4x10^6 cells, a high level of engraftment was not achieved even upon the infusion of the CTLs (1/6) (Fig 8).

Fig 8: Reduced donor type chimerism in sublethally (7Gy) irradiated hosts (C3H/HeJ) of 4x10^6 allogeneic BM (Balb/c-nude) and veto CTLs.

The insights gained on the mechanism of action of veto CTLs are important for the selection of additional agents that could be synergistic with the veto cells. Based on
previous work of Miller and colleagues\textsuperscript{24,33,34} on the importance of CD8 on the veto cell, and our more recent work\textsuperscript{36} showing the role of FasL, it has been established that deletion of effector T cells by the veto CTL is mediated through Fas upregulation on the effector cells. Considering that rapamycin blocks IL-2 signaling which occurs after Fas up-regulation in the T cell activation process, it could be predicted that rapamycin should not interfere with the veto activity. Moreover, cells, which escape deletion, by the veto cells could still be eliminated by rapamycin.

Thus we chose to test the potential synergy between rapamycin and veto CTLs. Since graft failure might reflect not only immune rejection but also stem cell competition, we tested the efficacy of the veto CTLs in a model, which adequately measures the isolated effect of our interventions on immune rejection. In this model (Fig 9), host mice are conditioned by supra-lethal TBI and radio protected with ‘Nude’ BM as a source of T cell depleted BM. Graft rejection is induced by infusion of 1x10\textsuperscript{4} purified host T cells. Thus, all the mice die in the radiation control group while the mice that were transplanted with BM survive the radiation. In an early study Lapidot et al. showed that the addition of host T cells in this model leads to graft rejection and consequently to lethal aplasia as evidenced by the blood cell counts\textsuperscript{14}.

\textbf{Fig 9:} A stringent mouse model for T cell mediated BM allograft rejection

As can be seen in Table 2, a marked synergism between veto CTLs and rapamycin was recorded. In 7 independent experiments only 8/48 or 2/46 mice that were infused with 10x10\textsuperscript{6} CTLs or treated with rapamycin alone were engrafted respectively, whereas treatment with rapamycin, together with CTL infusion, resulted in marked survival (34/47).
The failure of rapamycin as a single agent to ameliorate graft rejection and prevent aplasia under these conditions could not be associated with bone marrow toxicity, as it did not affect adversely the survival of mice receiving Balb/c-'nude' BM in the absence of host T cells (6/7).

Table 2: Synergism between rapamycin and host-non-reactive veto CTLs

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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Average %</th>
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<td>0/7</td>
<td>0/7</td>
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<td>0/5</td>
<td>0/7</td>
<td>0/47</td>
<td>0</td>
</tr>
<tr>
<td>Balb/c-Nu / BM 2 x 10^6</td>
<td>6/7</td>
<td>7/7</td>
<td>6/7</td>
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<td>8/8</td>
<td>5/5</td>
<td>7/7</td>
<td>45/48</td>
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<td>Balb/c-Nu / BM 2 x 10^6 + Rapamycin^b</td>
<td>-</td>
<td>6/7</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>Balb/c-Nu / BM 2 x 10^6 + HTC^a 1 x 10^4 + CTL (Balb/c--&gt;FVB) 10x10^6</td>
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<td>Balb/c-Nu / BM 2 x 10^6 + HTC^a 1 x 10^4 + CTL (Balb/c--&gt;FVB) 10x10^6 + Rapamycin^b</td>
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<td>2/7</td>
<td>4/6</td>
<td>6/7</td>
<td>6/7</td>
<td>7/7</td>
<td>34/47</td>
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</table>

^aHTC – Host (C3H/HeJ) T cells (CD4+ & CD8+)
^b Rapamycin 10 ug/mouse/day
A similar synergistic effect between veto CTLs and rapamycin was observed when anti-third party CTLs were generated from F1(C3H/HeJx Balb/C) origin, suggesting that the veto activity is not mediated by residual anti-host reactivity (Fig 10).

**Fig 10:** Enhancement of engraftment by anti-third party CTLs generated from (hostxdonor)F, mice compared to enhancement induced by CTLs of donor type.

Furthermore, as illustrated in Fig 11, the veto effect was found to be H-2 specific, since a CTL line, originating from a strain other than that of the BM donor, failed to prevent graft rejection.

**Fig 11:** Enhancement of engraftment of veto CTLs of the BM donor background (Balb/c) Vs veto CTLs of a different background (STL/J).
Considering that in sublethally irradiated mice infusion of $10 \times 10^6$ veto CTLs could effectively enhance engraftment of $5 \times 10^6$ nude BM cells, but only marginally facilitated engraftment of $4 \times 10^6$ BM cells, the potential synergy with rapamycin was further evaluated in this model. As can be seen in Fig.12, anti-third party CTLs and rapamycin by themselves exert a marginal effect on chimerism in hosts of $4 \times 10^6$ BM cells (1/6 and 2/5 mice were engrafted respectively). However, when treated with the two agents together, 11/13 mice were found to be donor type chimera at 4 month post transplant.

Fig 12: Synergistic enhancement of BM allografting of anti-third party CTLs and rapamycin in sublethally irradiated mice.
Discussion

Our results clearly show that anti-third party CTLs generated under IL2 deprivation afford a suitable source for effective veto cells which can enhance BM allografting without GVHD. In particular, these cells are effective if applied in conjunction with rapamycin. This synergism is compatible with the recent demonstration that veto CTLs operate via Fas-FasL triggering, upstream of rapamycin inhibition of IL2R signaling. Thus, rapamycin does not block the veto activity of CTLs but rather enhances their tolerizing effect, so that effector T cells which might have escaped deletion by the veto cells could still be eliminated by rapamycin. To analyze the relative contribution of each agent to BM allografting, we used a murine model which enables distinguishing between T cell mediated immune rejection and stem cell competition. In this model, rejection of T cell depleted BM allografts from ‘nude’ donors is induced in lethally irradiated mice by adoptive transfer of graduated numbers of purified host type T cells. Stem cell competition is minimal under this conditioning and, in the absence of T cell infusion, the host mice uniformly accept the allogeneic BM without GVHD. Thus, the observed graft rejection can be attributed to the adoptive transfer of purified host T cells.

It could be argued that the facilitating activity of the anti-third party CTLs is associated with a possible contamination of host-reactive T cell clones, which may not be sufficient to induce GVHD, but could potentially eliminate the infused host type T cells. This possibility is ruled out by two experiments: i) anti-third party CTLs which are not of donor origin, prepared by the same procedure used to generate the CTLs of donor origin, do not enhance engraftment of BM allografts. ii) Anti-third party CTLs of (host x donor) F1 origin, lacking alloreactivity due to their genetic background, enhance BM allografting similarly to the CTLs generated from the donor genetic background.

The effective number of the veto CTL that was found to synergize with rapamycin in the graft rejection model is rather high. Although state of the art technology enables large scale expansion of T cells, it is likely that the potential synergy between the combined administration of veto CTLs and other toleraizing cells such as CD4^+^CD25^-^ and/or Sca-1^-^Lin^-^ cells along with the use of maximal tolerable doses of rapamycin might further reduce the effective CTL dose.
An alternative approach to allow the use of anti-third party CTLs has been suggested recently by Fowler et al.\textsuperscript{41} who showed that TC2 CTLs are more effective veto cells compared to TC1 CTLs, and the former cells are also depleted of GVH reactivity\textsuperscript{42}. While we favor depletion of host reactive CTLp by IL2 starvation, the two approaches might be combined in the future to further enhance veto potency and reduce host reactivity of anti-third party CTLs.

As could be anticipated from the studies in the graft rejection model, marked synergism between rapamycin and veto CTLs was also observed upon transplantation of ‘nude’ Balb/c BM into sublethally irradiated C3H hosts. In this model we have previously shown that purified Sca-1\textsuperscript{+}Lin\textsuperscript{−} stem cell transplants can overcome rejection if very large numbers are administered. Thus, in evaluating the results in this model, it is important to note the contribution of veto cells within the stem cell compartment or other facilitating cells within the non-T cell compartment. Indeed, while administration of both rapamycin and veto CTLs was required to secure engraftment upon transplantation of 4×10\textsuperscript{6} Nude BM cells, a high level of chimerism could be obtained by using CTLs without rapamycin when the BM cell dose was increased by 20 percent. These results suggest a quantitative relationship between the residual host anti-donor CTL-p pool on one hand and the BM, Veto CTLs and rapamycin on the other. Preliminary results using (hostxdonor)F1 CTLs to distinguish between the infused CTLs and host or naïve donor derived T cells, suggest that the infused CTLs survive for at least a few weeks post transplant. In principle, when an adequate number of CTLs are infused and engraftment of donor cells prevails, it can be assumed that the majority of alloreactive host CTL-p have been eliminated. If the latter cells are not eliminated and are allowed to mature, graft rejection is more likely to take place.

This quantitative relationship between BM dose, veto CTLs and rapamycin could have immediate implications to allogeneic BMT, not only under reduced intensity conditioning but also in the treatment of leukemia patients undergoing full myeloablation. Presently, the conditioning of hosts of T cell depleted BM or of purified CD34 cells, includes ATG, the serum levels of which remain high for a prolonged period of time. While this agent might
reduce graft rejection and potential GVHD, it can also interfere with immune reconstitution during the early period post transplant. Considering the documented effect of short term treatment with rapamycin on GVHD and on graft rejection in murine models, we anticipate that the combination of anti-third party CTLs with rapamycin might successfully replace ATG in the conditioning of leukemia patients, even in the context of haploidentical transplants in which the risk for graft rejection and GVHD is significantly higher compared to HLA identical transplants.

Clearly, achieving engraftment under reduced intensity conditioning represents a major challenge due to the relatively large number of residual host T cells that might survive the conditioning. At present, such transplants are carried out predominantly in elderly patients who have an HLA identical sibling or an unrelated donor. Engraftment in these patients is greatly enhanced by the presence of a large number of alloreactive T cells in the graft, which are also associated with more than 20% GVHD lethality. The latter problem is even more pronounced in elderly patients with multiple myeloma or with B-CLL. Thus, the use of purified CD34 cells in conjunction with anti-third party CTLs and rapamycin may prove beneficial for such patients. If this approach will indeed prove to be associated with low transplant related mortality, it could be used as a prelude for cell therapy with donor lymphocyte infusions or with cell lines or clones directed against specific host type minor hematopoietic antigens or against tumor specific antigens. In this context, it is assumed that the CTLs are depleted of GVL reactivity, and they are predominantly used to induce tolerance for donor cells and, subsequently, other more specific cell lines or clones might be needed to eradicate disease. However, it is possible that anti-tumor or anti-viral clones within the infused CD8 cells are not completely diminished and might be expended upon proper stimulation in vivo. Further quantitative studies using the TcLandscape method developed by Guillet et al. to define the repertoire of these anti-third party CTLs,
are in progress. In addition, we found recently that anti-third party human CTLs generated
by a similar approach are endowed with an effective capacity to eradicate tumor cells from
patients with B-CLL. This TCR independent recognition between veto CTLs directed
against third party HLA and the BCLL cells, was shown to be mediated by LFA1- ICAM1
interaction (Arditti et al. unpublished results).
Finally, If successful, this approach could be further extended to treat diseases which are
lethal in the course of years but do not present an immediate threat, such as non-
malignant hematological diseases or in the induction of tolerance for organ transplantation.
References


Anti-third party veto CTLs overcome rejection of hematopoietic allografts: synergism with rapamycin and BM cell dose

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