Clonal Deletion of Postthymic T Cells: Veto Cells Kill Precursor Cytotoxic T Lymphocytes

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Summary

Veto cell-mediated suppression of cytotoxic T lymphocyte (CTL) responses has been proposed as one mechanism by which self-tolerance is maintained in mature T cell populations. We have previously reported that murine bone marrow cells cultured in the presence of high-dose interleukin 2 (IL-2) (activated bone marrow cells [ABM]) mediate strong veto suppressor function. To examine mechanisms by which ABM may suppress precursor CTL (p-CTL) responses, we used p-CTL generated from spleen cells of transgenic mice expressing a T cell receptor specific for H-2 Ld.

It was demonstrated that the cytotoxic response by these p-CTL after stimulation with irradiated H-2^d/k spleen cells was suppressed by DBA/2 (H-2^d) ABM, but not by B10.BR (H-2^k) ABM or dml (D^d, L^d mutant) ABM. Flow cytometry analysis with propidium iodide staining revealed that these p-CTL were specifically deleted by incubation with H-2^d ABM, but not with H-2^k ABM. These data indicate that ABM veto cells kill p-CTL with specificity for antigens expressed on the surface of the ABM, and that the mechanism for veto cell activity of ABM is clonal deletion of p-CTL.

The mechanisms by which T lymphocytes develop and maintain tolerance to normal self-antigens include the clonal deletion of thymocytes expressing autoreactive TCRs (1-4) and the induction of antigen unresponsiveness, or anergy, of postthymic T cells (5-7). Clonal elimination of T cells that have exited the thymus is less clearly established as a general mechanism of tolerance (8-10). Studies on the generation of cytotoxic CTL response have shown that these responses can be specifically suppressed by veto cells, and this suppression has been proposed as one mechanism by which self-tolerance is maintained in mature T cell populations (11-14). Whether veto cells exert their effect by the induction of clonal anergy or clonal deletion of precursor CTL (p-CTL) has not been clearly determined previously.

We have reported that murine bone marrow cells cultured in the presence of high-dose IL-2 (activated bone marrow cells [ABM]) mediate strong veto suppressor function in vitro and in vivo (15). Such cell populations do not express appreciable levels of CD4 or CD8 (15). To determine whether CD4^-CD8^- ABM suppress p-CTL responses by clonal anergy or deletion mechanisms, we examined CTL responses in MLCs in which the responding cells were from transgenic mice expressing high frequency TCR specific for H-2 Ld (16, 17).

Our experiments demonstrated that the anti-H-2^d cytotoxic response by these cell populations was specifically suppressed by H-2^d ABM, and that p-CTL were in fact deleted from the responding cell population by veto cells expressing the target antigen. These data indicate that ABM veto cells kill p-CTL with specificity for antigens expressed on the surface of the ABM, and that the mechanism for veto cell activity of ABM is clonal deletion of postthymic p-CTL, rather than induction of anergy.

Materials and Methods

Mice. C57BL/6, (B6, H-2b), DBA/2 (H-2^d), C3H/HeJ (C3H, H-2^k), B10.BR (H-2^k), (C57BL/6 × C3H/He)F1 (B6C3F1, H-2^b/k), (C3H/HeJ × DBA/2)F1 (C3D2F1, H-2^k/d), and (B10.BR × B10.D2)F1 (BRD2F1, H-2^k/d) were obtained from The Jackson Laboratory (Bar Harbor, ME) or were bred at National Cancer Institute-Frederick Cancer Research Center (Frederick, MD). The D^d/L^d mutant mice, dml, were kindly provided by Dr. Jay Berzofsky (National Institutes of Health, Bethesda, MD). Transgenic H-2^k mice expressing the α/β T cell receptor from the cytotoxic T cell (CTL) clone 2C (16, 17) were kindly provided by Dr. Dennis Y. Loh (Washington University School of Medicine, St. Louis, MO).

ABM. The methods of generation of ABM were previously described (15). Briefly, bone marrow cells were depleted of T cells by two rounds of treatment with mixture of anti-T cell mAbs (HO-13-4, 83-12-5, and C3PO) and guinea pig complement (Gibco Laboratories, Grand Island, NY). These T cell-depleted mouse bone marrow cells were cultured in the presence of high-dose IL-2 (activated bone marrow cells [ABM]) mediate strong veto suppressor function in vitro and in vivo (15).

Abbreviations used in this paper: ABM, activated bone marrow cells; CML, cell-mediated lysis; FCM, flow cytometry; p-CTL, precursor cytotoxic T lymphocytes; PI, propidium iodide.
marrow cells were cultured in complete medium (RPMI 1640 with 10% FCS, sodium pyruvate, nonessential amino acids, t-glutamine, 2-ME, penicillin, and streptomycin) containing 1,000 U/ml human rIL-2 (generously supplied by Cetus Corp., Emeryville, CA) for 4–10 d at 37°C in a humidified chamber containing 5% CO2. These cells were washed twice with media before further experiments.

**MLC.** MLC was set up using 4 × 10⁶ spleen cells from the indicated mice as responder cells and 2 × 10⁶ spleen cells from the indicated mice irradiated with 2,500 cGy as stimulator cells in 2 ml of complete media in a 24-well culture plate (Costar Data Packaging Corp., Cambridge, MA). To evaluate the veto suppressive activity of ABM cells, these cells were added to the MLC on day 2. After culture for 5 d, effector cells were harvested and tested for specific cytotoxicity.

**Cell-mediated Lysis (CML) Assay.** Allospecific cytotoxic activity of MLC was assessed by CML assay, as previously described (15). Target cells were prepared by culturing spleen cells for 2 d in the presence of 3 μg/ml Con A and labeling with Na[51Cr]O₄ (Amer- sham Corp., Arlington Heights, IL). CML assay was performed using variable numbers of effector MLC cells and 5 × 10⁶ target cells in 96-well U-bottomed plates. 51Cr release was measured after a 4-h incubation at 37°C. Results are expressed as a specific lysis calculated as follows: percent specific lysis = 100x [(experimental release – spontaneous release)/(maximum release – spontaneous release)].

NK cell activity of ABM was assessed similarly by CML assay in which ABM were used as effector cells and YAC-1 cells were used as target cells in a 4-h 51Cr-release assay. In addition, killing activity of ABM against the transgenic H-2b mice anti-BRD2F1 MLC was also assessed by CML assay using DBA/2 ABM and C3H ABM as effector cells, and MLC cells as target cells.

**Antigranule Antibody.** Antigranule antibody was prepared by producing rabbit antiserum against highly purified preparations of cytoplasmic granules from a rat large granular lymphocyte tumor line, as previously described (18). To evaluate the effect of antigranule antibody on veto mechanism, antigranule antibody (final concentration 1:200) was added to the MLC on day 2 of culture at the time of addition of ABM. Anti-KLH antibody was used as a control antibody.

**Evaluation of Cell Death by Flow Cytometry (FCM) with Propidium Iodide (PI)-stained Cells.** MLC was set up using 0.4 × 10⁶ transgenic H-2b mice spleen cells/well mixed with 3.6 × 10⁶ normal B6 spleen cells/well as responder cells, 2,500 cGy-irradiated C3D2F1 spleen cells used as stimulator cells. Cells were collected from the MLC on day 2, cleared of dead cells by centrifugation over lymphocyte separation media (Organon Technika, Durham, NC), and put back in culture. A portion of cells after the separation were used for flow cytometric analysis before further manipulation of cultures, then 10⁶ ABM/well were added to the culture as indicated. The cells were collected from the MLC 24 h after addition of ABM, washed, and incubated with 1B2 for 30 min at 4°C, followed by FITC-conjugated goat anti-mouse Ig (gamma specific; Fisher Scientific Co., Pittsburgh, PA). After addition of 0.8 mg/ml of PI (Fluka Chemical Corp., Ronkonkoma, NY) as an indicator of dead cells (19), cells were analyzed using FACScan® (Becton Dickinson & Co., Mountain View, CA). Fluorescence data were collected using a logarithmic amplification on 20,000 cells after gating out cell fragments or debris smaller than dead cells stained with PI by forward light scatter intensity. These small fragments were distinguished clearly from PI-stained dead cells. Two-color immunofluorescence data were displayed as contour diagrams in which log intensities of green (FITC) and red (PI) fluorochromes were plotted on the x-axis and y-axis, respectively. Lines used to define positive and negative cells were set against the outermost contour of staining controls in which FITC-conjugated goat anti-mouse Ig alone was used. Positive percentage shown in the boxes are values from which background (control staining) values have been subtracted (see Fig. 4 A). Absolute cell numbers of each cell population were calculated using the positive percentages by FCM analysis and live cell number/well of the culture assessed by trypan blue exclusion.

**Results and Discussion**

**Veto Cell Activity of ABM.** Recent evidence suggests that acquisition of anergy of p-CTL as the mechanism by which veto cells suppress T cell cytotoxic responses (21). Major difficulties in investigating mechanisms of veto cell function have been the unreliable suppressor activity of veto cell populations and the low precursor frequency of responding p-CTL susceptible to veto suppression during a primary response. As reported previously (15), we and others (23) have overcome the problem of poor reliability of veto cell function by incubating bone marrow cells, which have been reported to contain veto cells (24), with IL-2. To assess the veto activity of such ABM, C57BL/6 (B6, H-2b) ABM or C3H/He (C3H, H-2b) ABM were added to DBA/2 (H-2b) anti-C57BL6 × C3H/HeJF1 (B6C3F1, H-2b/k) MLC. As shown in Fig. 1 A, the addition of B6 ABM significantly suppressed the generation of an anti-H-2b cytotoxic response as compared with the response with added C3H ABM or no added ABM. Conversely, the generation of an anti-H-2b cytotoxic response of the same MLC was significantly suppressed by addition of C3H ABM, but not by addition of B6 ABM or no ABM (Fig. 1 B). The use of F1 stimulator cell populations excluded as a mechanism of suppression the possibility that veto activity was mediated by elimination of the stimulator cell population by ABM. Further studies demonstrated that the MLC responder population was susceptible to veto cell suppression from 1–3 d of culture with maximal veto cell suppression observed when veto cells were added 2 d after initiating the MLC (data not shown). Therefore, ABM-mediated suppression of cytotoxic responses was characteristic of veto cell populations in that the suppressive activity was antigen specific and acted upon cytotoxic cells soon after primary stimulation. The possibility that suppression of target cell lysis in the chromium release assay was due to cold target inhibition associated with the presence of target antigen-expressing veto cells in the effector population, was eliminated by treating the effector cells with appropriate antibody and complement (15). Additional studies demonstrated that the ABM cell population, after 7–10 d of culture with added IL-2, was relatively homogeneous and expressed Thy-1 and NK1.1, but not MAC-1, CD4, CD8, or CD3. ABM cells had substantial levels of both NK cell and LAK cell activity (data not shown).

**Antigranule Antibody Inhibits Veto Cell Activity.** If clonal deletion of p-CTL is the mechanism by which veto cells sup-
press the generation of cytotoxic responses, then lysis of p-CTL most likely is involved. Such lysis would presumably proceed via degranulation of either the veto cell population or perhaps the p-CTL (cellular "suicide"). To assess the possibility that a granule-mediated lytic event was involved in veto cell suppression of a CTL response, the effect of neutralizing antigranule antibody (18) on veto activity was investigated. Such antigranule antibody preparations have previously been demonstrated to inhibit lysis by NK effector cells (18, 25). As shown in Fig. 2 A, antigranule antiserum, but not a control anti-KLH antibody, inhibited B6 ABM-mediated suppression of an anti-H-2 b cytotoxic response in DBA/2 anti-B6 MLC and resulted in near complete restoration of CTL activity compared with control values (addition of antigranule antiserum alone). When C3H ABM were added to the same MLC to confirm the specificity of veto activity, no suppression of anti-H-2 b cytotoxic response was observed (Fig. 2 B). Preincubation of either the responder population or the ABM population with chloroquine, an inhibitor of lytic granule activity (26), demonstrated that the granule activity contributing to veto cell-mediated suppression resided in the ABM but not in the p-CTL population, and thereby indicated that a cellular suicide mediated by granules of p-CTL was unlikely (data not shown). These experiments strongly suggested that veto cell-mediated suppression of CTL responses required veto cell degranulation and that a lytic event was therefore critical to such suppression. These results would be consistent with previously reported observations correlating veto activity of cell populations with cytotoxic potential (12).

**ABM Can Lyse MLC Responder Cells.** To directly assess the possibility of clonal deletion of p-CTL by veto cells, it was necessary to circumvent the problem of inherently low p-CTL frequency in the responder cell population of a MLC. To do this, we used as responder cells spleen cells of transgenic H-2 b mice expressing the α/β TCR from the CTL clone 2C (16, 17) as a source of p-CTL. This TCR is specific for the class I MHC-encoded antigen L α and can be specifically identified by the mAb 1B2 (16). To test whether the anti-H-2 a response of the CTL from the transgenic mice could be suppressed by ABM, DBA/2 (H-2 b) ABM, B10.BR (H-2 k) ABM, or dml (a H-2L a and D a mutant of B10.D2), ABM were added to a MLC in which the responder cells were derived from the spleen of a transgenic mouse (H-2 b) and the stimulator cells were H-2 a/k (BRD2F1). As shown in Fig. 3 A, the addition of DBA/2 ABM significantly suppressed the generation of an anti-H-2 a cytotoxic response of the MLC as compared with the response with added B10.BR ABM, dml ABM, or no ABM. The assay of NK cell activity demonstrated comparable cytolytic activity of the various ABM populations (Fig. 3 B). Thus, an anti-H-2 a cytotoxic response generated from the transgenic H-2 b mice can be significantly suppressed by H-2 b ABM, but not by H-2 k ABM or D a/L a mutant ABM. Therefore, ABM can exert a veto-suppressive action on the anti-H-2 a CTL response generated by these transgenic responder cell populations.

Although it has been determined by functional assays that veto cells decrease the frequency of p-CTL that respond to stimulator cells in MLC (13, 14), it has been unclear whether veto cells kill p-CTL (resulting in clonal deletion), or induce antigen unresponsiveness (anergy) of this cell population, thereby rendering it incapable of differentiating into functional CTL. To assess the mechanism of veto cell suppression
of CTL responses, we first directly evaluated p-CTL lysis by ABM using a $^{51}$Cr-release assay. The transgenic H-2$^b$ mouse spleen cells were stimulated with irradiated BRD2F1 spleen cells for 2 d and then used as target cells. As shown in Fig. 3 C, the responder cells from the TCR transgenic mouse anti-BRD2F1 MLC were lysed by DBA/2 ABM, but not by B10.BR ABM or dml ABM, but the present specific cell lysis of the MLC was relatively low (14.9% at E/T ratio 100:1). These data suggested that ABM could kill MLC responder cells, but it remained unclear whether the degree of killing observed could account for a full suppression of the CTL response or whether the cells killed were in fact p-CTL. The use of purified T cell responder populations as target cells and of longer periods of incubation during the $^{51}$Cr-release assay failed to resolve these issues.

**ABM Veto Cells Mediate Clonal Deletion of p-CTL.** Because more than 95% of CD8-positive cells derived from these transgenic mice were 1B2 positive and no anti-H-2$^d$ response was generated from the TCR transgenic H-2$^b$ mouse anti-BRD2F1, MLC (data not shown), the specificity of veto activity of DBA/2 ABM versus a third-party ABM population (e.g., H-2$^d$) could not be defined. Therefore, we used as responder populations in MLC a mixture of spleen cells from transgenic and normal B6 (H-2$^b$) mice and H-2$^{d/k}$ stimulator cells to generate simultaneous anti-H-2$^d$ and anti-H-2$^k$ cytolytic responses in MLC to evaluate specificity of activity of the various ABM populations in the same MLC. The initial responder cell population was made up of 10% transgenic mouse spleen cells and 90% normal B6 spleen cells, and veto cell activity of H-2$^d$ ABM and H-2$^k$ ABM was assessed. The addition of DBA/2 ABM markedly suppressed the generation of an anti-H-2$^d$ response, as compared with the response with added C3H ABM or no added ABM. An anti-H-2$^k$ response, generated from the same MLC, was suppressed by addition of C3H, but not DBA/2, ABM (data not shown). To examine whether responder p-CTL could be killed by ABM, we performed flow cytometric analysis of responder cell populations derived from the transgenic mouse using the mAb 1B2 specific for the transgene-encoded TCR, and determined changes of the T cell population expressing this receptor in the MLC after addition of ABM.

In addition, we studied the cell population by staining with PI (19) as an indicator of dead cells. At 48 h of MLC and before addition of ABM, the responder population had 3.9% 1B2-positive, live cells and had 11.1% dead (PI-positive) cells. After another 24 h of culture with no ABM, the percentage of 1B2-positive live cells increased approximately fourfold with a more modest increase of dead cells (Fig. 4 A). In those MLCs to which DBA/2 ABM were added, live 1B2-expressing T cells were dramatically decreased to virtually undetectable levels (0.2%), accompanied by a substantial increase in dead cells as compared with control cultures. Addition of C3H ABM did not reduce the 1B2-positive cell population. In addition, the absolute cell number of 1B2-positive cells was also markedly reduced, and the dead cell number increased after addition of DBA/2 ABM, but not after addition of C3H ABM (Fig. 4 B). Because the cell number of 1B2-negative cells was not reduced by addition of DBA/2 ABM and the 1B2-positive cell population virtually disappeared, the increment of dead cells in the MLC in the presence of DBA/2 ABM was likely due to the death of 1B2-positive cells. Downregulation of TCR expression, rather than death of the p-CTL population, was ruled out by staining with anti-CD3 and -CD8 mAbs that demonstrated an absence of any new CD8$^+$/ CD3$^-$ population after exposure of the MLC population to veto cells. Because antibody nonspecifically associates with dead cells, no attempt to interpret the 1B2 staining of PI-positive cells was made in the experiments shown in Fig. 4. These data demonstrate that H-2$^d$, but not H-2$^k$, ABM eliminate live 1B2-positive (but not 1B2$^-$) cells from the responding population. Thus, p-CTL are clonally deleted by ABM in an antigen-specific manner. This appears mechanistically distinct from effects mediated through CD8 as an inhibitory ligand (27), in that the veto cells in the population...
used in these experiments do not express CD8 by fluorescence analysis (15), and also distinct from cell death induced by signaling through the TCR and class I MHC molecules that can be mediated by antibodies of the appropriate specificity in the absence of a cytotoxic veto cell population (22).

If the mechanism of veto cell–mediated suppression of CTL responses involves degranulation of veto cells and lysis and deletion of p-CTL, as indicated in these experiments, the question of how that degranulation is triggered must be considered. This consideration must take into account the observation that p-CTL, but not mature CTL, are susceptible to veto suppression, and that veto cell populations can mediate specific suppression of cytotoxic T cell responses even to acquired antigens on the surface of veto cells (11). One possibility we would propose is that the developing p-CTL acquires the capacity to deliver an excitatory signal to the veto cell after TCR-mediated recognition of antigen expressed on the surface of the veto cell. Specifically, the veto cell stimulus would result from p-CTL degranulation of immature granules containing limited cytolysin capable of mediating some ion (especially calcium) leakage with subsequent membrane repair by the veto cell. This ion leakage would stimulate veto cell degranulation resulting in lysis of the p-CTL, and so would be similar to “backwards” killing of cells bound to triggered cytotoxic lymphocytes (28).

Whatever the events might be that mediate triggering of ABM, the findings presented here provide clear evidence that this veto cell population mediates suppression of CTL responses by clonal deletion of p-CTL. Preliminary results of recent experiments suggest that veto cell populations in marrow not exposed to exogenous IL-2 in vitro similarly act by clonal deletion of p-CTL. These results, in combination with previous work showing antigen-specific effects of veto cell populations in vivo (15), suggest that clonal deletion of postthymic T cells by veto cells represents a mechanism of tolerance that is operative among unmanipulated cell populations in vivo.

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