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Thymocytes can tolerize thymocytes by clonal deletion *in vitro*

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Abstract

Clonal deletion of thymocytes bearing TCR for self antigens is one major mechanism of T cell tolerance induction. Peptide antigen-induced deletion of thymocytes from αβ TCR transgenic mice has been studied using single cell suspension cultures. The results show that antigen-presenting immature CD4$^+$CD8$^+$ thymocytes can tolerize antigen-reactive immature thymocytes *in vitro* by programmed cell death (apoptosis) 6 – 8 h after antigen exposure. Antigen-induced apoptosis of immature thymocytes was inhibited by antibodies specific for the αβ TCR, CD3, CD8, and LFA-1 molecules. This implies that clonal elimination of self-reactive CD4$^+$CD8$^+$ thymocytes does not depend on specialized deleting cell types in the thymus and occurs whenever the TCR of immature thymocytes bind antigen fragments presented by MHC molecules.

Introduction

During T cell development the TCR repertoire is shaped in the thymus by positive and negative selection events. It is now well established that clonal deletion of self-reactive T cells is the major mechanism of T cell tolerance induction for thymic self antigens. This has been demonstrated in normal mice for superantigens (1 – 3) and in αβ TCR transgenic mice for conventional MHC-restricted peptide antigens (4 – 6).

Clonal deletion is thought to occur via antigen-induced programmed cell death (apoptosis) of autoreactive thymocytes. Anti-CD3 treatment of fetal thymic organ culture has been proposed to be an *in vitro* model of negative selection (7,8). This view has been recently challenged by two studies which show that thymocytes which are resistant to anti-CD3-induced apoptosis are still susceptible to superantigen-induced deletion *in vivo* (9,10).

To learn more about the molecular and cellular requirement for tolerance induction by deletion, an assay system, originally described by Swat *et al.* (11,12), has been utilized which allows the study of antigen-induced deletion of thymocytes in single cell suspension cultures *in vitro*. This approach allows the definition of the minimal cellular requirement for clonal deletion and the identification of cell surface molecules involved in this process.

Methods

Mice

The TCR transgenic mice (line 327) have been previously described (6). Mice 6 – 12 weeks of age were used.

Thymocyte cultures

Single cell suspensions of thymocytes were prepared by squeezing the whole thymus through a wire screen. Clumps were allowed to settle and then discarded. The cells were >99% Thy-1$^+$, <0.1% Mac-1$^+$, <0.7% Mac-2$^+$, and <0.4% IgM$^+$. After washing, thymocytes (5 × 10^6 cells/ml) were cultured (37°C, 5% CO_2) in Iscove's modified Dulbecco's medium (IMDM) supplemented with 15% FCS in 24-well Costar tissue culture plates (1 ml/well) for 14 h if not otherwise indicated. The lymphocytic choriomeningitis virus (LCMV) glycoprotein aa33 – 42 (KAVYNFATCG) (13) peptide was used at 30 μM. Lymph node cells (2 × 10^6 cells/well) from αβ H-2^b TCR transgenic mice (>70% CD8$^+$) were added to the indicated cultures. FACS-sorted CD4$^+$CD8$^+$ thymocytes (>99.8% pure) were cultured (5 × 10^6 cells/ml) in 96-well tissue culture plates (200 μl/well) in the presence or absence of 30 μM LCMV peptide for 14 h. Afterwards, the cells were restained with anti-CD4 and -CD8 mAb.

Inhibition of antigen-induced deletion

Thymocytes were cultured in the presence of the supernatants of the following B cell hybridomas: 53-6.72, anti-CD8 (14); GK1.5, anti-CD4 (15); H129.37, anti-LFA-1-α (16); KT3, anti-CD3 (17); B20.1, anti-TCR $V_α2$ (18); KJ16, anti-TCR $V_β8$ (19), J11d and B2A2, anti-HSA (20,21); KM202, anti-CD44 (22); and M1/9 and 23G, anti-CD45 (23,24). Per cent deletion of CD4^{high}CD8^{high} thymocytes was calculated by:

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$$100 \times \left[1 - \left(\frac{\%CD4^{high}CD8^{high} \text{ thymocytes with LCMV peptide}}{\%CD4^{high}CD8^{high} \text{ thymocytes without LCMV peptide}}\right)\right]$$

Flow cytometry analysis

Thymocytes were double stained with anti-CD4 – phycoerythrin (PE) and anti-CD8 – fluorescein isothiocyanate (FITC) (Becton Dickinson, Mountain View, CA) mAb. CD4/CD8 expression of the anti-CE4 or anti-CD8 treated cultures was determined by restaining with anti-CD4 (15) or anti-CD8 (14) mAb and goat anti-rat IgG – FITC (TAGO, Burlingame, CA), followed by CD8 – biotin – avidin – PE or CD4 – PE (Becton Dickinson) respectively. Thymocytes were analyzed on an EPICS profile analyzer (Coulter, Hialeah, FL) with four logarithmic scales. Data were only collected from viable (i.e. propidium iodide negative) cells gated by a combination of forward light scatter (FS) and 90° side scatter (SS).

Results and discussion

The molecular and cellular requirements for clonal elimination of self-reactive $CD4^+CD8^+$ thymocytes from $\alpha\beta$ TCR transgenic mice by apoptosis was examined in single cell suspension cultures. These mice express an LCMV/H-$2D^b$-specific TCR ($V_\alpha 2/V_\beta 8.1$) on most thymocytes (70 – 80%) and peripheral $CD8^+$ T cells (6). Apoptosis of immature $CD4^+CD8^+$ thymocytes was analyzed by flow cytometry based on the fact that thymocytes undergoing programmed cell death express reduced levels of CD4 and CD8 molecules (11). Thus, the degree of cell death among cultured $CD4^+CD8^+$ thymocytes can be monitored by the shift from a $CD4^{high}CD8^{high}$ to a $CD4^{low}CD8^{low}$ phenotype (11,12). When thymocytes from TCR transgenic H-2^b mice were cultured in the presence of the LCMV peptide (glycoprotein aa33 – 42) recognized by the transgenic TCR, the population of immature thymocytes expressing CD4 and CD8 at a high level ($CD4^{high}CD8^{high}$) gradually decreased and a distinct apoptotic cell population bearing lower levels of CD4 and CD8 ($CD4^{low}CD8^{low}$) appeared (Fig. 1A). The disappearance of $CD4^{high}CD8^{high}$ thymocytes was MHC-restricted (Fig. 1B, top) and antigen-specific (Fig. 2, top), and was not observed in LCMV peptide-treated thymocyte cultures from non-transgenic H-2^b mice where only a minute fraction [$< 1/10^5$ (25)] of T cells bear an LCMV-specific TCR (Fig. 1B). Apoptosis of $CD4^+CD8^+$ thymocytes was not caused by direct cell lysis mediated by mature thymocytes because co-culture of normal H-2^b

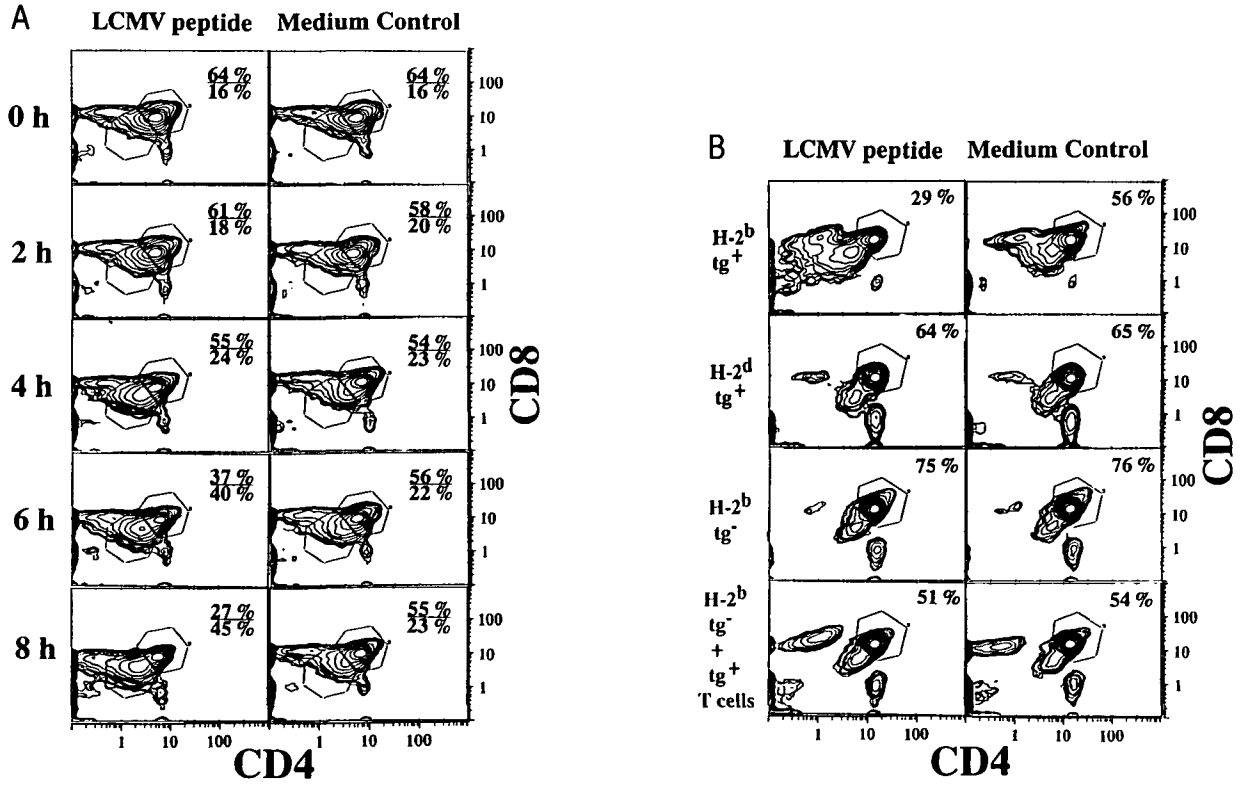

Fig. 1. Peptide antigen-induced apoptosis in thymocyte cultures. (A) Thymocytes from TCR transgenic mice (H-2^b) were cultured for the indicated time in the presence (left) or absence (right) of the LCMV peptide (GP aa33 – 42) and subsequently analyzed for expression of CD4 and CD8 molecules by flow cytometry. The percentages given in each plot indicate the relative number of $CD4^{high}CD8^{high}$ and $CD4^{low}CD8^{low}$ cells in the indicated gate. Most (70 – 80%) of the total input cell numbers were recovered as trypan blue negative thymocytes after 8 h in peptide-treated and control cultures. (B) Thymocytes from the indicated mice were cultured for 14 h with or without the LCMV peptide and analyzed for CD4 and CD8 expression. In the bottom row, peripheral $CD8^+$ T cells from TCR transgenic mice (H-2^b) were added to thymocyte cultures from normal H-2^b mice. The percentage given in each plot indicates the relative number of $CD4^{high}CD8^{high}$ thymocytes. Most (60 – 70%) of the total input cell numbers were recovered as trypan blue negative thymocytes. In LCMV peptide-treated H-2^b transgenic cultures, only 30 – 40% of the total input cells were recovered as trypan blue negative cells.

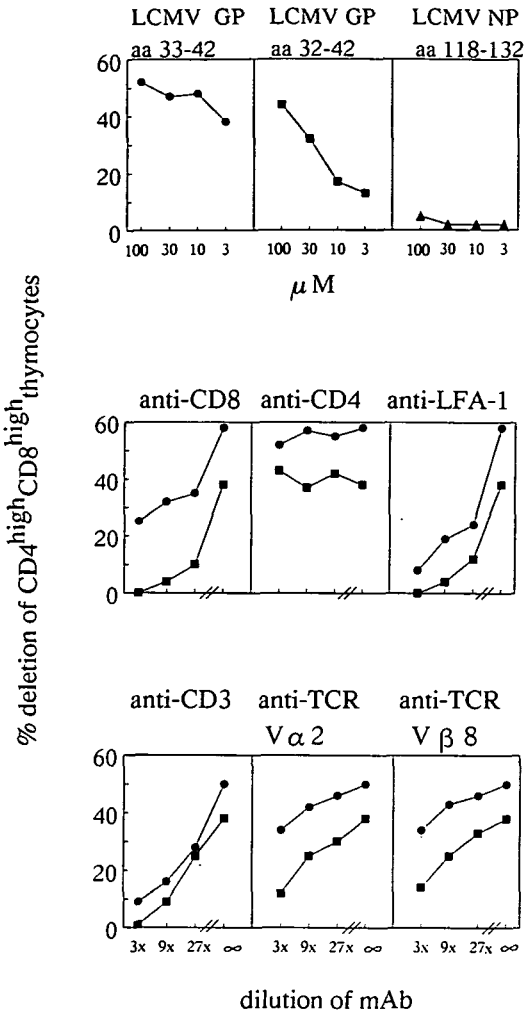

Fig. 2. Inhibition of LCMV peptide-induced deletion of $CD4^{high}CD8^{high}$ thymocytes with mAb. Apoptosis was induced either with the LCMV glycoprotein aa33 – 42 (100 μM ●) or with the suboptimal aa32 – 42 (100 μM ■) peptide.

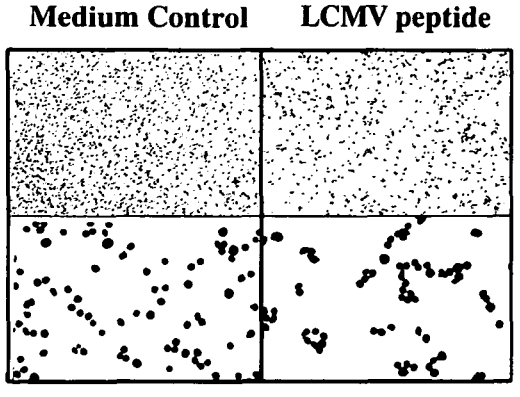

Fig. 3. Peptide antigen-induced cell aggregation. Thymocytes from TCR transgenic H-2^b mice were incubated at 37°C in the absence (left) or presence (right) of LCMV peptide for 3 h. Afterwards, the cell suspension was mounted on a glass slide, air dried, and stained using May – Grünwald – Giemsa eosine – methylene blue solution. Magnification: top panel ×21; bottom panel ×105.

thymocytes and mature transgenic $CD8^+$ T cells, in the presence of LCMV peptide, did not reduce the number of normal $CD4^{high}CD8^{high}$ thymocytes (Fig. 1B, bottom). In addition, unprimed TCR transgenic thymocytes and peripheral T cells did not show any cytolytic activity when tested on LCMV peptide-coated target cells in a ^{51}Cr release assay (not shown) and elimination of mature thymocytes by cell sorting did not abolish apoptosis of $CD4^+CD8^+$ thymocytes (see below).

Monoclonal antibodies were tested for their ability to interfere with LCMV peptide-induced apoptosis: mAb specific for transgenic TCR α and β chains, CD3, CD8, and LFA-1 molecules inhibited antigen-induced elimination of $CD4^{high}CD8^{high}$ thymocytes in a dose-dependent manner (Fig. 2). Inhibition was more pronounced when a suboptimally antigenic LCMV peptide (glycoprotein aa32 – 42) was used (Fig. 2, top). Antibodies specific for other thymocyte antigens, such as the CD44 and CD45 molecules, and the heat stable antigen (HSA), did not block apoptosis, whereas mAb H141-51 (26), specific for the LCMV peptide-presenting MHC class I molecules H-$2D^b$, were found to be inhibitory (data not shown). Neonatal anti-CD4 antibody treatment has been shown to prevent Mls- (27) and I-E-mediated (28) deletion of self-reactive T cells *in vivo*.

During the course of these experiments it was noted that the addition of LCMV peptide to thymocyte cultures from TCR transgenic H-2^b mice induced a transient cell aggregation 2 – 3 h after peptide addition. These aggregates often appeared

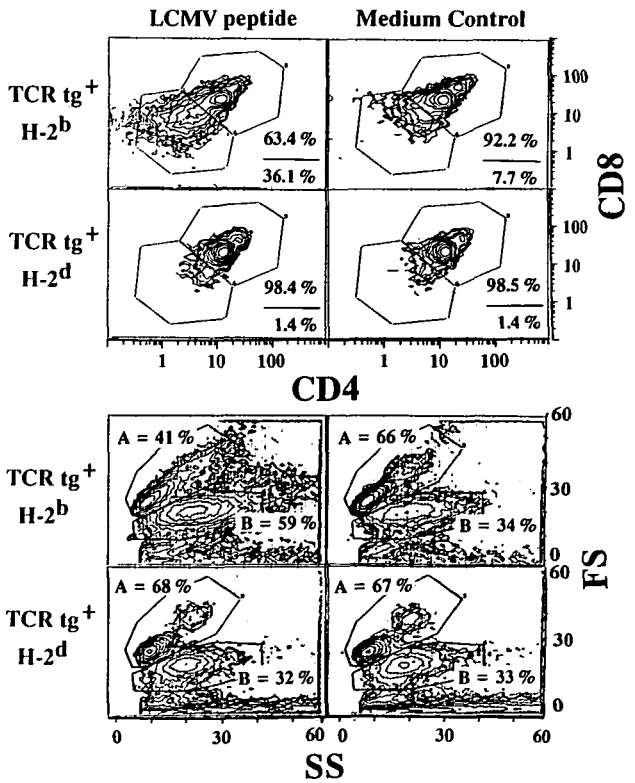

Fig. 4. $CD4^+CD8^+$ thymocytes induce antigen-specific apoptosis of $CD4^+CD8^+$ thymocytes. Fluorescence data were collected only from 'viable' cells with high FS and low SS (population A; bottom) which did not stain with propidium iodide. Cells from population B were mostly propidium iodide positive.

as characteristic thymocyte chains (Fig. 3). A similar effect was not observed when LCMV peptide-treated thymocyte cultures from normal H-2^b or TCR transgenic H-2^d mice were examined (not shown). This observation suggests that LCMV peptide-coated thymocytes may act as antigen-presenting cells and therefore induce visible cell – cell binding via the LCMV-specific transgenic TCR present on most thymocytes. Alternatively, it is possible that antigen-induced TCR cross-linking on thymocytes stimulates adhesiveness through LFA-1 molecules as shown for mature T cells (29).

To formally demonstrate that immature $CD4^+CD8^+$ thymocytes are able to present the antigenic LCMV peptide to themselves and thereby induce apoptosis, $CD4^+CD8^+$ thymocytes from TCR transgenic mice were purified by cell sorting, cultured in the presence or absence of the LCMV peptide for 14 h, restained with CD4 and CD8 mAb, and analyzed by flow cytometry. Thymocytes from TCR transgenic H-2^d mice served as a specificity control because H-2^d MHC molecules do not present the LCMV peptide to the transgenic TCR (not shown). LCMV peptide-induced apoptosis occurred only in thymocyte cultures derived from transgenic H-2^b but not from H-2^d mice, as judged by the disappearance of $CD4^{high}CD8^{high}$ cells and the appearance of the $CD4^{low}CD8^{low}$ population (Fig. 4, top). Fluorescence data were collected only from 'viable' cells which did not stain with propidium iodide.

In addition, plots of FS and 90° SS of the recovered cells revealed an increase (34 to 59%) of the cell population with low FS and high SS (population B in Fig. 4, bottom) in LCMV peptide-treated thymocyte cultures derived from TCR transgenic H-2^b mice. Most of these cells were stained by propidium iodide, indicating permeable cytoplasmic membranes (not shown). A similar effect was not observed in LCMV peptide-treated thymocyte cultures derived from TCR transgenic H-2^d mice (Fig. 4, bottom). These results show that the addition of the LCMV peptide to purified $CD4^+CD8^+$ thymocytes from TCR transgenic H-2^b mice also specifically decrease cell viability from 66 to 41% (Fig. 4, bottom).

The possibility that the observed antigen-induced apoptosis of immature thymocytes in these experiments was a result of the few contaminating cells in the purified $CD4^+CD8^+$ population cannot be completely ruled out. However, it is considered unlikely that the <0.2% contaminating cells in the preparations were able to induce CD4/CD8 down-regulation in 30% of 'viable' thymocytes and to decrease the viability of the recovered cells from 61 to 41% in this *in vitro* culture system within 14 h. In addition, the extent of deletion of purified $CD4^+CD8^+$ thymocytes (Fig. 4) was comparable to the results obtained with total thymocyte preparations (Fig. 1).

FACS-purified double negative ($CD4^-CD8^-$) thymocytes injected into the thymus of irradiated host mice have been shown to induce T cell tolerance to allogeneic MHC class I antigens (30). The results of this study directly demonstrate that $CD4^+CD8^+$ thymocytes induce clonal deletion of self-reactive immature $CD4^+CD8^+$ thymocytes by apoptosis *in vitro*. This complements a recent report by Swat *et al.* (12) which revealed that adherent cell preparations from thymus and spleen are able to induce antigen-specific clonal deletion *in vitro*. Mice, neonatally infected with LCMV, delete LCMV-specific $CD8^+$ T cells in the thymus (6). Because LCMV does not infect thymocytes, clonal deletion was induced in this system either by infected thymic epithelia or bone marrow-derived macrophages/dendritic cells. In this study the LCMV peptide has been used as a model antigen for self determinants expressed or passively acquired (31,32) by thymocytes. This approach allowed the demonstration that tolerance induction, via antigen-triggered self destruction of $CD4^+CD8^+$ thymocytes, only requires the presence of the appropriately processed self peptides presented by MHC molecules.

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Abbreviations

FITC	fluorescein isothiocyanate
FS	forward light scatter
HSA	heat stable antigen
IMDM	Iscove's modified Dulbecco's medium
LCMV	lymphocytic choriomeningitis virus
PE	phycoerythrin
SS	side scatter

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