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Peptide-induced deletion of CD8 T cells *in vivo* occurs via apoptosis *in situ*

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Abstract

The ultimate fate of T cells undergoing antigen-induced cell death *in vivo* remains controversial. Whereas apoptosis of $CD4^+$ T cells driven by superantigen is readily detectable in lymphoid organs, $CD8^+$ T cells have been reported to disappear from the lymphoid organs and accumulate in the liver where they undergo apoptosis. Using transgenic mice that produce large numbers of ovalbumin-specific $CD8^+$ T cells (OT-I cells), we were able to investigate the events that follow soluble peptide administration in an independent $CD8^+$ T cell system. Here we show that the OT-I cells undergo proliferation and apoptosis *in situ* in lymphoid organs in response to antigenic stimulation with no evidence for liver involvement. This is similar to the course of events found for $CD4^+$ T cell activation and counters the view that the liver is a general site for $CD8^+$ T cell clearance following antigen-specific activation.

The fate of stimulated T cells *in vivo* has been a point of active discussion ever since the early studies of Sprent and Miller (1–3). Using radiolabeled cells and adoptive transfer, these workers showed that although some activated T cells could home to the gut, many homed to the spleen, and they concluded that the cells died *in situ* and were engulfed by phagocytes. Use of superantigens (with corresponding V_β on the TCR) or mice with transgenic TCR has allowed more direct approaches to be used to assess the fate of responding T cells. $CD4^+$ T cells expand after antigen stimulation *in vivo* and undergo antigen-induced cell death in peripheral organs where they are activated (4–7). The fate of $CD8^+$ T cells is more equivocal. One report (8) described that in TCR anti-SV40 transgenic mice, $CD8^+$ T cells underwent blastogenesis upon peptide stimulation without expansion in lymphoid organs. Because apoptotic T cells were found in the liver but not in the lymphoid organs, it was concluded that activated T cells left the lymphoid organs and accumulated to this site where they underwent apoptosis. Why $CD8^+$ T cells should have a different fate than $CD4^+$ T cells remains unexplained. We have used another $CD8^+$ TCR transgenic system (9) to investigate the proposed route of $CD8^+$ T cell destruction. T cells in the OT-I mouse are skewed towards $CD8^+$ cells bearing receptors specific for an H-$2K^b$-restricted ovalbumin peptide (OVAp). OT-I cells were activated by peptide exposure which generally resulted in their proliferation and subsequent depletion. We show that depletion is the result of peptide-induced apoptosis in the lymphoid organs, challenging the view that $CD8^+$ T cells migrate to the liver to die.

To elicit antigen-induced cell death, OVAp (OVA257–264 peptide sequence SIINFEKL) was given i.v. to OT-I mice at two doses, 2 and 20 nmol. Insulin peptide (INSp) (INS8–15 peptide sequence GSHLVEAL) which is also capable of binding K^b (10) was used as a control. To compare separate experiments, cell numbers were expressed as a percentage of those from age-matched control-treated mice. The number of thymocytes was dramatically reduced (by ~80% on day 2, Fig. 1A). In the periphery, however, there was evidence of proliferation. The OT-I cells expressed high levels of the activation marker CD44 (Fig. 1B), and an increase in the numbers of OT-I cells was observed in the spleen (P = 0.02) and lymph nodes (P = 0.004) (Fig. 1A). This peptide-induced proliferation of OT-I cells in the periphery was followed by a significant decrease in OT-I cells by day 7 in spleen and lymph nodes (Fig. 1C). The activation, proliferation and then deletion of antigen-specific cells is similar to findings observed in many systems (4–7,11–21).

Depletion of OT-I cells in response to soluble OVAp was

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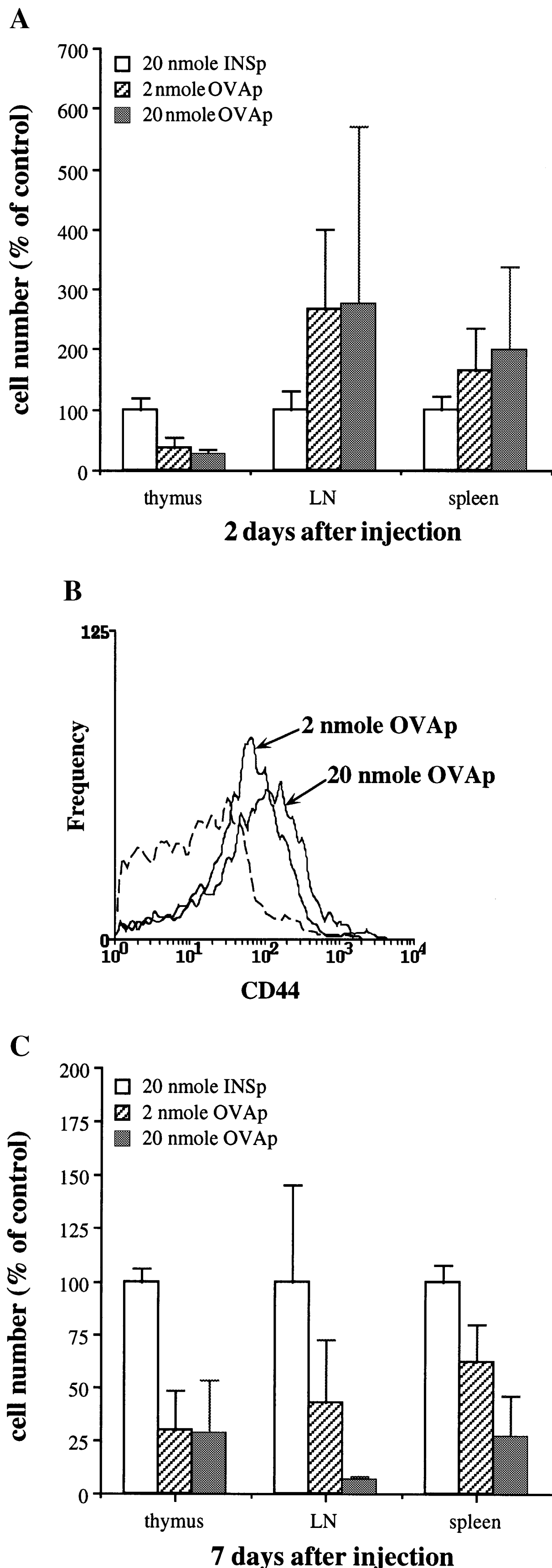

revealed by TUNEL (TdT-mediated dUTP-biotin nick-end labelling of DNA fragmentation) staining to occur by apoptosis in the lymphoid organs i.e. thymus, spleen and lymph node. Specificity of TUNEL staining was confirmed by the lack of staining when TdT was omitted (Fig. 2A) and by the lack of staining when control peptide was injected (Fig. 2B). Results for peptide treatment after 4 days are shown in Fig. 2(B). The frequency of apoptotic events was similar 2 and 6 days after peptide treatment (Fig. 2C), and when 20 nmol of peptide was used (data not shown). The TUNEL$^+$ cells did not overlap with anti-B220 staining; thus, apoptosis was confined to the T cell areas in the lymph node and spleen (Fig. 3) and to CD8$^+$ cells (data not shown). Antigen-induced apoptosis of mature OT-I cells occurred in the spleen and lymph nodes, thus providing an explanation for the reduction in absolute numbers observed in these organs. There was no accumulation of apoptotic cells near blood vessels, further suggesting that apoptotic cells are not exported but die *in situ*. Some apoptotic cells were detected in control thymi (not seen in Fig. 2B). This was expected though, since physiological cell death occurs in the thymus, an organ with high cell turnover (22). In mice treated with OVAp the number of apoptotic cells in the thymus was greatly increased compared to control mice (Fig. 2B).

In our OT-I system, the lymphoid organs were the major sites of apoptosis. This is in agreement with most CD4$^+$ T cell models, including class II-restricted TCR transgenic mice and superantigen systems (4–7), but is in contradiction to other reports on CD8$^+$ T cells (8,23) where non-lymphoid organs such as the liver, lung and kidney are proposed as the main sites for CD8$^+$ T cell apoptosis. Huang *et al.* (8) found apoptotic CD8$^+$ T cells in the liver but not in lymphoid organs and suggested that this may be a peculiarity of CD8$^+$ T cells

Fig. 1. (A) The number of OT-I ($V_\alpha 2^+CD8^+$) cells in the thymus and peripheral organs 2 days after OVAp and INSp injection i.v. Cell suspensions were stained for $V_\alpha 2$ and CD8, and the proportion of double-positive cells determined. The change in absolute cell number induced by OVAp treatment is expressed as percentage of the absolute cell number in control INSp-treated mice. (B) The CD44 activation profile of $V_\alpha 2^+CD8^+$ lymph node cells 2 days after peptide injection. Dashed line represents the profile obtained when control peptide was injected. (C) The number of OT-I ($V_\alpha 2^+CD8^+$) cells in the thymus and peripheral organs 7 days after OVAp injection i.v. As above the change in absolute cell number induced by OVAp treatment is expressed as percentage of the absolute cell number in control INSp-treated mice. Transgenic mice (OT-I mice) bearing $V_\alpha 2V_\beta 5$ TCR specific for OVAp (sequence SIINFEKL) (9) were bred and housed at the WEHI animal facilities. OVAp and INSp were dissolved in PBS and 2 or 20 nmol (~2 or 20 μg respectively) injected i.v. as a single dose. At 2–7 days after injection, thymus, spleen and lymph nodes (inguinal, brachial and axillary) were harvested for analysis. Then 10^6 thymus, spleen or lymph node cells were incubated for 30 min on ice. The mAb used were FITC-conjugated anti-CD8 (Caltag, San Francisco, CA) and biotinylated anti-$V_\alpha 2$ (clone B20.1; PharMingen) and biotinylated anti-CD44 (clone IM7.81). Streptavidin–phycoerythrin and streptavidin–TriColor (Caltag) were used to visualize biotinylated antibodies. All samples were stained with propidium iodide (Sigma, St Louis, MO) at 20 μg/ml, and analyzed with a FACScan and Lysys II software (Becton Dickinson, San Francisco, CA). Viable lymphocytes were selected for analysis using a combination of forward/side light scatter and negative propidium iodide staining.

migrating to the liver to die. We find little evidence to support this. We found no apoptotic cells in the kidney (data not shown) and the few apoptotic cells found in the liver could be explained by occasional cells trafficking through this organ; such cells were also evident in control mice (Fig. 2B). The few TUNEL$^+$ cells found in the liver were not CD8$^+$ (data not shown). It is not known whether these cells were originally CD8$^+$ but lost CD8 expression during death. No significant lymphocytic accumulations were found in the liver compared to control mice. Even though the liver and kidney may not be representative of all non-lymphoid organs, it should be noted that mild lymphocytic inflammation can occasionally be seen in normal mice.

Our data suggest that the site for antigen-induced death is identical for CD4$^+$ and CD8$^+$ T cells. CD4$^+$ T cells die in lymphoid organs after i.v. (5,7) or i.p. (14) peptide administration. Previous reports on CD8$^+$ T cells (8,23) used i.p. administration. To eliminate this as the reason for our differences, mice were given single i.v., single i.p. or three daily i.p. injections of peptide. The results following i.p. administration

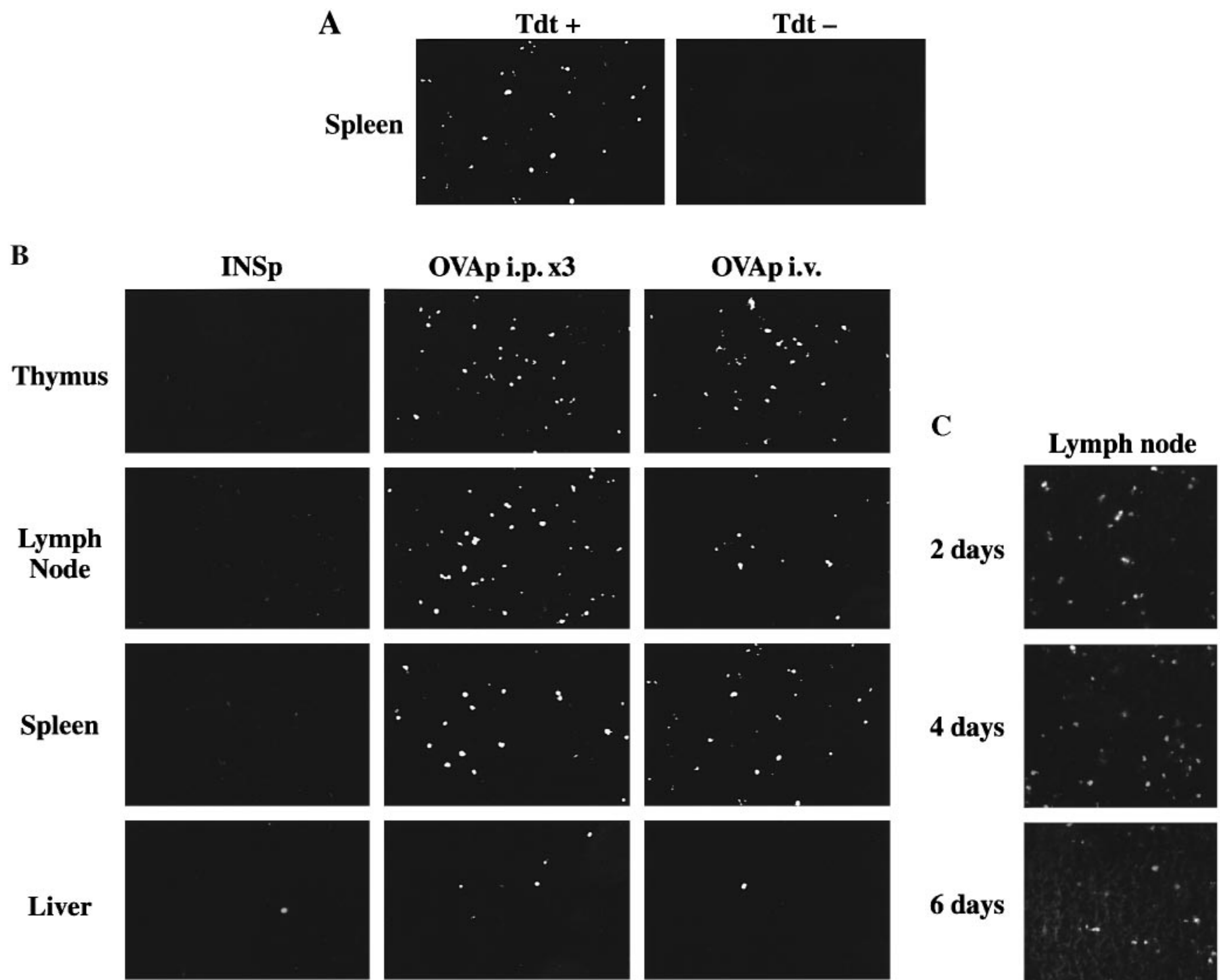

Fig. 2. Apoptosis detection by TUNEL. (A) Spleen section (×400) from OVAp i.v. treated mouse incubated with the TUNEL reaction with (+) or without (–) the TdT shows that staining is specific for fragmented DNA. White dots represent fragmented DNA labeled with biotin-16-dUTP and then visualized using streptavidin–Texas Red. Background staining without TdT is minimal. (B) TUNEL staining of thymus, spleen and liver (×400) 4 days after treatment with 2 nmol OVAp. i.v. or 2 nmol OVAp i.p. daily for 3 days. Also shown are organs from mice treated with control INSp. (C) TUNEL staining of lymph node (×400) 2, 4 and 6 days after treatment with 2 nmol OVAp i.v. Thymus, spleen, lymph node (inguinal), kidney and liver samples were taken 2, 4 and 6 days post-peptide injection and fixed in 4% paraformaldehyde. Paraffin embedded sections were cut and placed on 3-aminopropyl-triethoxy-silane (Sigma) coated slides. Results shown in (C) are from frozen sections of lymph node (fixed in OCT compound; Tissue-Tek). Apoptotic cells were detected using TUNEL. Sections were pretreated with a biotin blocking system (Dako, Carpinteria, CA) and then incubated in a moist chamber (60 min, 37°C) with 10 μM biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany), 25 mM $CoCl_2$ and 24 U TdT (Promega). Subsequently, sections were washed in PBS, incubated with streptavidin–Texas Red (1:600 dilution, Caltag) in the dark for 60 min, washed in PBS and mounted in fluorescent mounting medium (S3023; Dako).

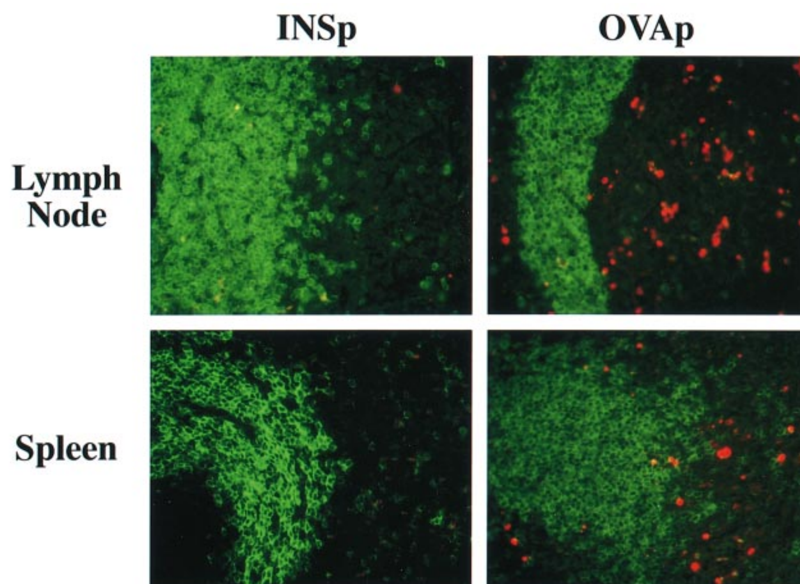

Fig. 3. TUNEL and anti-B220 staining of lymph node and spleen sections (×400) 4 days after treatment with 2 nmol OVAp or INSp i.v. $TUNEL^+$ cells are shown in red and anti-$B220^+$ cells are shown in green. Apoptotic cells were detected using TUNEL as shown in Fig. 2. After the TUNEL reaction was performed sections were incubated with anti-B220 antibody (16 B2) for 60 min at room temperature. Sections were then washed in PBS, incubated with streptavidin–Texas Red to visualize the $TUNEL^+$ cells (1:600 dilution, Caltag) and rabbit anti-rat FITC to visualize the anti-$B220^+$ cells (1:50 dilution, Vector Laboratories) in the dark for 60 min, washed in PBS, and mounted in fluorescent mounting medium (S3023; Dako).

of peptide were similar to those following i.v. administration, although the apoptotic cells were even more abundant when three injections were given (Fig. 2B). There was a marked increase in $TUNEL^+$ cells in the thymus, lymph node and spleen but TUNEL staining was unremarkable in the liver. Whereas previously it has been reported (8,23) that the major sites of $CD8^+$ T cell death are the non-lymphoid organs including the liver, here we have found that the major sites of antigen-induced death of OT-I cells are the lymphoid organs. The difference may be due to some intrinsic difference between the various peptide–TCR systems either in terms of antigen or transgenic animals. For example, it is noteworthy that in the SV40 T antigen system (8) there was a lack of proliferation/expansion in the lymphoid organs (but ample expansion in the liver) and since proliferation is necessary for antigen-induced cell death (6,18), this would explain the lack of apoptosis in the lymphoid organs. Interestingly, a very recent report described transgenic $CD8^+$ T cells against influenza nucleoprotein (23) that proliferated in the spleen after peptide stimulation and subsequently some apoptosis was detected there (the findings in lymph nodes were not reported). We therefore conclude that the lymphoid organs are sites for antigen-induced cell death for both $CD4^+$ and $CD8^+$ T cells.

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Abbreviations

INSp	insulin peptide 8–15
OT-I cells	OVA-specific $CD8^+$ T cells
OVAp	ovalbumin peptide 257–264
TUNEL	TdT-mediated dUTP-biotin nick-end labeling of DNA fragmentation

References

1 Sprent, J. 1976. Fate of H-2-activated T lymphocytes in syngeneic hosts I. Fate in lymphoid tissues and intestines traced with ^{3}H-thymidine, ^{125}I-deoxyuridine and ^{51}chromium. *Cell. Immunol.* 21:278.
2 Sprent, J. and Miller, J. F. 1976. Fate of H-2 activated T lymphocytes in syngeneic hosts. II. Residence in recirculating lymphocyte pool and capacity to migrate to allografts. *Cell. Immunol.* 21:303.
3 Sprent, J. and Miller, J. F. 1976. Fate of H-2 activated T lymphocytes in syngeneic hosts. III. Differentiation into long lived recirculating memory cells. *Cell. Immunol.* 21:314.
4 Webb, S., Morris, C. and Sprent, J. 1990. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell* 63:1249.
5 Kawabe, Y. and Ochi, A. 1991. Programmed cell death and extrathymic reduction of $V_\beta 8^+ CD4^+$ T cells in mice tolerant to *Staphylococcus aureus* enterotoxin B. *Nature* 349:245.
6 Renno, T., Hahne, M. and MacDonald, H. R. 1995. Proliferation is a prerequisite for bacterial superantigen-induced T cell apoptosis *in vivo*. *J. Exp. Med.* 181:2283.
7 Liblau, R. S., Tisch, R., Shokat, K., Yang, X., Dumont, N., Goodnow, C. C. and McDevitt, H. O. 1996. Intravenous injection of soluble

antigen induces thymic and peripheral T-cell apoptosis. *Proc. Natl Acad. USA* 93:3031.
8 Huang, L., Soldevila, G., Leeker, M., Flavell, R. and Crispe, I. N. 1994. The liver eliminates T cells undergoing antigen-triggered apoptosis *in vivo. Immunity* 1:741.
9 Hogquist, K. A., Jameson, S. C., Heath, W. R., Howard, J. L., Bevan, M. J. and Carbone, F. R. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76:17.
10 Sheil, J. M., Shepherd, S. E., Klimo, G. F. and Paterson, Y. 1992. Identification of an autologous insulin β chain peptide as a target antigen for H-2K^b restricted cytotoxic T lymhocyte *J. Exp. Med.* 175:545
11 Mamalaki, C., Norton, T., Tanaka, Y., Townsend, A. R., Chandler, P., Simpson, E. and Kioussis, D. 1992. Thymic depletion and peripheral activation of class I major histocompatibility complex-restricted T cells by soluble peptide in T-cell receptor transgenic mice. *Proc. Natl Acad. Sci. USA* 89:11342.
12 Kisielow, P., Bluthmann, H., Staerz, U. D., Steinmetz, M. and von Boehmer, H. 1988. Tolerance in T cell receptor transgenic mice involves deletion of nonmature CD4$^+$8$^+$ thymocytes. *Nature* 333:742
13 Murphy, K. M., Heimberger, A. B. and Loh, D. Y. 1990. Induction by antigen of intrathymic apoptosis of CD4$^+$8$^+$TCR^{lo} thymocytes *in vivo. Science* 250:1720.
14 Kyburz, D., Aichele, P., Hengartner, H., Zinkernagel, R. M. and Pircher, H. 1993. T cell immunity after a viral infection versus T cell tolerance induced by soluble viral peptides. *Eur. J. Immunol.* 23:1956.
15 Critchfield, J. M., Racke, M. K., Zuniga-Pflucker, J. C., Cannella, B., Raine, C. S., Goverman, J. and Lenardo, M. J. 1994. T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. *Science* 263:1139.
16 Moskophidis, D., Laine, E. and Zinkernagel, R. M. 1993. Peripheral clonal deletion of antiviral memory CD8$^+$ T cells. *Eur J. Immunol* 23:3306.
17 Kearney, E. R., Pape, K. A., Loh, D. Y. and Jenkins, M. K. 1994. Visualisation of peptide-specific T cell immunity and peripheral tolerance induction *in vivo. Immunity* 1:327.
18 Lenardo, M. J. 1991. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature* 353:858.
19 Zhu, L. Q. and Anasetti, C. 1995. Cell cycle control of apoptosis in human leukemic T cells. *J. Immunol.* 154:192
20 Moskophidis, D., Lechner, F., Pircher, H. and Zinkernagel, R. M. 1993. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* 362:758.
21 Boehme, S. A. and Lenardo, M. J. 1993. Propriocidal apoptosis of mature T lymphocytes occurs at S phase of the cell cycle. *Eur. J. Immunol.* 23:1552.
22 Surh, C. D. and Sprent, J. 1994. T cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* 372: 100.
23 Wack, A., Corbella, P., Harker, N., Crispe, I. N. and Kioussis, D. 1997. Multiple sites of post-activation CD8$^+$ T cell disposal. *Eur. J. Immunol.* 27:577