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Differential TCR Signaling Regulates Apoptosis and Immunopathology during Antigen Responses In Vivo

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Summary

Clonal selection theories postulate that lymphocyte fate is regulated by antigen receptor specificity. However, lymphocyte apoptosis is induced through non-antigen-specific receptors such as Fas (CD95/APO-1) or TNFR. We define a selective TCR that controls apoptosis by Fas or TNFR stimulation. Variant ligands can deliver this "competence to die" signal without the full TCR signals necessary for cytokine synthesis. These partial agonists regulate T cell deletion in vivo even when Fas or TNF is provided by T cells of unrelated specificity, but they do not cause the liver necrosis that is associated with T cell elimination by the full agonist. Thus, selective signaling ligands regulate T cell deletion and immune damage in vivo and may be important for peripheral T cell tolerance.

Introduction

A central tenet of clonal selection theories of lymphocyte homeostasis and tolerance is that lymphocyte fate is regulated by the antigen receptor specificity. This concept has been employed to explain self-tolerance induced during B and T lymphocyte development and, more recently, deletional tolerance involving mature T cells in the peripheral immune system (Schwartz, 1993). How peripheral T cells that are triggered during protective immune responses and have cross-reactivity with self-peptides are deleted or otherwise regulated in a clonotype-specific manner is not well understood. During immune responses, the interaction of activated T cells that are cycling in IL-2 with cognate antigen provokes an indirect mechanism of programmed death caused by apoptosis-inducing molecules such as Fas ligand (FasL) and tumor necrosis factor (TNF) and their receptors (Fas, TNFR) (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995; Zheng et al., 1995). The molecular structure of these molecules makes them part of a large superfamily of ligand and receptor genes including CD27, CD30, CD40, the low-affinity nerve growth factor (NGF) receptor, OX40, DR3–5, and others. Members of this gene superfamily play crucial and diverse roles in immunity, especially in processes involving apoptosis (Nagata, 1997). However, Fas and TNF receptors are expressed on most activated T cells and have no intrinsic antigen clonotype specificity. The nature of peptide-induced antigen receptor signals that govern the death of activated T cells by FasL or TNF (Hornung et al., 1997; Wong et al., 1997) and ensure clonotype specificity of T cell elimination is not presently known.

Although multiple mechanisms participate in controlling mature T cell immune responses, recent investigations have placed special emphasis on antigen-induced apoptosis (Lenardo et al., 1995). It is now generally thought that apoptosis of lymphocytes mediated by the Fas pathway contributes to peripheral immune homeostasis and tolerance. Compelling support for this inference comes from the observation that severe lymphadenopathy and autoimmune disease in mice and humans results from deleterious mutations in the *apt1* (Fas) gene (Watanabe-Fukunaga et al., 1992; Fisher et al., 1995; Rieux-Laucat et al., 1995). However, the expression of Fas and its ligand occur as part of an overall antigen response involving lymphokine secretion and other molecular events characteristic of lymphocyte activation. This raises the possibility of collateral damage as a secondary event either through Fas ligand or other damaging lymphokines acting upon host tissues in the proximity of dying T cells. The strategies that the immune system employs to limit damage to the host as it achieves peripheral lymphocyte homeostasis and tolerance through deletion are not completely understood. We therefore explored this question in our studies of the antigenic regulation of Fas- and TNF-induced apoptosis of mature T cells.

Results

TCR-Induced Apoptosis Is Clonotype Specific

To verify that T cell death induced by a full agonist peptide is clonally restricted, we first analyzed a mixture of cells containing a pigeon cytochrome c (PCC)-specific Th1 clone, A.E7 (Hecht et al., 1983), and a heterogeneous population of MHC-matched concanavalin A blast (CAB) T cells (Boehme and Lenardo, 1993). Both cell populations had been activated and were briskly cycling in response to culture in medium containing IL-2, making them highly susceptible to T cell receptor (TCR)-induced death (Lenardo, 1991; Combadière et al., 1998). Restimulation of these cultures with the PCC 88–104 peptide in the presence of I-E^k-expressing, antigen-presenting cells (APC) caused the death of A.E7 in a dose-dependent fashion (Figure 1Aa). Death of this T cell clone was preceded by up-regulation of both Fas-ligand (Figure 2A) and TNF (Figure 2B) (Boehme and Lenardo, 1993; Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995; Lenardo et al., 1995; Zheng et al., 1995; Combadière et al., 1998). Nonetheless, the neighboring cycling CAB T cells that expressed an equivalent or

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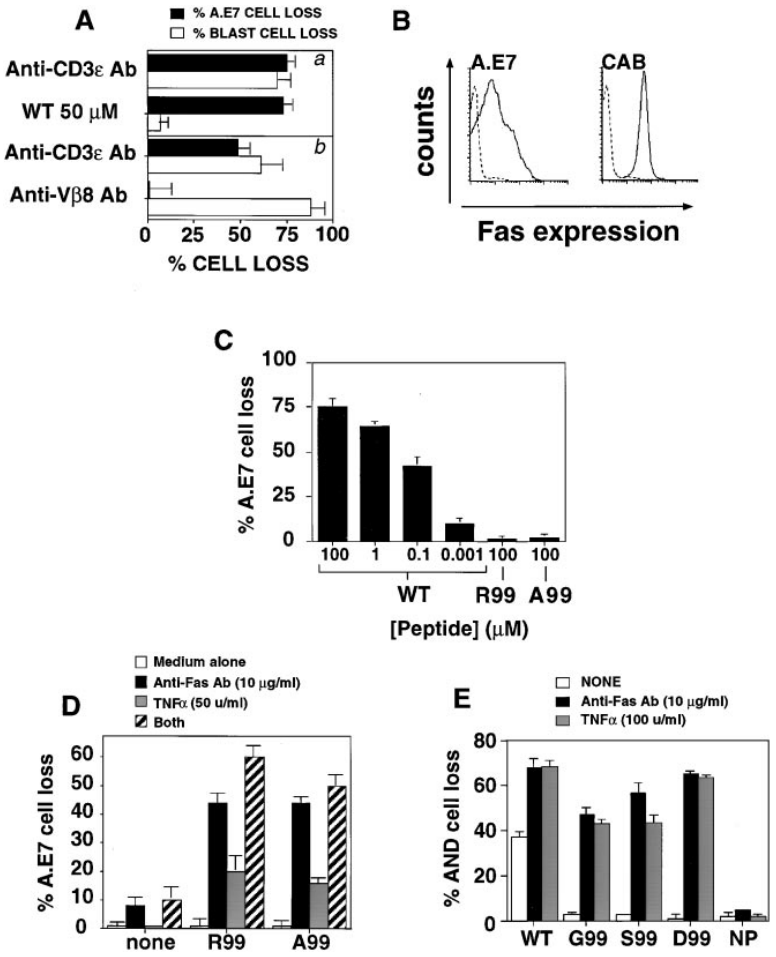

Figure 1. Antigen-Induced Death Is Clonotype Specific

(Aa) Cell death assay using three types of cells: cycling A.E7, CMFDA-labeled P13.9 APC, and d-He-labeled cycling CAB from B10.A mice (Boehme et al., 1995). Cells were incubated for 24 hr in medium alone or in the presence of PCC 88–104 (wt) peptide or immobilized anti-CD3ε MAb (2C11).

(Ab) Cell death assay using two types of cells: cycling CMFDA-loaded A.E7 and Vβ8$^+$ T cell blasts. Cells were incubated with either immobilized anti-CD3ε MAb (2C11) or biotinylated anti-Vβ8 and 1 μg/ml of avidin. Cell loss for each cell type was measured by flow cytometric counting of viable cells.

(B) Expression of CD95/Apo-1/Fas receptor on A.E7 cells or CAB by flow cytometry: control hamster Ig (dotted line) and anti-Fas (solid line). One of three representative experiments is shown.

(C) Dose–response analyses of cycling A.E7 cells stimulated with different doses of PCC (88–104) wt peptide or with variant peptides R99 or A99 at 100 μM. T cell apoptosis was measured after stimulation for 24 hr and after addition of 7 amino-actinomycin D (7-AAD). All error bars shown represent one SD from the mean.

(D) R99 or A99 stimulation render A.E7 susceptible to Fas- or TNFR-mediated apoptosis. Cycling A.E7 cells were stimulated with P13.9 APC preincubated with 100 μM of R99 or A99 in the presence of either medium alone or biotinylated anti-mouse Fas (Jo2, 10 μg/ml), avidin (1 μg/ml), and/or TNFα (50 U/ml). After 24 hr incubation at 37°C, A.E7 cells were stained with fluoresceinated anti-CD4 to differentiate them from APC and cell death was assessed after addition of 7-AAD by flow cytometry.

(E) Cycling AND T cells specific for PCC 88–104 were stimulated with CMFDA-loaded P13.9 APC preincubated with 100 μM of wt, G99, S99, or D99 in the presence of either medium alone or biotinylated anti-mouse Fas (Jo2, 10 μg/ml), avidin (1 μg/ml), and/or TNFα (50 U/ml). T cell apoptosis was measured after 24 hr.

greater starting level of Fas and TNF receptors (TNFRs) were spared (Figures 1A and 1B; data not shown). Control experiments showed that the CAB T cells were able to undergo apoptosis upon direct stimulation through the TCR/CD3 complex (Figure 1A, anti-CD3ε) (Boehme and Lenardo, 1993). In a reciprocal experiment, we also found that the death of Vβ8.2$^+$ blasts stimulated with anti-Vβ8 Ab does not cause the death of bystander cycling A.E7 cells, although both T cells died in response to anti-CD3ε (Figure 1Ab). We therefore hypothesized that engagement of the TCR prior to or at the time of Fas cross-linking generated a "competence to die" signal, without which T cells were spared.

Variant Peptides Can Selectively Deliver a Competence to Die Signal in the Absence of Cell Activation

In pioneering studies, investigators have used altered ligands, which are typically single amino acid variants of antigenic peptides, to dissect various components of TCR signaling (Evavold et al., 1993; Sloan-Lancaster et al., 1994; Jameson and Bevan, 1995; Madrenas et al., 1995; Madrenas and Germain, 1996). We studied the putative competence to die signal in A.E7 cells by testing a large number of variants of the PCC 88–104 peptide that contain single amino acid changes for lysine-99 (K99) (Combadière et al., 1998). Substitutions at position 99 do not affect binding to the I-E^k molecule but rather alter TCR recognition of the peptide–MHC complex (Jorgensen et al., 1992; Madrenas et al., 1995). Stimulation of cycling A.E7 T cells with wild-type (wt) PCC 88–104 peptide induced apoptosis in a dose-dependent manner (Figures 1A and 1C) as well as the secretion of the T_H1 cytokines IL-2, IL-3, and IFNγ (Combadière et al., 1998). We have previously described two variants, C99 and Y99, that selectively induced the programmed cell death (PCD) of A.E7 cells without inducing synthesis of T_H1 cytokines (Combadière et al., 1998). By contrast, two other variants, R99 and A99, did not stimulate lymphokine secretion on their own (Combadière et al., 1998) nor induce death of cycling A.E7 cells (Figure 1C). Despite this failure of the R99 and A99 peptides to induce apoptosis or activation cytokines per se, both peptides had the property of dramatically amplifying T cell apoptosis in response to antibody cross-linking of Fas, TNFR, or both together (Figure 1D). Control experiments

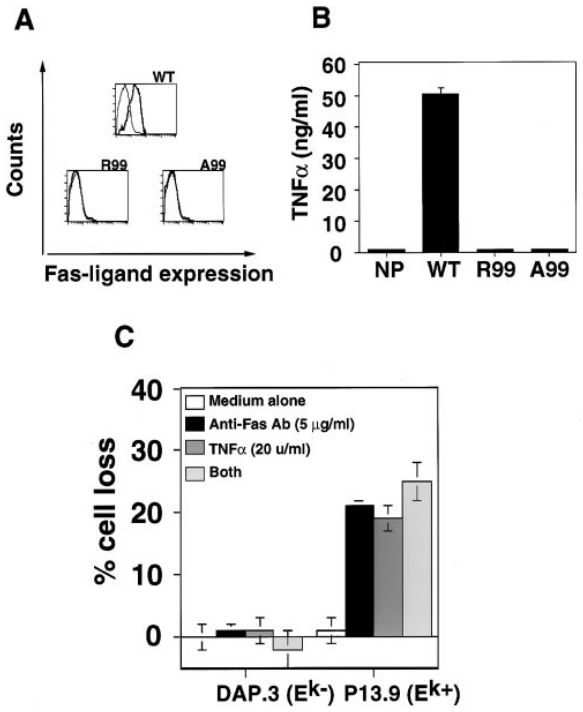

Figure 2. Variant Ligands R99 and A99 Do Not Induce Death Molecules

(A) Cycling A.E7 cells were stimulated with variants or wt peptide (100 μM) for 12 hr in presence of metalloproteinase inhibitor and were stained with PE-conjugated anti-FasL and FITC-conjugated anti-CD4. Dead cells were gated out and viable $CD4^+$ A.E7 cells were analyzed for FasL expression.

(B) TNFα production in the same experiment was measured by ELISA. Results are representative of at least three independent experiments.

(C) Variant peptides must be presented on APCs to promote cell death. Cycling A.E7 cells were stimulated with either CMFDA-loaded DAP.3 (H-$2E^{k-}$) (left side) or P13.9 (H-$2E^{k+}$) APC (right side) preincubated with either medium alone (no peptide) or 100 μM of R99 in the presence of cross-linked anti-mouse Fas Ab (5 μg/ml) and/or 20 U/ml of TNFα. Cells were incubated for 24 hr at 37°C. Percent cell death was measured by flow cytometry after the addition of PI. All error bars are shown and represent one SD from the mean.

showed that stimulation of Fas and/or TNFR alone does not kill cycling A.E7 cells (Figure 1D) and R99 or A99 did not induce Fas-ligand up-regulation or TNFα secretion (Figures 2A and 2B). Thus, A99 and R99 variant peptides appear to deliver a selective signal that renders cycling A.E7 cells competent to die by the Fas and TNFR pathways.

We then investigated whether this phenomenon applied to other T cells. We prepared T cells from AND TCR (Vα11/Vβ3) transgenic mice that recognize PCC 81–104/E^k but bear a structurally different TCR from A.E7 cells and, consequently, have a different fine specificity. Three variants of the PCC 88–104 peptide, S99, G99 and D99, were found that did not induce the death of AND T cells or stimulated cytokine secretion. However, each of these variant peptides greatly amplified AND T cell apoptosis in the presence of antibody cross-linking of Fas and/or TNFR (Figure 1E). Thus, two distinct cell populations with different TCRs selected unique sets of competence to die peptides.

To ensure that this result was not due to a nonspecific toxic effect of the peptide preparations but rather had the hallmarks of a response to the peptides as antigens, we performed additional tests. We examined whether MHC presentation of the R99 and A99 peptides was required for inducing susceptibility to apoptosis. We found that incubation of A.E7 cells with R99 in the presence of DAP.3 (E^{k-}) cells, which are the parental cells for P13.9 (E^{k+}) APC and lack MHC class II molecules, failed to allow A.E7 cells to die in response to Fas or TNFR stimulation (Figure 2C). By contrast, incubation with R99 in the presence of P13.9 did not induce apoptosis in the absence of other stimuli but allowed the cells to die in response to anti-Fas cross-linking or TNFα. We also evaluated phosphorylation events that might indicate direct signaling by the variant peptides through the TCR (data not shown). Consistent with our previously published phosphotyrosine analyses (Combadière et al., 1998), we found that stimulation with the wt peptide induces prominent tyrosine-phosphorylated species corresponding to the three isoforms of phospho-ζ (p18, p21, and p23), CD3ϵ, and ZAP-70, whereas the R99 and A99 peptides only induced the p21 tyrosine-phosphorylated form of TCR-ζ chain without stable phosphorylation of ppζ23, CD3ϵ, and ZAP-70 (data not shown). Thus, R99 and A99 peptides combine with MHC to deliver a genuine TCR signal, which is only part of that seen in response to full agonist stimulation.

Bystander T Cells that Receive a Competence to Die Signal Can Undergo Apoptosis When Exposed to Other Activated T Cells

It was necessary to exclude the possibility that the effects of the R99 and A99 peptides only enhanced the effect of artificial stimuli such as the Fas cross-linking antibody or large amounts of soluble TNF. We therefore investigated whether the competence to die signal could regulate interactions between T cells of different specificity by establishing the three-cell system illustrated in Figure 3A. Both the wt PCC 88–104 and the R99 variant act as full agonists for cycling AND T cells and cause death through Fas and TNFR (Figure 3B) as well as IL-2 synthesis (data not shown). We mixed cycling AND and A.E7 T cells together with I-E^{k+} APC to see if FasL and TNF expressed by AND T cells in response to recognition of R99 would eliminate A.E7 T cells that simultaneously received the R99 competence to die signal (Figure 3A). AND T cells underwent apoptosis in response to both wt and R99 peptides, irrespective of the presence of A.E7 T cells (Figure 3B). By contrast, the R99 peptide caused the death of A.E7 cells only in the presence of AND T cells, whereas the wt peptide caused A.E7 cell death with or without AND-PCC T cells (Figure 3B). For both the wt and R99 peptides, the death of A.E7 or AND-PCC T cells was abrogated by blocking both FasL and TNF, indicating that apoptosis in this system was due to these molecules expressed naturally by the AND T cells (Figure 3D). Thus, T cells that receive a selective competence to die signal can undergo apoptosis when they encounter physiological levels of FasL or TNFα produced by T cells of a different specificity in the same microenvironment.

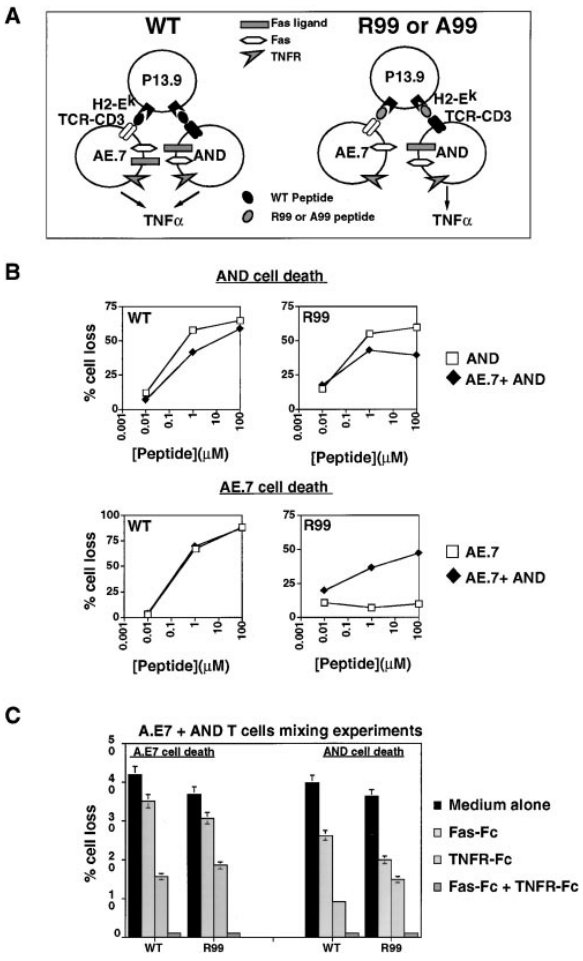

Figure 3. Cells that Receive a Competence to Die Signal Undergo Apoptosis in the Presence of Third-Party T Cells Expressing FasL or TNF In Vitro

(A) Scheme of the three-cell system. Activated, cycling AND T cells recognize wt PCC 88–104 and the R99 variant as agonists, upregulate FasL and TNFα, and die by apoptosis. Shown are the hypothetical interactions between the A.E7 and AND T cells tested in this experiment.

(B) Cycling T cells from AND-TCR transgenic mice and CMFDA-loaded cycling A.E7 cells were added to P13.9 APC preincubated with wt or variant peptides at various concentrations. AND T cells were stained with PE-conjugated anti-H-2K^b prior to flow cytometry. Cell death was measured after the addition of 7-AAD (Sigma Chemical Co., St Louis, MO) by flow cytometry.

(C) To block cell death, Fas-Fc (10 μg/ml) and TNFR-Fc (10 μg/ml) or both were included in the assay described above. Error bars represent one SD from the mean.

Deletion of T Cells that Receive a Competence to Die Signal In Vivo

In the course of immune responses, different T cell clones are juxtaposed in lymphoid tissues, perhaps even encountering antigen on the same APC. Therefore, T cells expressing death molecules in vivo could elicit the death of T cells of a different specificity if the latter received a competence to die signal through the TCR. On the other hand, the presentation of agonist and partial agonist peptides may not be sufficiently coincident to induce the competence to die signal in T cells that are in proximity to other T cells that express death cytokines. To rigorously test these possibilities, it was necessary to perform antigen challenges in vivo (Figure 4A). We adoptively transferred cycling A.E7 T cells and challenged them in vivo with the A99 and R99 peptides in the presence or absence of activated T cells specific for an unrelated antigen, the 46–61 peptide of hen egg lysozyme (HEL), presented by a different MHC class II molecule, I-A^k (Babbitt et al., 1985). Activated T cells against HEL were generated in B10.A mice by immunizing with the HEL 46–61 peptide and boosting on day 14 (Figure 4A). Control mice received only PBS injections and therefore lacked activated anti-HEL T cells. The adoptively transferred A.E7 T cells were labeled with a fluorescent dye and given intravenously on day 15 to both groups of mice. Six hours later, the HEL-46–61 peptide was administered by intraperitoneal injection either alone or together with the wt, R99, or A99 PCC peptides (Figure 4A). On day 16, the mice were sacrificed and various tissues were analyzed. In mice that received no PCC peptides, labeled A.E7 cells were detected in the spleen and liver but not in lymph nodes (Figures 4B, 5B, and 5C; data not shown). Mice injected with wt PCC 88–104 peptide had over 90% fewer labeled A.E7 cells in their spleens compared to mice that received no PCC peptide (Figure 4B; compare wt with PBS). A few TUNEL$^+$ cells remained, but most were eliminated, indicating very rapid apoptotic death similar to that known to occur during thymocyte deletion (Figure 4B). Moreover, the disappearance of A.E7 cells was independent of HEL-reactive T cells because it took place in both the HEL-injected and control (PBS-injected) mice (Figure 4B). By contrast, injection of either the R99 or A99 peptide caused significant depletion of A.E7 cells only in the spleens of animals immunized with HEL 46–61 (48% and 65% cell loss with the R99 and A99 peptides, respectively) (Figure 4B, compare PBS group and HEL group). The loss of A.E7 cells induced by R99 and A99 in HEL-immunized mice was due to apoptosis since 40%–60% of the remaining cells were TUNEL$^+$, thereby indicating that a sizable fraction of cells had been programmed to die. Thus, the R99 and A99 peptides promoted A.E7 cell apoptosis in the spleen only when other stimulated T cells were present in the mice. Considered together with our in vitro observations, these data suggest that the in vivo induction of A.E7 cell death was due to the combined effects of the A99 and R99 partial agonists, which deliver a competence to die signal in propinquity to highly activated HEL-specific T cells that express death-inducing cytokines.

Deletion of A.E7 by Partial Agonists Occurs without Induction of Severe Liver Necrosis

Programming the death of A.E7 cells in vivo by the full agonist peptide or the partial agonist peptides allowed us to compare the effects of these different stimuli on the whole animal. During routine histopathological analysis of internal organs, we were surprised to observe the dramatic appearance of focal sclerotic lesions in the livers of mice receiving A.E7 cells and the wt agonist peptide (Figure 5A). As noted above, activated A.E7 cells homed primarily to the spleen and liver of adoptive recipients. The lesions that we observed consisted of striking

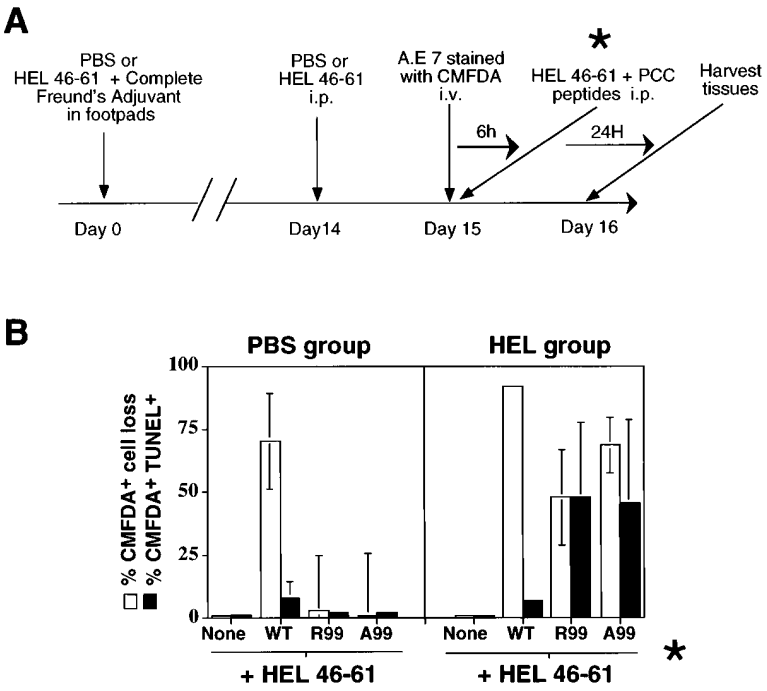

Figure 4. Effect of the A99 and R99 Variant Peptides on A.E7 Cells In Vivo during Immune Responses to an Unrelated Antigen

(A) Experimental scheme. Time course of injections of cells and antigens. The asterisk indicates the time at which death-inducing peptide challenges were given, as indicated in (B). See Experimental Procedures for details.

(B) Spleen cell suspensions were analyzed for the presence of TCR^+CMFDA^+ T cells. Events (1.5 million) were acquired by flow cytometry after staining of the T cells with PE-conjugated anti-TCRβ. Percent TCR^+CMFDA^+ A.E7 cell loss was calculated compared to control HEL + PBS injected mice in each group. A fraction of spleen cells was further analyzed for the presence of remaining apoptotic cells by using a TUNEL assay as described by the manufacturer (Trevigen, Gaithersburg, MD).

areas of liver cell necrosis that were associated with venous thrombosis (regions marked by asterisks in Figures 5A and 5B). By contrast, liver damage did not occur in mice harboring A.E7 cells and injected with the R99 or A99 variant peptides or PBS, independently of whether the mice had been immunized against HEL (Figure 5B; data not shown). A striking aspect of the liver disease was that there was no evidence of hepatocyte apoptosis such as has been previously described with CD95 cross-linking. Rather, the type of thrombosis and liver damage that we observed is similar to that previously reported to be caused by tissue factor and procoagulant activity induced by Th1 cytokines (Bierhaus et al., 1995; DelPrete et al., 1995; Schmid et al., 1995). Thus, we considered the idea that liver damage was due to cytokines secreted by A.E7 cells after wt peptide stimulation. To investigate this interpretation, we also challenged animals with the Y99 and C99 variant PCC peptides, which are partial agonists that induce FasL, TNF, and A.E7 death in the absence of Th1 cytokines such as IL-2 and IFNγ (Combadière et al., 1998). We found that Y99 and C99 caused the elimination of A.E7 cells in the livers of adoptive recipients (Figure 6A). Furthermore, only a slight mononuclear infiltrate but no liver necrosis remained after treatment with these peptides (Figure 6B). Since Y99 and C99 induce the FasL and TNF death cytokines but not other Th1 cytokines (Combadière et al., 1998), we conclude that the necrotic liver damage and venous thrombosis is most likely due to Th1 cytokine release during TCR-induced T cell elimination, rather than a consequence of apoptotic T cell death induced by death cytokines. Unlike the wt, C99, and Y99 peptides, the A99 and R99 ligands allowed A.E7 deletion in the spleens but not in the livers of HEL-immunized mice (Figure 5C). This is most likely due to the absence of HEL-activated T cells in the liver, since HEL immunization per se never caused T cell infiltration of that organ (data not shown). Thus, T cell deletion by the cytokine-inducing full agonist wt peptide is accompanied by end-organ damage to the liver, whereas the partial agonists A99, R99, Y99, and C99 permitted antigen-specific T cell deletion but did not induce liver immunopathology.

Discussion

Our data reveal a novel facet of antigen regulation of FasL- and TNF-induced apoptosis and provide insights into the biological function of partial agonist peptides in vivo. Antigen-induced FasL- and TNF-mediated apoptosis is important for the maintenance of normal lymphocyte homeostasis and peripheral tolerance (Zheng et al., 1996). Clonal selection theory postulates that lymphocyte fate is regulated in an antigen-specific manner. For T cell apoptosis, which depends on death cytokines and receptors that are not clonally restricted, our investigation reveals a novel TCR signal that can regulate T cell elimination by FasL and TNF pathways. Full agonist stimulation leads to various effector outcomes including cytokine secretion, up-regulation of death receptors, and death of the T cell. By using variant peptide ligands to dissect the array of signals emanating from the TCR, we show that one consequence of antigen stimulation is the delivery of a competence to die signal that is separable from other effector functions and may involve a minimal set of tyrosine phosphorylation events. Cells stimulated by the selective competence to die peptides do not up-regulate death molecules. Rather, we found that "competent" T cells are susceptible to death ligands expressed on neighboring cells, both in vitro and in vivo. We are investigating the molecular basis of this effect and so far our data suggest that BCL-Xl is not involved in

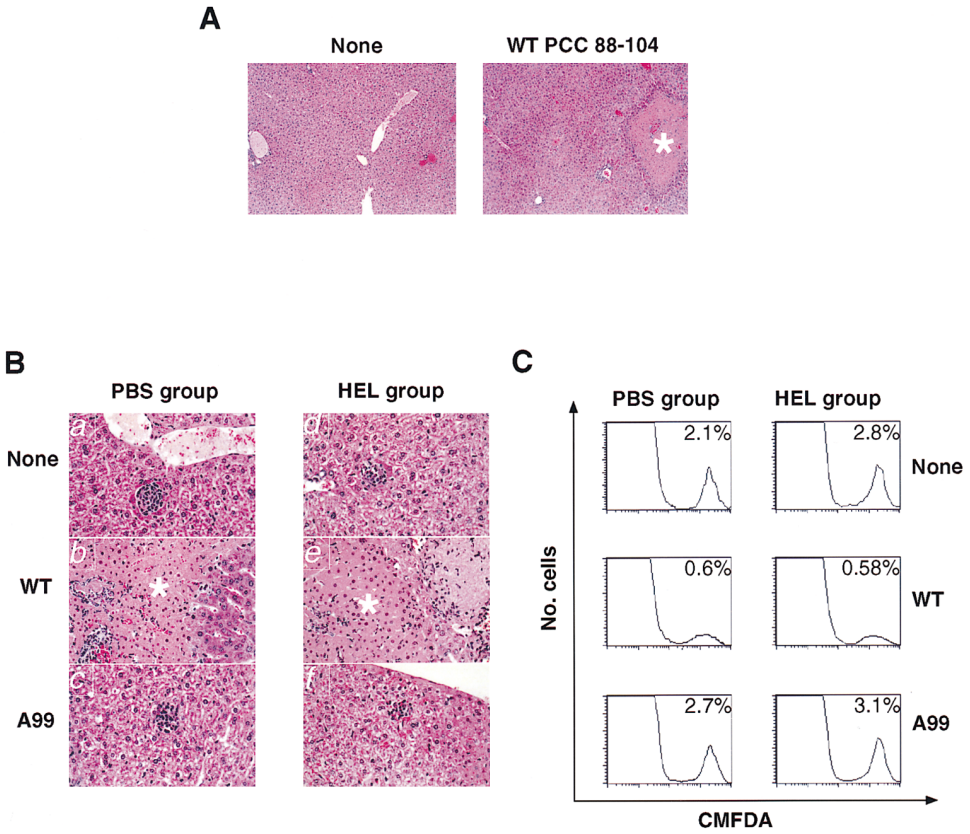

Figure 5. The Wt Agonist Peptide, but not Partial Signaling Peptides, Induces Liver Damage

Paraffin sections of liver after hematoxylin and eosin stain (American HistoLabs, Gaithersburg, MD) from the experiment described in Figure 4. (A) Liver section (100× original magnification) from the PBS group injected with A.E7 cells plus either PBS (left panel) or wt PCC 88–104 peptide (right panel), together with HEL 46–61. Liver infarction comprising venous thrombosis and hepatocyte necrosis present throughout the whole liver are seen after wt peptide treatment only and are indicated by an asterisk. (Ba), (Bb), and (Bc) correspond to the PBS group, and (Bd), (Be), and (Bf) to the HEL group. Enlargement of liver sections (630× original magnification) from mice injected with HEL 46–61 plus the indicated PCC peptides: no peptide (Ba and Bd), wt (Bb and Be), and A99 (Bc and Bf). No areas of liver damage were observed after the injection of variant peptide A99 in either group; large areas of liver damage after treatment with wt peptide are indicated by an asterisk. (C) Detection of A.E7 cells by flow cytometric analysis of cell suspensions prepared from the liver. Percent $CMFDA^+$ cells (A.E7) are shown for each plot. (A), (B), and (C) are from the same experiment.

this phenomenon (Combadière et al., 1998). We presume that the competency signal for death also occurs in the context of full signaling by agonists and this could explain the exquisite antigen selectivity found in many models of T cell deletion (Kawabe and Ochi, 1991; Russell et al., 1991; Critchfield et al., 1994; Lenardo et al., 1995; Alexander-Miller et al., 1996; Brocke et al., 1996; Hornung et al., 1997; Wong et al., 1997). Thus, our data establish that peptide antigen provides two important signals for death. The first is needed for death ligand expression and the second is needed to facilitate the apoptosis signaling pathway at the time of Fas or TNFR engagement. It is the latter signal whose absence prevents Fas- or TNFR-expressing bystander cells from undergoing death and preserves the specificity of clonal selection.

Although partial agonism has been of interest in the experimental analysis of TCR signaling, the role of partial agonist ligands in immune responses in vivo has been enigmatic. Our data show how partial agonists could take part in mature T cell apoptosis regulation in vivo. The A99 and R99 partial agonists only permitted clonotype-specific apoptosis in the presence of other activated T cells. T cell deletion induced by the full agonist was associated with severe immunopathology due to T cells that had homed to the liver; however, the A99 and R99 peptides caused T cell deletion (in the spleen) but did not stimulate full Th1 responses in the liver and thus avoided end-organ damage. Liver damage in our model appears to be related to the phenotype of activated A.E7 cells that home to the liver in healthy mice and is not seen in all T cell transfer models (Critchfield et al., 1994). The partial agonists Y99 and C99, which induce T cell apoptosis directly by the selective production of FasL and TNF without effector cytokines such as IL-2, IL-3, or IFNγ (Combadière et al., 1998), did not cause liver damage (Figure 6). This, coupled with the fact that liver cell death was necrotic (and associated with venous thrombosis) rather than apoptotic, suggests that effector cytokines, rather than FasL or TNF, caused the liver immunopathology in the present experiments. In addition, preliminary data has shown that these necrotic

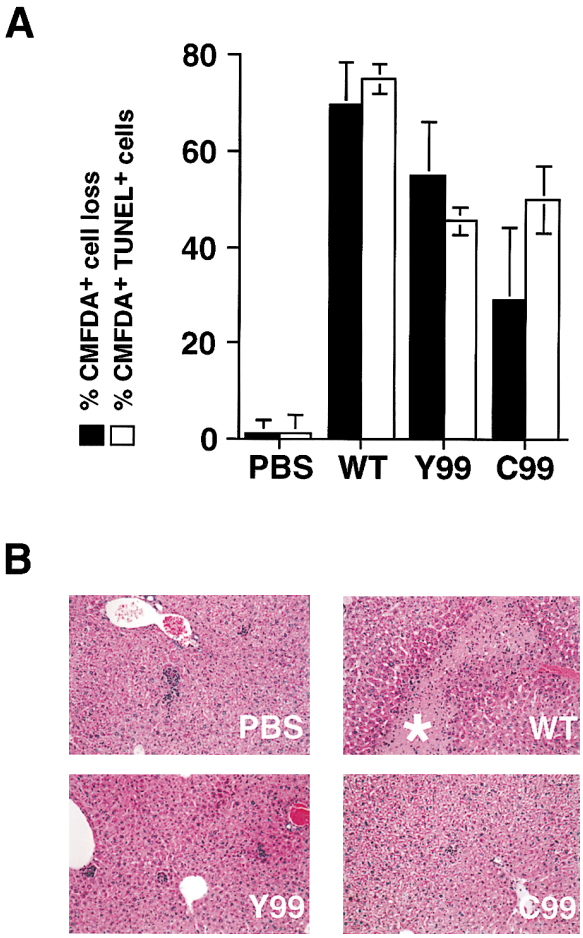

Figure 6. Variant Peptides that Selectively Promote T Cell Apoptosis through FasL and TNF Do Not Induce Liver Damage In Vivo

(A) Cell suspensions of spleen were analyzed for the presence of TCR^+CMFDA^+ T cells. Events (1.5 million) were acquired by flow cytometry after staining of the T cells with PE-conjugated anti-TCRβ. Percent TCR^+CMFDA^+ A.E7 cell loss was calculated and compared to control PBS-injected mice in each group. A fraction of spleen cells was further analyzed for the presence of remaining apoptotic cells by using a TUNEL assay as described by the manufacturer (Trevigen, Gaithersburg, MD). Similar results were obtained in the liver (data not shown).

(B) Liver sections (100× original magnification) from the PBS group injected with A.E7 cells plus either PBS, wt PCC 88–104, Y99, or C99 peptides. Liver infarction comprising venous thrombosis and hepatocyte necrosis that were seen only with wt peptide treatments are indicated by an asterisk and are present throughout the entire liver.

lesions cannot be prevented by zVAD, an inhibitor of apoptosis (Jaeschke et al., 1998). Thus, our experiments show that the outcome of partial agonist stimulation in vivo can be correlated with the T cell responses to various peptides in vitro.

An important implication of our data is that partial TCR signaling could serve to specifically eliminate T cells in vivo that are stimulated during a protective immune response (where the agonist peptide is derived from the pathogen) but have significant cross-reactivity with self (where the self-peptide is a related structure with partial agonist properties). Self-peptides that selectively deliver a competence to die signal could promote apoptosis of the responding T cells when the latter are exposed to FasL or TNF produced in *trans* by other T cells. Other self-peptides might directly induce apoptosis without activation of cross-reactive T cells, analogous to the response of A.E7 to the C99 and Y99 altered ligands. By this concept, cross-reactions with many self-peptides would not be damaging, because these partial agonists would not provoke cytokine release and effector function but could eliminate cross-reactive T cells. This model is supported by our finding that splenic A.E7 cells were deleted and persisting A.E7 cells in the liver caused no damage in mice injected with the A99 and R99 peptides. Further support for our idea is that many PCC-related peptides with amino acid substitutions in the K99 position induce either the competence to die signal or apoptosis directly and fail to induce effector cytokines such as IL-2 or IFNγ (B. C. and M. J. L., unpublished data). If the propensity of partial agonist peptides to induce T cell death in our model is found to be representative of other antigen systems, then the elaboration of partial signals by the TCR could promote tolerance by the benign deletion of activated T cells. However, we speculate that such a mechanism might also have the untoward complication of allowing the immune response to fail when confronted with a rapidly mutating pathogen such as the human immunodeficiency virus.

Experimental Procedures

Cells

A.E7 is a Th1, $CD4^+$ T cell clone specific for the 88–104 fragment of pigeon cytochrome c (PCC) presented by $I\text{-}E^k$ (Hecht et al., 1983). A.E7 cells were stimulated with (5 μM) PCC in the presence of irradiated splenocytes from B10.A mice ($H\text{-}2^a$). After 48 hr, cells were isolated by Ficoll gradient centrifugation and were transferred to fresh medium containing IL-2 (50–100 IU/ml recombinant human IL-2 [Chiron, Emeryville, CA] or 10%–15% T-Stim [Collaborative Biomedical Products, Bedford, MA]) and incubated for an additional 48–96 hr to generate cycling A.E7 cells that are predisposed to apoptosis following TCR engagement (Lenardo, 1991). Cycling concanavalin A blasts (CAB) were prepared as described (Boehme et al., 1995) from the spleens of B10.A mice. Cycling Vβ8 blasts were obtained by stimulating B10.A splenocytes with biotinylated-anti-Vβ8 Ab (1 μg/ml) and avidin (1 μg/ml) for 48 hr, followed by transfer to fresh medium containing IL-2 (50–100 U/ml). A PCC (88–104) specific T cell line from $H\text{-}2^b$ AND TCR transgenic mice (TCR against PCC 81–104 presented by $I\text{-}E^k$) was generated by sequential stimulation of lymph node cells with irradiated $H\text{-}2^k$ splenocytes and PCC 88–104 peptide. P13.9, L cell transfectants expressing $I\text{-}E^k$, ICAM-1, and B7.1 (CD80) molecules or DAP-3, the parental L cell line, were used as antigen-presenting cells (APC) (Madrenas et al., 1995; Combadière et al., 1998).

Peptides

The sequence of PCC (88–104) is KAERADLIAYLKQATAK. Peptides Y99, C99, R99, A99, S99, G99, and D99 are PCC (88–104) variants in which lysine in position 99 is changed to tyrosine (Y99), cysteine (C99), and arginine (R99) or alanine (A99), serine (S99), glycine (G99), and aspartate (D99), respectively. All peptides were synthesized by the Peptide Synthesis Facility, NIAID, NIH, Bethesda, MD.

Apoptosis Assay

Cycling A.E7 cells (5×10^4) and CAB were incubated with 5×10^4 P13.9 cells in the presence of the indicated concentrations of PCC

peptides. For direct stimulation via the TCR complex, cells (5×10^4) were incubated on plates coated with anti-CD3ε antibody (2C11) or were exposed to biotinylated-anti-Vβ8.2 followed by avidin. In addition to TCR stimulation, in some experiments cross-linking of Fas and/or TNFR was also carried out by incubating the cells in the presence of 5 or 10 μg/ml of biotinylated anti-mouse Fas Ab (Jo2) (Pharmingen, San Diego, CA) plus 1 μg/ml of avidin (Sigma) and/or 20 or 50 U/ml m-TNFα (Genzyme, Cambridge, MA). Fas-Fc (10 μg/ml), TNFR-Fc (10 μg/ml), or both were included in some assays to block apoptosis.

In all apoptosis assays, conditions were set up in quadruplicate in 96-well round-bottom plates. Cells were harvested after 24 hr incubation, 2 of each 4 wells were pooled, and propidium iodide (PI) or 7 amino-actinomycin D (7-AAD) was added to a final concentration of 10 μg/ml. Ungated cells were acquired for a fixed time at a constant flow rate using a FACScan equipped with CellQuest software (Becton-Dickinson, Mountain View, CA). For in vivo analysis, acquisition was set for a fixed number of events (1.5×10^6) rather than fixed time to ensure statistical significance. The number of viable cells from duplicate tubes was averaged and the results were converted to percent apoptosis as previously described (Boehme and Lenardo, 1993). In some experiments, P13.9 cells were preloaded with 0.1 μM carboxyfluorescein diacetate-acetylester (CMFDA; Molecular Probes, Eugene, OR) to allow APC to be excluded from the analysis by gating on fluorescein-negative cells. In other experiments, A.E7 cells were stained with fluoresceinated-anti-CD4 before flow cytometry to differentiate them from other cells; AND T cells were stained with phycoerythrin (PE)-conjugated anti-H-$2K^b$. In experiments with A.E7 and B10.A CAB, the latter were preloaded with dihydroethidium (d-He) to differentiate them from other cell types.

Assays for Death Molecules

Supernatants from the apoptosis assays were harvested at 24 hr, and TNFα was measured by ELISA (Genzyme, Cambridge, MA) according to the manufacturer's instructions. Fas was measured by flow cytometry after staining with PE-conjugated anti-Fas (Pharmingen, San Diego, CA). To measure the up-regulation of FasL, cycling A.E7 cells were stimulated with APC and variant or wt peptides for 12 hr in the presence of metalloproteinase inhibitor (KB8301), as described elsewhere (Kayagaki et al., 1995), and stained with PE-conjugated anti-FasL and FITC-conjugated anti-CD4 Abs (Pharmingen).

Adoptive Transfer of A.E7 Cells and Stimulation In Vivo

Female B10.A mice were procured from the Jackson Laboratory and used between 6 and 12 weeks of age. Each mouse was injected with either PBS or 50 μg of HEL 46–61 peptide in complete Freund's adjuvant (CFA) in the hind footpads (Figure 4A). Mice were boosted 2 weeks later with an intraperitoneal injection of either PBS (PBS group) or 100 μg of HEL-46–61 (HEL group). Twenty-four hours later, 2 mM CMFDA-loaded cycling A.E7 cells (15×10^6) were given intravenously to each mouse. This was followed 6 hr later by an intraperitoneal injection of 100 μg HEL-46–61 + PBS or 1 mg of the indicated PCC peptides (asterisk in Figure 4A). Two mice were used per treatment group. Mice were sacrificed 24 hr later and tissues (spleen, lymph nodes, and liver) were harvested for analysis. Cell suspensions of liver or spleen were prepared and analyzed by flow cytometry for the presence of $CMFDA^+TCR^+$ A.E7 viable cells (7-AAD exclusion) and stained for DNA fragmentation by the TUNEL method using the Trevigen TACS2 kit as per manufacturer's instructions (Trevigen, Gaithersburg, MD). Livers were fixed in formalin and paraffin sections were cut and stained with hematoxylin and eosin (American Histolabs, Gaithersburg, MD).

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