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T cell apoptosis by tryptophan catabolism

F Fallarino[1], U Grohmann[1], C Vacca[1], R Bianchi[1], C Orabona[1], A Spreca[1], MC Fioretti[1] and P Puccetti*,[1]

[1] Department of Experimental Medicine, University of Perugia, Italy
* Corresponding author: P Puccetti, Department of Experimental Medicine, Pharmacology Section, University of Perugia, Via del Giochetto, I-06126 Perugia, Italy. Tel: +39-075-5857463; Fax: +39-075-5857473; E-mail: plopcc@tin.it

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Abstract

Indoleamine 2,3-dioxygenase (IDO) is a tryptophan-catabolizing enzyme that, expressed by different cell types, has regulatory effects on T cells resulting from tryptophan depletion in specific local tissue microenvironments. Different mechanisms, however, might contribute to IDO-dependent immune regulation. We show here that tryptophan metabolites in the kynurenine pathway, such as 3-hydroxyanthranilic and quinolinic acids, will induce the selective apoptosis *in vitro* of murine thymocytes and of Th1 but not Th2 cells. T cell apoptosis was observed at relatively low concentrations of kynurenines, did not require Fas/Fas ligand interactions, and was associated with the activation of caspase-8 and the release of cytochrome *c* from mitochondria. When administered *in vivo*, the two kynurenines caused depletion of specific thymocyte subsets in a fashion qualitatively similar to dexamethasone. These data suggest that the selective deletion of T lymphocytes may be a major mechanism whereby tryptophan metabolism affects immunity under physiopathologic conditions.
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Keywords: apoptosis; tryptophan catabolism; kynurenines; T cells; Th1/Th2 cells

Abbreviations: AA, anthranilic acid; DC, dendritic cell(s); 3-HAA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenine; IDO, indoleamine 2,3-dioxygenase; KYN, L-kynurenine; PI, propidium iodide; QUIN, quinolinic acid.

Introduction

The discovery that inhibition of indoleamine 2,3-dioxygenase (IDO) activity reduces the survival of MHC-mismatched fetuses in mice,[1] and that the risk of fetal allograft rejection correlates with the degree of parental tissue incompatibility,[2] has led to the hypothesis that IDO activity protects fetal allografts from maternal T cell-mediated immunity. In the case of trophoblasts and macrophages, it has been proposed that these cells may veto T cell proliferation by removing tryptophan, the rarest essential amino acid, from local tissue microenvironments.[3,4] Our group has previously reported that murine dendritic cells (DC), in addition to macrophages, express high levels of IDO activity, which may account for the tolerogenic function of the CD8α^+ subset of splenic DC, particularly following activation by IFN-γ.[5,6] More recently, macrophages and DC were found to differ significantly in their patterns of IDO expression, with CD8$^+$ DC being highly effective in mediating IDO-dependent apoptosis of T cells *in vitro*.[7]

While inhibition of T cell proliferation by tryptophan depletion likely represents a critical component of the nonspecific immunosuppression mediated by macrophages and IDO,[3,4] other mechanisms, including T cell apoptosis by DC, could contribute to a general immunoregulatory role of the enzyme.[8] Macrophages and DC might have distinct *in vivo* functions, and expression of IDO by DC, and its modulation by cytokine signals, could be a common mechanism required for the maintenance of T cell homeostasis and self tolerance.[5,6,9]

Here we provide direct *in vitro* and *in vivo* evidence that tryptophan catabolites can induce apoptosis of thymocytes and terminally differentiated T helper cells. In particular, Th1 but not Th2 clones rapidly undergo cell death in the presence of kynurenine concentrations in the low micromolar range, through Fas-independent mechanisms involving the activation of caspase-8 and the release of cytochrome *c* from mitochondria. Because of such selective effects on Th1 cells, T cell apoptosis by tryptophan catabolism may not only be involved in the maintenance of T cell homeostasis and self tolerance, but may also contribute to certain disease states through an imbalance in the T helper response.

Results

Induction of apoptosis in thymocytes by 3-HAA

L-kynurenine (KYN), anthranilic acid (AA), 3-hydroxykynurenine (3-HK), 3-hydroxyantranylic acid (3-HAA), and quinolinic acid (QUIN) are L-tryptophan metabolites formed along the metabolic route known as the kynurenine pathway (Figure 1), in which IDO catalyzes the initial and rate-limiting step.[10–13] Among these kynurenines, QUIN, 3-HK, and to a lesser extent 3-HAA, represent potential neurotoxins that may cause neuronal death by necrosis and/or apoptosis at relatively elevated concentrations in the high micromolar range.[14–18] In addition, 3-HAA has recently been shown to induce apoptosis in monocyte/macrophage cell lines, according to a mechanism that presumably involves production of hydrogen peroxide.[19] Based on our previous observation that IDO-expressing murine DC will mediate apoptosis of T cells and that the effect is reversed by the addition of an IDO inhibitor,[5,6,9] we investigated the possible induction of T cell apoptosis by kynurenines. Preliminary experiments had

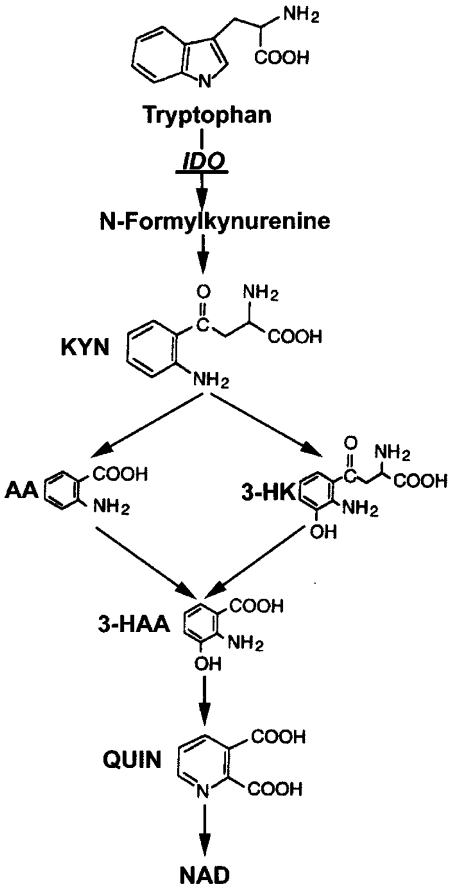

Figure 1 IDO-dependent tryptophan catabolism in the kynurenine metabolic pathway. AA, anthranilic acid; 3-HAA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenine; IDO, indoleamine 2,3-dioxygenase; KYN, L-kynurenine; QUIN, quinolinic acid

shown that DC exposed to physiological concentrations of tryptophan (i.e., 10–100 μM) will produce kynurenine levels in the 5–50 μM range, and that murine thymocytes, a cell type commonly used in studies of T cell apoptosis, would be highly susceptible to kynurenine-induced cell death. Thymocytes were thus incubated for 24 h with 10 μM 3-HAA. Apoptosis was demonstrated by intranucleosomal fragmentation resulting in a typical 'ladder' of 180-bp fragments and multiples thereof in agarose gel electrophoresis, according to a pattern qualitatively similar to that of 1 μM dexamethasone (Figure 2A). Apoptosis of 3-HAA-treated thymocytes was confirmed by typical condensation or fragmentation of cell nuclei, as revealed by acridine orange staining in fluorescence microscopy (Figure 2B), by electron microscopy (Figure 2C), and by the presence of subdiploid DNA peaks in the PI fluorescence assay (Figure 2D).

Induction of apoptosis by kynurenines in different cell types

A range of 3-HAA concentrations was tested, demonstrating that significant apoptosis of thymocytes was evident at 10 μM (Figure 3A), whereas apoptosis of macrophages required $>$10-fold higher concentrations (Figure 3A,B). Results similar to macrophages were obtained with splenic DC positively selected by the expression of CD11c (data not shown). Different kynurenines, namely KYN, AA, 3-HK, and QUIN, were tested in parallel with 3-HAA for ability to induce apoptosis of thymocytes, demonstrating that only QUIN, in addition to 3-HAA, would induce significant apoptosis at the concentration of 10 μM (Figure 3C). However, concentrations of 3-HK as high as 100 μM resulted in apoptosis of thymocytes (approximately 20% *vs* 13% of controls) that could be increased to over 40% (*vs* 20% of controls) by prolonging the exposure time from 24 to 72 h (data not shown).

Differential susceptibility of Th1 and Th2 clones to apoptosis induced by kynurenines

Th1 and Th2 cells can be differentially regulated to undergo apoptosis, suggesting that the rapid death of Th1 effectors leading to selective Th2 survival may provide a critical mechanism for differential regulation of the two subsets.[20,21] To investigate any possible difference in apoptosis mediated by kynurenines between Th1 and Th2 cells, we made use of well-polarized clonal effectors specific for OVA or the tumor/self antigen, P815AB. Addition of 10 μM 3-HAA or QUIN to antigen-stimulated T cells for 24 h resulted in significant DNA fragmentation of Th1 but not Th2 cells in the PI fluorescence assay (Figure 4).

Lack of Fas involvement in apoptosis induced by kynurenines

Differences in sensitivity to apoptosis of Th1 and Th2 cells have been attributed either to an intrinsic defect in the Fas signal transduction pathway in Th2 cells[20,22,23] or to the interception of this pathway by signals generated through CD3.[24] Augmented interaction of pre-existing cell surface Fas and Fas ligand might therefore contribute to the induction of apoptosis in Th1 cells by kynurenines. Although we failed to detect expression of Fas ligand on the latter cells treated or not with kynurenines (data not shown), we wanted to examine the possibility that interaction of Fas with undetectable levels of Fas ligand might play a role in the apoptosis of T cells by tryptophan metabolites. Therefore, we used Fas-deficient *lpr/lpr* mice for assessment of kynurenine-induced apoptosis in thymocytes. Cultures of thymocytes were established as described above and were incubated with 10 μM 3-HAA or QUIN for 24 h. Apoptosis was measured in the PI fluorescence assay. Figure 5 shows that no significant differences in the percentage of apoptotic cells were found in thymocyte cultures treated with either kynurenine. Therefore, the genetic deficiency of the Fas molecule would not impair the kynurenine apoptotic potential. As a corollary to this, it should be noted that the levels of apoptosis induced by kynurenines in C57BL/6 *lpr/lpr* and wild-type mice were higher than those observed in the DBA/2 strain. Although a systematic analysis of any strain-related differences in mouse susceptibility to kynurenine effects has not been undertaken yet, preliminary evidence does suggest the occurrence of reproducible variations among the different strains of mice tested so far.

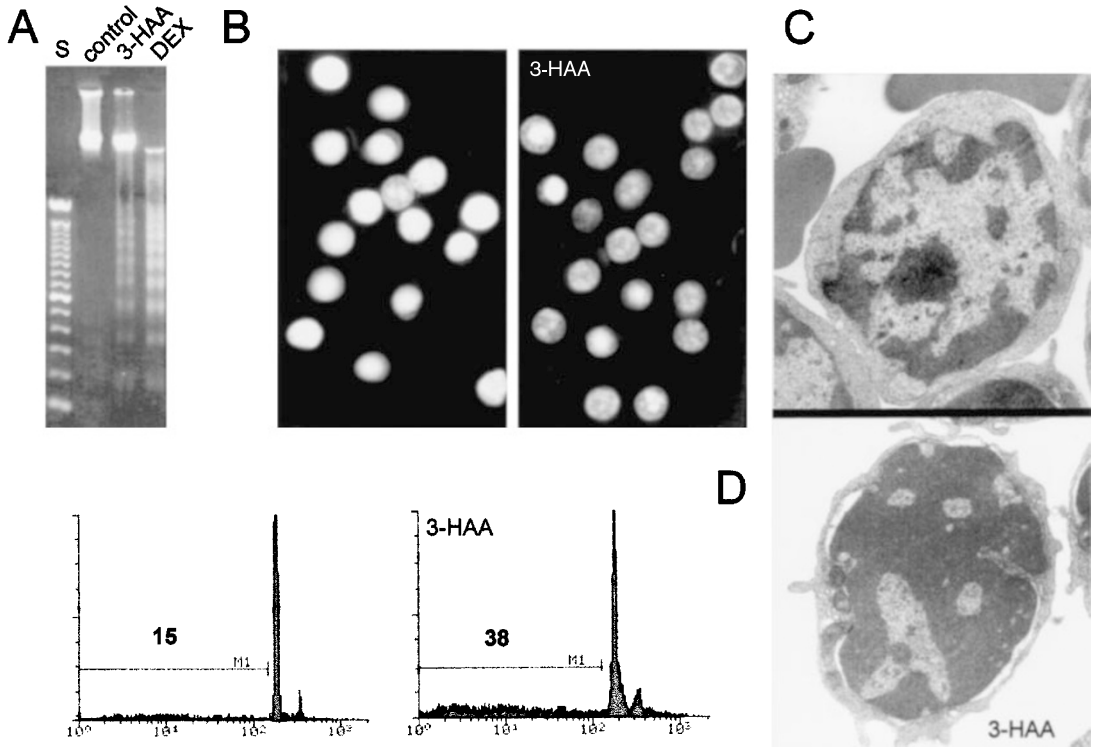

Figure 2 Induction of apoptosis in thymocytes by 3-HAA. Thymocytes were incubated for 24 h with medium or 10 μM 3-HAA. (**A**) DNA fragmentation was evaluated by electrophoresis on 1.7% agarose gel, controls including cells exposed to 1 μM dexamethasone (DEX). S, standard consisting of 100 base pair ladder. (**B** and **C**) Morphology of thymocytes after acridine orange staining and by electron microscopy, respectively, without 3-HAA or with 3-HAA (indicated). (**D**) Flow cytometric analysis of PI-stained nuclei using thymocytes either untreated or exposed to 3-HAA (indicated). Numbers above histograms indicate the percentages of apoptotic nuclei (broad apodiploid peak). Data are representative of several independent experiments

Effects of caspase inhibitors on kynurenine-induced T cell apoptosis

Although different apoptotic stimuli initially activate distinct pathways leading to cell death, these converge on a common execution mechanism involving the activation of a set of cysteine proteases, the caspases. Cleavage of a restricted set of cellular substrates then results in the demise of the cell.[25,26] The mechanisms of 3-HAA- and QUIN-induced T cell apoptosis and the possible involvement of specific caspases were analyzed using caspase inhibitors. We made use of Z-VAD, a generalized caspase inhibitor, and of selective inhibitors of caspase-8 and -9, the two major upstream caspases involved in extrinsic and intrinsic cell death signaling pathways, respectively (Figure 6). Complete inhibition of apoptosis of P815AB-specific Th1 cells was induced by the use of Z-VAD or the caspase-8 inhibitor. With 3-HAA as an inducer of apoptosis, marginal inhibition of cell death ($\sim$20%) was also observed using the caspase-9 inhibitor (P=0.002, presence *vs* absence of the inhibitor). Although this finding would suggest a minor role of caspase 9 in the apoptosis induced by HAA, the overall contribution of this caspase to kynurenine-induced cell death remains to be determined.

The involvement of caspase-8 in kynurenine-induced T cell apoptosis was further investigated by direct measurement of caspase activity (Figure 7). Thymocytes either untreated or treated for different times with QUIN were assayed for caspase-8/FLICE activity. Using anti-CD3 (2-C11) agonist mAb as a positive control, we found that significant induction of caspase-8 activity was observed following thymocyte treatment with the kynurenine. The effect appeared to be the greatest at 3–6 h with both the anti-CD3 mAb and QUIN.

Cytochrome *c* protein levels in mitochondria and cytosol of kynurenine-treated thymocytes

The release of cytochrome *c* is a requirement for initiating apoptosis in many different apoptotic models and microinjection experiments with cytochrome *c* have confirmed the important role of this mitochondrial protein in the initiation of the apoptotic pathway via activation of the caspase enzymes.[27,28] Protein extracts were prepared from the mitochondrial and cytosolic fractions of thymocytes treated with 3-HAA or QUIN, and equal amounts of protein from the two fractions were analyzed by Western immunoblotting with cytochrome *c*-specific mAb. Figure 8 depicts cytochrome *c* levels for each fraction at different times from exposure to either kynurenine. It is apparent that early after treatment, significant cytochrome *c* levels were present in the mitochondrial but not in the cytosolic fraction. However, the amount of cytochrome *c* detectable in the cytosol increased over time reaching a peak at about 5 h, whereas mitochondrial cytochrome *c* levels appeared to progressively decrease from 3 h post-treatment. One interesting observation in this experiment is that cytochrome *c* was no longer detectable at

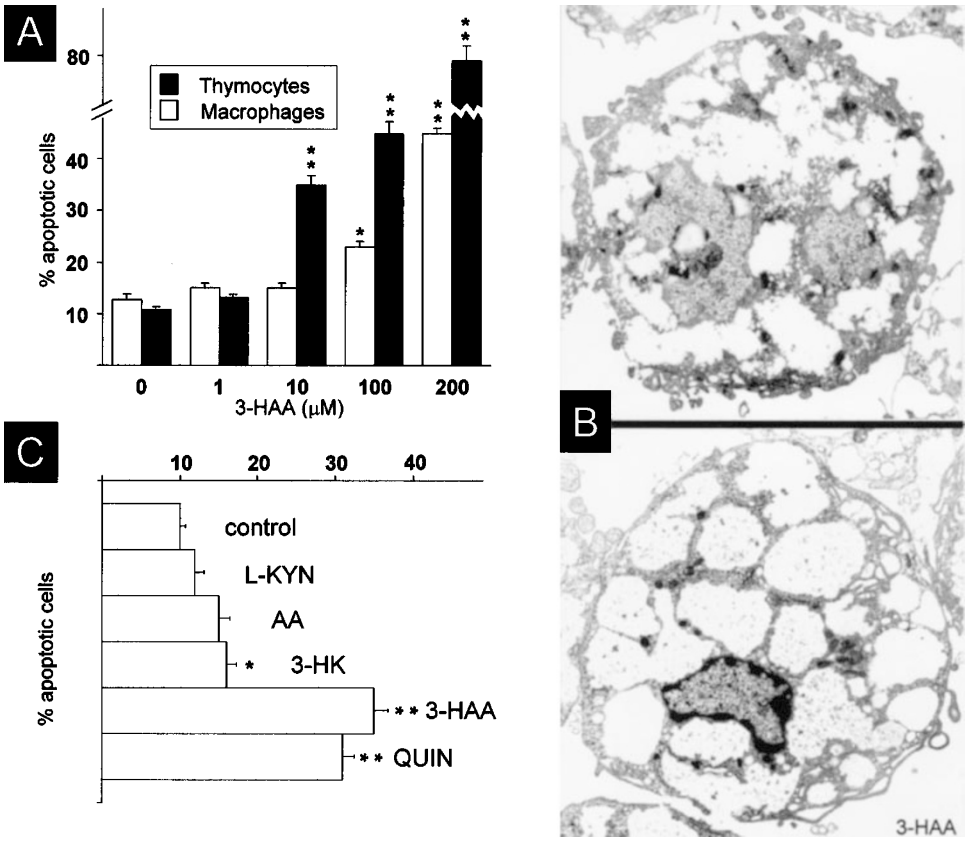

Figure 3 Induction of apoptosis by kynurenines in different cell types. (**A**) Thymocytes and macrophages were exposed for 24 h to a range of 3-HAA concentrations and the percentage of apoptotic cells was enumerated in a PI fluorescence assay. Values are the mean ± S.E. of three independent experiments. $^{*}P<0.01$ and $^{**}P<0.001$ (treated cells *vs* untreated). (**B**) Electron microscopy (× 7000) of macrophages treated for 24 h with medium or 200 μM 3-HAA (indicated). (**C**) Different kynurenines were assayed at 10 μM for induction of apoptosis in a PI fluorescence assay, control representing cells incubated with medium alone. Values are the mean ± S.E. of several independent experiments. $^{*}P<0.05$ and $^{**}P<0.001$ (kynurenine treatment *vs* control)

18 h in the cytosolic fraction, at a time when high levels of apoptosis were observed in the experiments above. This could reflect the occurrence of early, long-lived effects of cytochrome *c* release on caspase activation leading to apoptosis by kynurenines. However, it should also be noted that recent evidence indicates that cytochrome *c* is not required for all apoptotic pathways.[29]

Effects of kynurenine treatment on thymocytes *in vivo*

We also became interested in examining the effect of *in vivo* treatment with kynurenines on thymocytes. Mice were injected intraperitoneally with DEX (10 mg/kg), 3-HAA (20 mg/kg), or QUIN at 20 mg/kg, the latter dosage roughly corresponding to 1/50 of the mean lethal dose of the kynurenine.[30] Total yields of cells in the thymus and percentages of phenotypically distinct cell subsets were analyzed after 24 h (Figure 9). As expected,[31] the percentage of $CD4^{+}CD8^{+}$ immature thymocytes was significantly decreased after treatment with DEX. Remarkably, both 3-HAA and QUIN decreased the percentage of double-positive immature thymocytes to an extent similar to DEX, and comparable effects were also displayed by the three drugs on total yields of thymocytes recovered after treatment. Therefore, these data demonstrate that the two kynurenines display immunomodulatory activity *in vivo* which might reflect the induction of apoptosis *in vitro*.

Discussion

Although kynurenines have long been known to be potentially neurotoxic, the respective contributions of necrosis and apoptosis to neuronal death are unclear, as is the interplay between excitotoxic and apoptotic events.[14–18] For QUIN, an agonist of NMDA glutamate receptors, both necrosis[18] and NMDA receptor-triggered apoptotic cascades could play a role,[32] and signaling through this receptor might also occur in non-neuronal tissues.[33] For 3-HK and presumably other kynurenines, neuronal apoptosis likely depends on transporter-mediated cellular uptake and on the intracellular generation of oxidative stress.[16] In Th cell clones, we have previously demonstrated that IDO-dependent apoptosis follows incubation of activated T cells with tolerogenic DC exposed to IFN-γ,[5,6,9] implying a possible role for metabolic starvation[34] or for the generation of proapoptotic molecules by DC.

Inhibition of T cell proliferation by tryptophan catabolism protects the fetus from maternal immune responses.[1,2] The expression, however, of IDO activity by different cell types including macrophages and DC could have broader

immunological significance in tolerance and autoimmunity. Although inhibition of T cell proliferation by macrophage tryptophan catabolism may be pivotal in suppressing unwanted T cell responses under physiopathologic conditions,[3,4,35] the ability of murine DC in the CD8α^+ subset to mediate IDO-dependent apoptosis of T cells suggests that tryptophan metabolism may also act to regulate T cell homeostasis through the selective deletion of T cells.

We found that tryptophan metabolites in the kynurenine pathway, such as 3-HAA and QUIN, will induce the selective apoptosis *in vitro* of murine thymocytes and of Th1 but not Th2 cells. The effect could be demonstrated *in vitro* by different approaches, including intranucleosomal fragmentation in agarose gel electrophoresis, condensation or fragmentation of cell nuclei in fluorescence microscopy and electron microscopy, and the presence of subdiploid DNA peaks in a fluorescence assay. T cell apoptosis was independent of Fas/Fas ligand interaction and was associated with activation of caspase-8 and release of cytochrome *c* from mitochondria.

Caspases are currently considered as the central executioners of many, if not all, apoptotic pathways.[25,26] Caspases are synthesized as inactive proenzymes that are proteolytically processed to form an active tetrameric complex. The activation mechanism of caspases is mostly

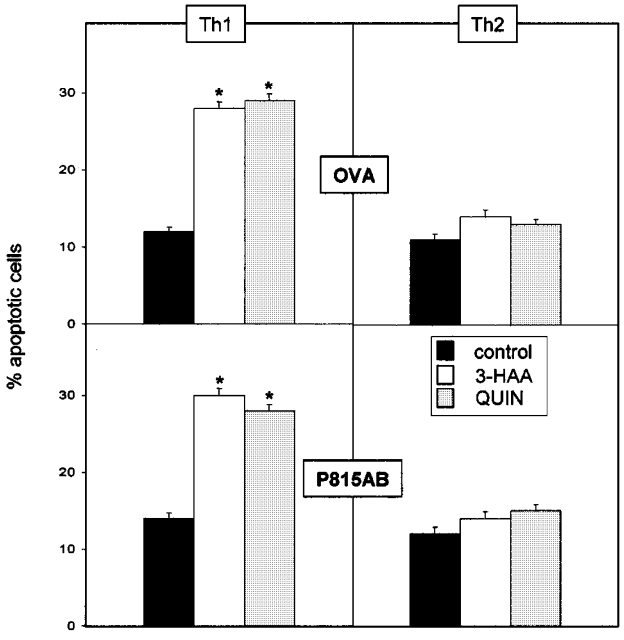

Figure 4 Differential susceptibility of Th1 and Th2 clones to apoptosis induced by kynurenines. OVA-specific and P815AB-specific Th1 and Th2 cell clones were exposed for 24 h to 10 μM 3-HAA or QUIN. Data are percentages of apoptotic cells in a PI fluorescence assay (mean $\pm$ S.E. of three independent experiments). *$P<0.001$ (kynurenine treatment *vs* control)

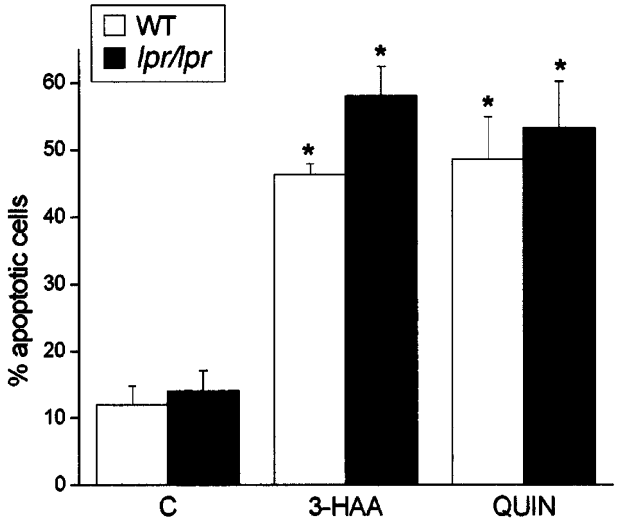

Figure 5 Induction of apoptosis by kynurenines in *lpr/lpr* mice. Thymocytes from C57BL/6 wild-type (WT) or *lpr/lpr* mice were incubated with medium (C) or 10 μM 3-HAA or QUIN. Flow cytometric analysis of PI-stained nuclei was performed at 24 h. Data are the means $\pm$ S.E. of three individual mice per group. One experiment of two. *$P<0.001$ (kynurenine treatments *vs* respective controls)

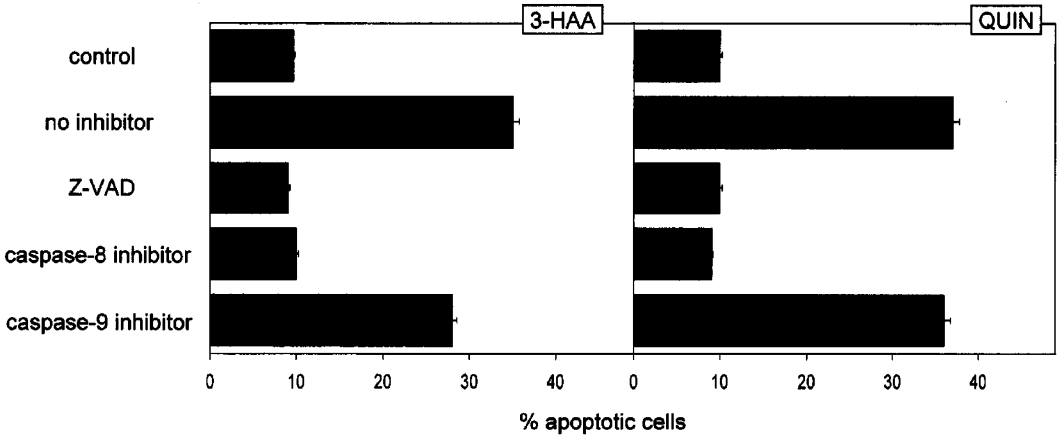

Figure 6 Effects of caspase inhibitors on kynurenine-induced T cell apoptosis. The P815AB-specific Th1 (F76) cells were pretreated with Z-VAD (100 μM), caspase-8 inhibitor (75 μM), or caspase-9 inhibitor (100 μM) for 1 h prior to the addition of medium alone (control) or medium containing 10 μM 3-HAA or QUIN. After an additional 24-h incubation, apoptosis was measured in a PI fluorescence assay. Data are means $\pm$ S.E. of three experiments

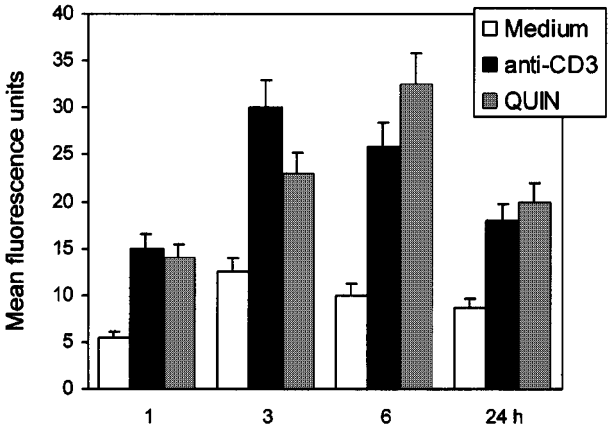

Figure 7 Induction of caspase-8 activity by QUIN. Thymocytes untreated or treated for different times with medium, anti-CD3 mAb, or QUIN were assayed for caspase-8/FLICE activity as described in Materials and Methods. Results are expressed as means $\pm$ S.E. of replicate samples. At all time points, values for anti-CD3 or QUIN treatments were significantly higher than those for control (medium) treatment ($P<0.01$). Data are representative of three independent experiments. The apoptosis percentages at 24 h were 7% for control cultures (medium), and 20% and 31% for anti-CD3 and QUIN treatments, respectively

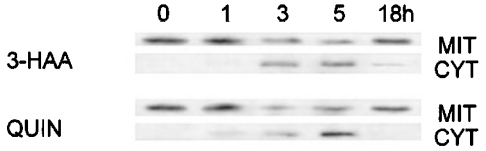

Figure 8 Relative levels of cytochrome *c* in the mitochondrial and cytosolic fractions of thymocytes treated with 3-HAA or QUIN. Mitochondrial and cytosolic extracts were prepared from thymocytes cultured for different times (indicated) with either kynurenine. Equal amounts of protein were processed for Western immunoblotting with anti-cytochrome *c* mAb, resulting in the detection of 15.3-kDa cytochrome *c* protein bands. Time 0 indicates control thymocytes with no treatment. Data are representative of three independent experiments

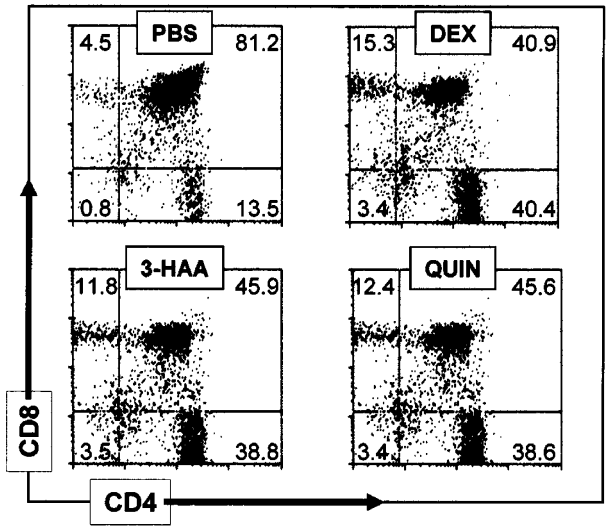

Figure 9 CD4/CD8 staining profiles of thymocytes from mice treated with kynurenines. Mice were injected intraperitoneally with 10–20 mg/kg of DEX, 3-HAA, or QUIN. Representative CD4/CD8 profiles of thymocytes from drug-treated or control, PBS-treated mice are shown. The percentages of cells present in each area are also indicated. The yields ($\times 10^6 \pm$ S.E.) of thymocytes in the different experimental groups (three mice per group) were as follows: PBS, 82 ± 4.1; DEX, 16 ± 2.6; 3-HAA, 20 ± 3.5; QUIN, 22 ± 4.6

unknown,[36–38] but it has been proposed that drug-induced apoptosis occurs through the Fas pathway.[39–41] Recent evidence, however, indicates that caspase-8 can be activated independently of Fas receptor/ligand interaction.[42–45] Using a variety of cytotoxic stimuli, mostly anticancer agents, it has been possible to demonstrate that the apoptosis of tumor cells induced by drugs or γ radiation does not require *de novo* synthesis of death ligands or Fas interaction and that caspase-8 can be activated in the absence of a death receptor signaling. In our experimental model with kynurenines and thymocytes, we obtained evidence that kynurenine treatment would not induce detectable expression of Fas ligand. More interestingly, thymocytes from Fas-deficient mice were as susceptible as the control cultures to kynurenine-induced apoptosis. Similar to the conditions of tumor cells treated with cytotoxic drugs, caspase-8 might play a crucial role in the regulation of kynurenine-induced apoptosis in thymocytes. Different mechanisms, largely controlled by the release of mitochondrial components, have been invoked to explain Fas-independent activation of caspase-8 and have been recently reviewed with considerable detail.[42,45]

Although the mechanism of activation remains obscure, QUIN did induce significant caspase-8/FLICE activation and functional caspase-8 activity appeared to be required for T cell apoptosis mediated by 3-HAA and QUIN. The sequence of activation of the caspases is believed to include the release of cytochrome *c* from mitochondria, which results in the activation of caspase-9 and subsequent activation of several downstream or 'effector' caspases.[46] We found that both 3-HAA and QUIN induced the release of cytochrome *c* from thymocyte mitochondria into the cytosol in a time-dependent fashion, with similar kinetics for the two kynurenines, possibly initiating long-lived effects on caspase activation.

We also attempted to assess the biological relevance of kynurenine-induced apoptosis under physiopathologic conditions. Following preliminary observations on the amounts of kynurenines produced *in vitro* by DC (i.e., concentrations in the 5–50 μM range), we performed experiments using kynurenines in the low micromolar range. *In vivo*, resorting to externally administered kynurenines to detect T cell apoptosis may not be completely informative, because endogenous tryptophan catabolites likely reach high physiological concentrations in specific local tissue microenvironments. Nevertheless, we observed similar effects on thymocytes using comparable doses of DEX and kynurenines administered to mice as single injections. In experiments not reported here, we also found that the same dosages of kynurenines lacked detectable effects on

mature peripheral T cells from the spleen or lymph nodes. Although we have not examined the effect of thymocyte activation on the ability of kynurenines to induce apoptosis, the data with terminally differentiated, antigen-activated T helper cell clones tend to suggest that both immature and activated T cells may be a major target for kynurenine-induced immune regulation.

Therefore, the current finding of Th1 cell apoptosis by tryptophan catabolites indicates that, in addition to generalized inhibition of proliferation, IDO-dependent tryptophan catabolism could contribute *in vivo* to the regulation of T cell responses via selective effects on specific lymphocyte subsets, according to mechanisms also at work in kynurenine-induced neuronal death. This phenomenon may have great biological significance because, at least for 3-HAA and QUIN, T cell apoptosis was observed at concentrations significantly lower than those required for neurotoxicity or for apoptosis of macrophages and DC.

The immunoregulatory role of tryptophan metabolites may be important for maintaining peripheral lymphocyte homeostasis and for minimizing the accumulation of autoreactive lymphocytes. However, antigen-induced deletion of Th cells could lead to an imbalance in Th1 and Th2 responses under pathologic conditions. Using clonal effectors specific for OVA or the tumor/self antigen, P815AB, we obtained evidence for differential susceptibility of Th1 and Th2 clones to apoptosis induced by kynurenines. Although unequal death of Th1 and Th2 cells has previously been proposed to contribute to certain disease states,[20,21] we present here a novel mechanism of how this phenomenon may occur.

There is a huge burst of IDO activity concomitant with the powerful immune response that accompanies injury and infection with many viruses including HIV, herpesvirus and poliovirus.[4,47] The biological significance of T cell apoptosis by tryptophan catabolism becomes, perhaps, most evident when considered in the context of local inflammatory reactions initiated by pathogens and dominated by IFN-γ. Under conditions in which production of IFN-γ and IDO induction represent an important antimicrobial mechanism,[13] DC may discriminate self from non-self and initiate cellular immune responses to the pathogen while preserving tolerance to self.[5–9] Apoptosis of potentially autoreactive lymphocytes by IDO-expressing DC activated by IFN-γ may represent a critical means of maintaining peripheral tolerance. Lymphocyte apoptosis, and the selection of Th2 responses, could also serve to downmodulate the extent of the local inflammatory reaction. However, unequal death of Th1 and Th2 cells might lead to immune deviation under pathologic conditions. In addition, the ability of an intracellular pathogen to inhibit IDO as an evasive strategy might result in deficiency of apoptosis and autoimmune disease.

In conclusion, this study provides a paradigm to further investigate how the many and apparently disparate activities of IDO, including inhibition of microbial infections, immunosuppression in pregnancy, production of metabolically active molecules and neurotoxins, could be expanded to accommodate a critical and perhaps unifying role in immunoregulation.

Materials and Methods

Cells

Thymocytes, from 4–6-week-old DBA/2 mice (Charles River, Calco, Milan, Italy), were enriched by passage through nylon wool columns. In selected experiments, C57BL/6-*lpr/lpr* mice were used that were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Plastic adherent peritoneal macrophages were obtained as previously described following the injection of endotoxin-free, 10% thioglycollate medium.[48] Viability was >95% and the cells consisted principally of macrophages (>99%), as demonstrated by microscopy and staining for nonspecific esterase. DC (>96% $CD11c^+$) were prepared from collagenase-treated spleens (collagenase type IV, Sigma Chemical Co., St. Louis, MO, USA) as previously described.[49] F76 (Th1-type) and F2 (Th2-type) T cell clones with specificity for the P815AB tumor peptide were previously obtained in our laboratory.[50] OVA-specific Th1-type and Th2-type T cell clones were obtained through the courtesy of Dr TF Gajewski, University of Chicago, IL, USA. To expand cell numbers, all T cell clones were maintained in IL-2, with biweekly stimulation of antigen and autologous APC. Experimental cultures for assessment of apoptosis were established at three days of restimulation.

Reagents and treatments

L-kynurenine (KYN), anthranilic acid (AA), 3-hydroxykynurenine (3-HK), 3-hydroxyantranylic acid (3-HAA), and quinolinic acid (QUIN) were purchased from Sigma Chemical Co. Cells were routinely incubated with 10 μM KYN, AA, 3-HK, 3-HAA, QUIN or 1 μM dexamethasone for 24 h in RPMI medium containing 10% FCS. Z-VAD, the caspase-8 inhibitor I, and the caspase-9 inhibitor I were purchased from Calbiochem (La Jolla, CA, USA), and used at concentrations of 75–100 μM. The inhibitors were present in the cultures starting from 1 h before the addition of kynurenines. For CD4/CD8 staining profiles of thymocytes from mice treated with kynurenines, PE-labeled anti-mouse CD8α and FITC-labeled anti-mouse CD4 (PharMingen, San Diego, CA, USA) were used.[50]

Apoptosis evaluation by propidium iodide staining

Apoptosis was measured by flow cytometry as described.[51] After culturing, cells were centrifuged, and the pellets were gently resuspended in 0.3 ml hypotonic propidium iodide solution (PI, 50 μg/ml in 0.1% sodium citrate plus 0.1% Triton X-100; Sigma Chemical Co.). The tubes were kept at 4°C in the dark for 1 h. The PI-fluorescence of individual nuclei was measured by flow cytometry with standard FACScan equipment (Becton Dickinson, Mountain View, CA, USA). The nuclei traversed the light beam of a 488-nm argon laser. A 560-nm dichroid mirror (DM 570) and a 600-nm band pass filter (band width 35 nm) were used to collect the red fluorescence caused by PI DNA staining, and the data were recorded in logarithmic scale in a Hewlett Packard (HP 9000, model 310; Palo Alto, CA, USA) computer. The percentage of apoptotic nuclei (subdiploid DNA peak in the DNA fluorescence histogram) was calculated with specific FACScan research software (Lysis II). In selected experiments, anti-CD3 mAb (2-C11, PharMingen) was used to induce thymocyte apoptosis by culturing cells overnight at 4°C in mAb-coated plates.

Agarose gel electrophoresis for DNA fragmentation

For analysis of DNA fragmentation, 1×10^7 cells were resuspended in 1.5 ml of lysis buffer containing 10 mM Tris-HCl, pH 7.5, 400 mM

NaCl, 2 mM EDTA, pH 8.2, 0.7% SDS, and 10 μg/ml proteinase K (Boehringer, Mannheim, Germany) and incubated at 55°C for 3 h. After centrifugation of lysates at 3300 *g* for 30 min, DNA in the supernatant was precipitated with 2.5 volumes of isopropanol and washed in 70% ethanol. The DNA pellet was dissolved in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.5 mg/ml RNase (Boehringer). After determination of the DNA content, 2 μg of DNA per lane were separated on 1.7% agarose gels containing ethidium bromide at 60 V for 2 h. The DNA fragmentation pattern was detected by UV transillumination.

Transmission electron microscopy and fluorescence microscopy

For transmission electron microscopy, cells were incubated with 3-HAA for 24 h. A total of 5×10^6 cells were pelleted at 1200 r.p.m. for 5 min, washed twice with PBS, and fixed in cold 2.5% glutaraldehyde in 0.1 M sodium cacodylate/1% sucrose buffer for 2 h. The cells were postfixed in 1% osmium tetroxide (50 min), encapsulated in 1% agar, stained with uranyl acetate and phosphotungstic acid, and dehydrated in a series of graded ethanolic solutions finishing with propylene oxide before finally being embedded in Epon 812-Araldite mixture. Ultrathin sections (50 nm) were cut on an ultramicrotome (LKB Wallac) and placed under 200-mesh standard copper grids, contrasted with uranyl acetate and lead citrate, and examined with a Philips TEM 400 transmission electron microscope. Apoptosis of thymocytes is shown at 15000 $\times$.

For fluorescence microscopy, cells were stained with 10 μg/ml acridine orange (Sigma Chemical Co.) in PBS and immediately examined with an Olympus BX20 fluorescence microscope.

Measurement of caspase-8 activity

Measurement of caspase-8 activity was performed using a Caspase-8/FLICE Fluorometric Protease Assay Kit according to the manufacturer's instructions (BioVision Research Products, Mountain View, CA, USA). Briefly, T cells (5×10^6) were lysed and, after centrifugation at 16 000 *g* for 20 min, the supernatants were collected and protein concentrations measured by BCA assay. Two hundred micrograms of protein in a 100-μl volume were mixed with an equal volume of 2 $\times$ reaction buffer and 5 μl of 4 mM IETD–AFC substrate (AFC: 7-amino-4-trifluoromethyl coumarin). The mixture was incubated at 37°C for 2 h and then read in a fluorometer.

Determination of cytochrome *c* release

The release of cytochrome *c* into the cytosol of thymocytes treated with kynurenines was measured as described elsewhere.[52] Briefly, 6×10^7 cells were washed in PBS and resuspended in 150 μl of ice-cold buffer containing 20 mM HEPES, 200 mM sucrose, 2 mM EDTA, 20 μg/ml PMSF, 2 μg/ml leupeptin, and 10 μg/ml aprotinin, pH 7.1. Cells were homogenized and centrifuged for 3 min at 3000 *g* to remove nuclei and unbroken cells. The supernatants were then centrifuged for 30 min at 12 000 *g* to isolate the mitochondrial fraction. Mitochondria were resuspended in 20 μl loading buffer whereas the concentrated supernatants (10 μl) were admixed with an equal volume of loading buffer. For Western blot detection of cytochrome *c*, the supernatants from the last centrifugation (fraction S100) and mitochondrial fractions were subjected to 12% SDS–PAGE. The proteins were then transferred onto a PVDF membrane (Millipore, Bedford, MA, USA) and membranes were blocked and incubated overnight at 4°C with cytochrome *c*-specific mAb (clone 7H8.2C12, PharMingen). Blots were extensively washed and developed with HRP-conjugated goat anti-mouse IgG Ab (1 : 2500). After washing, bands were visualized by ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Statistical analysis

Results are presented as mean values $\pm$ standard error (S.E.). Statistical analysis was performed by Student's *t*-test.

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