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T Cell Proliferation Induced by Autologous Non-T Cells Is a Response to Apoptotic Cells Processed by Dendritic Cells

Anna D. Chernysheva, Kyriakos A. Kirou, and Mary K. Crow

Self-reactive T cells are present in the mature immune repertoire as demonstrated by T cell proliferation induced by autologous non-T cells in the autologous mixed lymphocyte reaction. This reaction generates regulatory T cells in vitro and may reflect immune regulatory pathways in vivo, but the antigenic peptides recognized remain uncharacterized. We revisited this issue in light of the importance of apoptosis in immune regulation. We found that apoptosis among peripheral blood non-T stimulator cells is associated with augmented induction of autologous T cell proliferation. Our data show that caspase activity in the non-T stimulator population is essential for induction of autologous T cell proliferation, suggesting that cellular components in the non-T cell fraction are enzymatically modified, most likely by effector caspases, and have a direct or indirect effect on autoreactive T cell activation. Furthermore, exposure of macrophage-derived dendritic cells to apoptotic non-T cells augments autologous T cell proliferation, and blockade of α,β integrin, but not α,β integrin, inhibits the capacity of irradiated non-T cells or dendritic cells to stimulate autologous T cell proliferation. These experiments, using an entirely autologous system, suggest the interpretation that autoreactive T cells may recognize self-Ags modified through the actions of caspases and presented to T cells by dendritic cells. Induction of an in vivo autologous mixed lymphocyte reaction by caspase-modified self-Ags present in apoptotic cells may represent a mechanism to maintain peripheral immune tolerance. The Journal of Immunology, 2002, 169: 1241–1250.

The capacity of the immune system to respond to microbial pathogens in preference to self-Ags is highly dependent on the process of thymic selection, in which T cells with high affinity for peptides derived from self-Ags are deleted. However, the persistence in the T cell repertoire of cells with some reactivity with self-peptides, through presumably low affinity interactions between peptide-MHC class II complexes and TCRs, is well documented (1–7). In the case of patients with autoimmune disease, T cells specific for peptide components of the targeted tissue have been characterized. For example, in multiple sclerosis T cells with specificity for myelin-derived peptides contribute to CNS inflammation, and in systemic lupus erythematous T cells reactive with peptides derived from nucleosomes, small nuclear ribonucleoprotein particles, or ribosome particles circulate in peripheral blood and are associated with the presence of autoantibodies specific for components of those particles (3, 5, 7–15). Occasionally, healthy individuals have also been shown to have T cells with low reactivity with specific self-Ags (3–5, 16). In contrast to these examples of T cell responses directed toward characterized components of disease-targeted tissue, the basis of in vitro proliferation of T cells cultured with autologous APC is not understood (17, 18). In this autologous MLR (AMLR),3 low level T cell proliferation can generate effector cells that either suppress polyclonal Ab production or mediate autotolycytotoxic activity (19–23). The AMLR is MHC class II restricted (18, 23), but the antigenic peptides that are involved are not known.

A popular concept that has evolved in the context of autoreactivity involves cleaved fragments of self-Ags produced through the process of apoptosis. Some of the Ags targeted by lupus autoantibodies are clustered in the cell membrane blebs that form in cells undergoing apoptosis (24–27). It has been suggested that cryptic epitopes, revealed after enzymatic cleavage of those proteins by caspases or granzymes, might activate persisting self-reactive T cells and contribute to induction of autoimmune disease (26–28). However, no data have directly addressed the role of caspases in the activation of autoreactive T cells. To elucidate the cellular Ags that trigger autologous T cell proliferation in the AMLR and to gain insight into the relevance of that interaction to immunoregulation, we studied the role of apoptosis and caspase activity in the induction of T cell proliferation by autologous dendritic cells (DC).

Materials and Methods

T and non-T cell isolation and cell culture

PBMC were obtained from heparinized blood of healthy volunteers by Ficoll-Hypaque (Pharmacia, Peapack, NJ) centrifugation after approval of the research protocol by the Hospital for Special Surgery Institutional Review Board. PBMC T and non-T cell fractions were isolated using a magnetic bead separation system (Pan T Cell Isolation kit; Miltenyi Biotec, Auburn, CA). Cells were incubated at 37°C, 5% CO2, in culture medium containing RPMI 1640 (Life Technologies, Gaithersburg, MD), 10% heat-inactivated autologous human serum, 2 mM L-glutamine, and 50 U/ml penicillin and streptomycin (Life Technologies).

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked \author{Anna D. Chernysheva, Kyriakos A. Kirou, and Mary K. Crow} \affil{Mary Kirkland Center for Lupus Research, Hospital for Special Surgery, Weill Medical College and Graduate School of Medical Sciences of Cornell University, New York, NY 10021} \email{crowm@hss.edu} \textsuperscript{1}This work was supported by research grants from the National Institutes of Health (R01AI42185 and R01AI45011 to M.K.C.), the SLE Foundation, Inc. (to M.K.C. and K.A.K.), and the Arthritis Foundation (to M.K.C.). \textsuperscript{2}Address correspondence and reprint requests to Dr. Mary K. Crow, Hospital for Special Surgery, 335 East 70th Street, New York, NY 10021. E-mail address: crowm@hss.edu

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Caspar inhibitors (CI)

The broad spectrum CI N-benzylxoycarbonyl Cbz-Val-Ala-Asp fluoromethylketone (Z-VAD.fmk, targeting caspase-1, -3, -4, and -7) was purchased from Calbiochem (San Diego, CA). Additional CI were used in some experiments: N-benzylxoycarbonyl Cbz-Asp-Glu-Val-Asp fluoromethylketone (Z-DEVD.fmk, predominantly targeting caspase-3 and -7, as well as caspase-8, -9, and -10); N-benzylxoycarbonyl Cbz-D-Leu-Glu-Val-Asp fluoromethylketone (Z-IETD.fmk, predominantly targeting caspase-8 and granzyme B, as well as caspase-6 and -10), and N-acetyl-Tyr-Val-Ala-Asp aldehyde (Ac-TYVAD.cho, targeting caspase-1 and -4) were obtained from Calbiochem. The designated selectivities of the CI are derived from Calbiochem and BIOMOL (www.biomol.com) catalogs. CI were used at a 25 μM concentration unless otherwise indicated. All inhibitors were kept as stock solutions of 20 mM in DMSO and diluted 1/10 (2 mM) in culture medium before addition to cell cultures. The final concentration of DMSO in the cultures was ≤0.5%.

Induction of apoptosis

Non-T cells were gamma-irradiated with 250–4600 rad from a cesium source (intensity, 770 rad/min) and assayed for apoptosis or used as stimulators in AMLR culture. Alternatively, non-T cells were incubated for 24 h at 37°C and 5% CO₂ with staurosporine (0.5 μg/ml) for induction of apoptosis or with staurosporine (0.5 μg/ml) for 16 h for use as stimulators in AMLR. The lower concentration of staurosporine was used in AMLR cultures because higher concentrations proved toxic to T cells in those cultures. In some experiments, apoptosis of Jurkat T cells was induced by incubation with anti-Fas Ab (0.5 μg/ml; clone CH11; Upstate Biotechnol., Lake Placid, NY) for 3 h.

Determination of apoptosis

Non-T cell apoptosis was assessed by cell cycle analysis and poly(ADP-ribose) polymerase (PARP) cleavage flow cytometric analysis. For cell cycle analysis, cells were washed, alcohol fixed, stained with 50 μg/ml propidium iodide (PI), and analyzed using a FACSscan (BD Biosciences, San Diego, CA) flow cytometer for DNA content, as previously described (29). The percentage of mononuclear cells containing subdiploid DNA reflects the proportion of apoptotic cells. In the PARP cleavage assay, cells were fixed in 1% formaldehyde, washed, fixed in alcohol, permeabilized in 0.25% Triton, and stained with polyclonal rabbit anti-PARP p89 Ab (anti-PARP-85 fragment; Promega, Madison, WI), followed by secondary fluorescein-conjugated anti-rabbit Ig Ab (DAKO, Carpinteria, CA) (30). Cells were washed, and 20 μg/ml PI were added to the samples 20 min before analysis using a FACSscan flow cytometer.

T cell proliferation assays

The proliferative response of T lymphocytes cultured with autologous non-T cells (AMLR) was evaluated by [³H]thymidine incorporation. Non-T stimulator cells were untreated or induced to undergo apoptosis by gamma irradiation or treatment with staurosporine and then cocultured at a 1:1 or 2:1 ratio with 1 × 10⁵ autologous responder T cells in culture medium containing 10% autologous heat-inactivated human serum in round-bottom 96-well microtiter plates. [³H]Thymidine (1 μCi/well) was added to each well at day 5. After 16 h, cells were harvested, and DNA-associated radioactivity was counted by liquid scintillation (MicroBeta TRILUX; Wallac, Turku, Finland) and expressed as a mean cpm ± SEM of quadruplicate cultures. In some experiments, the non-T cells were cultured with 50 μg/ml mAb to α₃β₇ or α₅β₂ integrin (Chemicon International, Temecula, CA) or with control anti-TNP mAb for 1 h before induction of apoptosis and washed from the cell preparation before initiation of the AMLR culture.

The proliferative response of T cells to 50 ng/ml phorbol dibutyrate (PDB; Sigma, St. Louis, MO) and 500 ng/ml ionomycin (Sigma) was assessed after 5 days of culture by addition of [³H]thymidine. The proliferative response of T cells to the soluble Ag tetanus toxoid (TT) was assessed after 5 days by addition of [³H]thymidine to the cultures of T cells with irradiated (1000 rad) autologous non-T cells premixed for 16 h with 0.5 or 10 μg/ml TT (Massachusetts Department of Public Health Biological Laboratories, Jamaica Plain, MA).

Generation and use of DC as stimulators of AMLR

DC were generated from the T cell-depleted fraction of PBMC by culturing non-T cells at 0.67 × 10⁶/ml in six-well plates for 6–7 days in the presence of GM-CSF (Sigma), IL-4 (Sigma), and 1% autologous plasma. GM-CSF (30 ng/ml) and IL-4 (20 ng/ml) were added to the cultures on days 0, 2, and 4. At days 6–7, the cells had typical DC morphology and expressed CD83, HLA-DR, and α₅β₂ integrin. An aliquot of non-T cells, autologous to those

used to generate DC, was incubated for 24 h untreated or after gamma irradiation with 1000 rad and then added at a 1:1 ratio to the non-T cells exposed to GM-CSF and IL-4 for the prior 24 h. In some experiments, mAbs to α₃β₇ or α₅β₂ integrin at 10 μg/ml were added to DC induction cultures, together with the nonirradiated or gamma-irradiated non-T cells, 24 h after the initiation of culture. At days 6–7, DC were collected, washed, and used as stimulators in AMLR at various stimulator-responder cell ratios. T cell proliferation was assessed by [³H]thymidine incorporation at day 5.

Fluorescence flow cytometric analysis

The phenotype of cells in non-T or DC cultures was assessed on day 0, 24 h after induction of apoptosis, or at day 6 or 7 of culture with DC-inducing cytokines. Cells were reacted for 30 min on ice with mAb specific for CD83 (Immunootech, Marseille, France), HLA-DR, CD14, CD19 (BD Pharmingen, San Diego, CA), or α₅β₂ (Chemicon) and then analyzed using a FACSscan flow cytometer. The initial non-T cell preparation contained 27.8%, 24.3%, and 2.1% CD19, CD14, and CD83⁺ cells, respectively.

Results

Induction of non-T cell apoptosis by gamma irradiation and staurosporine

Among the well-described triggers of apoptotic cell death are ionizing radiation and inhibition of protein kinases by agents such as

[FIGURE 1. Induction of non-T cell apoptosis by gamma irradiation and staurosporine. Peripheral blood non-T cells were gamma irradiated with 250–4600 rad and assessed for apoptosis by PI cell cycle analysis after 24 h of culture. A, Mean percentage of cells expressing subdiploid DNA, ± SEM, based on data from four experiments. B, To assess apoptosis by cleavage of PARP, non-T cells were gamma irradiated with 500, 1000, or 2000 rad; after 20 h of culture, the cells were fixed, permeabilized, and stained with polyclonal rabbit anti-PARP p89 Ab, followed by secondary fluorescein-conjugated anti-rabbit Ig. The mean percentage of cells expressing the p89 cleavage fragment of PARP from four experiments is shown. C, Non-T cells were cultured for 24 h with the indicated concentrations of staurosporine and apoptosis assessed by PI cell cycle analysis. Mean data from six experiments are shown.]
staurosporine. To determine the experimental conditions that induce apoptosis of human peripheral blood non-T cells, including B cells, monocytes, and DC, PBMC were negatively depleted of T cells and exposed to varying doses of gamma irradiation. After 24 h of culture, the non-T cells were assessed using two measures of apoptosis, quantitation of cells containing subdiploid DNA by PI cell cycle analysis and detection of cells expressing the p89-kDa caspase cleavage fragment of PARP. Both assays reflect the coordinate action of a series of caspase enzymes. As shown in Fig. 1A, gamma irradiation increased the percentage of non-T cells containing subdiploid DNA from a baseline level of 9.1% to a maximum of 30.5% after 1000 rad. Induction of apoptosis by gamma irradiation showed a dose-related effect to the maximum at 1000 rad, followed by a dose-related decrease, such that 4600 rad induced no apoptosis over the baseline level, consistent with a previous report (31). These results were confirmed by FACS analysis of the percentage of non-T cells containing the p89 caspase cleavage fragment of PARP, again demonstrating maximum PARP cleavage after 1000 rad of gamma irradiation, with a 5-fold increase in the percentage of p89-positive cells observed (Figs. 1B and 2). Immunofluorescence analysis of CD14, CD19, and CD83 expression to detect monocytes, B lymphocytes, and DC, respectively, along with PI incorporation, 24 h after gamma irradiation showed that B cells were preferentially affected, compared with monocytes and DC, by 1000 rad gamma irradiation, consistent with a prior report (32). Of non-T cells expressing the B cell lineage marker CD19, 22.2% incorporated PI after gamma irradiation, indicating cell death, whereas only 3.3% of the untreated CD19 non-T cells incorporated PI (data not shown).

Similar experiments were performed to document the effective dose of staurosporine for induction of non-T cell apoptosis. Incubation of non-T cells for 24 h with staurosporine at a concentration of 0.5 or 1.0 g/ml increased the proportion of non-T cells containing subdiploid DNA from a baseline level of 5.6% to 13.7% and 43.5% respectively (Fig. 1C). These data were confirmed by induction of the PARP p89 cleavage fragment (data not shown).

Augmented T cell proliferation induced by gamma-irradiated or staurosporine-treated autologous non-T cells

We hypothesized that induction of apoptosis among non-T cells might modify self-proteins such that they become available for effective presentation to autologous T cells, resulting in T cell activation and proliferation. To investigate this possibility, T cells
were isolated from PBMC using a magnetic bead separation system that depletes non-T lymphoid populations, avoiding exposure of T cells to stimuli with activating potential, such as SRBC, activating Abs, or FCS. Non-T stimulator cells were induced to undergo apoptosis by gamma irradiation or treatment with staurosporine and cocultured with autologous T cells in autologous serum-supplemented medium. Treatment of stimulator cells with 250-4600 rad demonstrated a dose-related increase in AMLR-stimulatory capacity, concurrent with increased apoptosis, with 800-1000 rad inducing maximum apoptosis and optimal augmentation of T cell proliferation (Fig. 3A). Similarly, preculture of non-T cells with 0.5 μg/ml staurosporine for 16 h induced a 4-fold increase in proliferation of T cells cultured at a 1:2 ratio with autologous non-T cells (Fig. 3B). Data in some systems have suggested that apoptotic cells trigger maturation of APC, as assessed by increased expression of MHC class II or costimulatory molecules (33, 34). However, immunofluorescence analysis of cell surface MHC class II expression showed no increase in the irradiated non-T cell population (mean channel fluorescence, 689), containing increased apoptotic cells, compared with the untreated non-T cells (mean channel fluorescence, 675) after 24 h culture. Thus, induction of apoptosis in the non-T cell stimulator population, by either gamma irradiation or staurosporine, increased the capacity of those cells to induce autologous T cell proliferation in the absence of xenogenic proteins and without apparent maturation of potential stimulator cells among the non-T cells.

Abrogation of gamma irradiation-induced non-T cell apoptosis and AMLR stimulation by caspase inhibitors

Induction of apoptosis among cells of the AMLR non-T cell stimulator population might modify self-Ags or nonspecifically augment Ag presenting function of surviving cells. Among several possible mechanisms, caspase-mediated cleavage of self-proteins might expose cryptic epitopes on self-proteins or promote Ag processing, inducing activation and proliferation of circulating T cells with autoantigen-specific TCRs. To investigate the possibility that apoptosis increases induction of autologous T cell proliferation through effects of caspase enzymes, non-T cells were incubated with CI (25 μM) for 1 h before gamma irradiation and then cultured for 24 h, and the percentage of apoptotic cells was determined by PI cell cycle analysis (histograms shown in Fig. 4) or PARP cleavage (histograms shown in Fig. 2). Z-VAD.fmk, which inhibits a broad spectrum of caspases including caspase-3 and -7, inhibited gamma irradiation-mediated non-T cell apoptosis by 74% (Fig. 4A). Z-IETD.fmk (relatively selective for caspase-6, -8, and -10 and granzyme B) and Z-DEVD.fmk (relatively selective for caspase-3 and -7) blocked 55 and 49% of gamma irradiation-mediated apoptosis, respectively, while the caspase-1 (IL-1-converting enzyme-like protease) inhibitor Ac-YVAD.cho was not effective (Fig. 5A). Although the CI are only relatively specific for the designated enzymes, the data are consistent with previous studies of gamma irradiation-induced apoptosis in other cell types and suggest that the effector caspase-3 or -7, as well as caspase-6, -8, or -10, mediate gamma irradiation-induced non-T cell apoptosis under these culture conditions (35).

To investigate a role for caspases in the augmented AMLR stimulatory capacity of gamma-irradiated non-T cells, those cells were incubated with CI for 1 h before gamma irradiation, cultured for 16 h, washed, and placed in AMLR cultures (Fig. 5B). Z-VAD.fmk inhibited the induction of AMLR T cell proliferation by 92%, and Z-IETD.fmk and Z-DEVD.fmk were also effective, decreasing the magnitude of the AMLR response by 63% and 61%, respectively. In contrast, Ac-YVAD.cho, the caspase-1 inhibitor, did not substantially affect proliferation. In separate experiments, Z-VAD.fmk did not impair viability of monocyte-derived CD83⁺ DC, ruling out loss of effective APC as an explanation for the decreased stimulation of AMLR (data not shown).

A dose-response analysis of the effect of the CI on non-T cell apoptosis and stimulation of the AMLR response confirmed the efficacy of Z-VAD.fmk, but not Ac-YVAD.cho, at concentrations as low as 6.25 μM (Fig. 5, C and D). A modest inhibition

**FIGURE 3.** Induction of apoptosis among non-T cells is associated with increased AMLR stimulatory capacity. A, Peripheral blood non-T cells were gamma irradiated with 250 to 4600 rad, and both were assessed for the percentage of subdiploid DNA by cell cycle analysis (■) or used as stimulators in AMLR (□). Results are expressed as a percentage of the optimal result (observed with 800 rad in this experiment). B, Non-T cell apoptosis was induced with 1000 rad or 0.5 μg/ml staurosporine, and those cells were used as stimulators in AMLRs at a 1:1 (■) or 2:1 (□) ratio of stimulator to responder cells (S:R). Mean cpm ± SEM of 11 experiments for gamma irradiation treatment and six experiments for staurosporine treatment are shown.

**FIGURE 4.** Abrogation of gamma irradiation-induced non-T cell apoptosis by caspase inhibitors. Cytofluorograph histograms demonstrate PI cell cycle analysis of non-T cells treated with 25 μM CI before 1000 rad gamma irradiation. The percentage of cells expressing subdiploid DNA (in the M1 region) after 24 h of culture is indicated. The experiment is representative of seven performed.
of the T cell proliferative response, but not apoptosis, was observed after treatment of gamma-irradiated non-T cells with high doses of Ac-YVAD.cho. Caspase-1 acts on IL-1 to generate immunostimulatory IL-1β, and inhibition of that effect might account for the observed decrease in AMLR at 50 and 100 μM Ac-YVAD.cho.

Abrogation of staurosporine-induced non-T cell apoptosis and AMLR stimulation by CI

To assess the effect of caspase inhibition on the augmented AMLR induced by staurosporine-treated non-T cells, similar experiments were performed. As was noted with the gamma-irradiated non-T cells, the broad spectrum CI Z-VAD.fmk, as well as Z-DEVD.fmk, markedly inhibited both non-T cell apoptosis (Fig. 6A) and T cell proliferation induced by staurosporine-treated non-T cells (Fig. 6B). Preculture of non-T cells with the caspase-1 inhibitor Ac-YVAD.cho did not reproducibly inhibit AMLR stimulatory capacity at a 1:1 stimulator-responder cell ratio. Taken together, the data described thus far suggest that induction of apoptosis among the non-T stimulator population is accompanied by caspase-dependent modifications in components of that population that are recognized by cocultured autologous T cells. It is likely that several caspases are involved in the apoptotic events that confer non-T cell stimulatory capacity. Although the CI used are broadly active, the data are consistent with an important role for effector caspases, including caspase-3, -6, or -7, in AMLR stimulation.

Treatments of responder T cells with caspase inhibitors does not significantly inhibit the AMLR induced by gamma irradiation- or staurosporine-modified non-T cells or the T cell response to polyclonal activators

In view of recent data indicating that caspases may be required for optimum T cell proliferation induced by mitogens or anti-CD3 Ab stimulation (36, 37), we considered the possibility that the inhibition of AMLR activity after preincubation of non-T cells with CI might be attributable to persistent CI in the AMLR culture and direct inhibition of T cells. Experiments parallel to those just described were performed in which the T cell fraction, rather than the non-T cell fraction, was preincubated with CI for 1 h and the CI was washed from the cells before culture in the AMLR. As demonstrated in Fig. 7A, although Z-VAD.fmk had a modest inhibitory effect on T cell proliferation stimulated by gamma-irradiated non-T cells in the AMLR (29% inhibition with 1:1 T-non-T cell ratio and 39.2% inhibition with a 2:1 non-T-T cell ratio), the other CI had no effect, and none of the CI substantially modified the T cell response stimulated by staurosporine-treated non-T cells (Fig. 7B). To investigate
whether CI have an inhibitory effect on T cell proliferation triggered by polyclonal activators, T cells were incubated with Z-VAD.fmk or Ac-YVAD.clo and stimulated with PDB and ionomycin. At the concentration (25 μM) used, neither CI had an effect on [3H]thymidine incorporation in this assay (Fig. 7C).

It appeared unlikely, then, that the CI were acting directly on the responder T cells to inhibit the proliferative response to either autologous non-T cells or polyclonal activators.

**FIGURE 6.** Abrogation of staurosporine (ST)-induced non-T cell apoptosis and AMLR stimulation by caspase inhibitors. Peripheral blood non-T cells were preincubated with 25 μM CI for 1 h before culture with 0.5 μg/ml staurosporine. After 16 h, the non-T cells were washed and assessed for apoptosis by PI cell cycle analysis (A; mean of three experiments) and for AMLR stimulatory capacity by [3H]thymidine incorporation (B; mean of nine experiments). Non-T cells were placed in culture with autologous T cells at a 1:1 (●) or 2:1 (○) stimulator-responder cell ratio (S:R). Mean [3H]thymidine incorporation of three experiments is shown.

**FIGURE 7.** Caspase activity is not required for the T cell response to autologous non-T stimulator cells or mitogens, or for non-T cell presentation of optimal concentrations of soluble Ag. A and B, T cells were preincubated with 25 μM CI for 1 h, washed, and then cultured with autologous non-T cells gamma irradiated (XR) with 1000 rad (A) or treated with 0.5 μg/ml staurosporine (ST) (B) at a 1:1 (●) or 2:1 (○) stimulator-responder cell ratio (S:R). Mean [3H]thymidine incorporation data from six experiments for gamma irradiation treatment and from three experiments for staurosporine treatment are shown. C, T cells were incubated with PDB and ionomycin (Iono), along with 25 μM CI, for the duration of the culture. Incorporation of [3H]thymidine was measured on day 5 of culture. The mean cpm ± SEM of three experiments is shown. D, Non-T cells were precultured for 1 h with 25 μM CI before gamma irradiation with 1000 rad and addition of TT at 0, 0.5, or 10 μg/ml for an additional 16 h. After two washes, AMLR cocultures were established with autologous T cells (2:1 stimulator-responder cell ratio). T cell proliferation for each condition was compared with the corresponding value for non-T cells preincubated without CI pretreatment and expressed as a percentage. Mean [3H]thymidine incorporation cpm values in the samples without CI were 3,701 (AMLR), 24,689 (TT 10 μg/ml), and 8,370 (TT 0.5 μg/ml). Means and error bars (SEM) were derived from five experiments for the AMLR and TT 10 μg/ml conditions and from three experiments for 0.5 μg/ml.
Caspase inhibitors do not abrogate the capacity of non-T cells to present optimal concentrations of soluble Ag to T cells

In the next set of experiments, the capacity of gamma-irradiated non-T cells to stimulate Ag-specific T cell proliferation was studied using TT. None of the CI used, including Z-DEVD.fmk, Z- IETD.fmk, and Ac-YVAD.cho, substantially modified the capacity of gamma-irradiated non-T cells to present an optimal concentration (10 μg/ml) of TT to autologous T cells (Fig. 7D), whereas in the same cultures, the CI abrogated non-T cell induction of autologous T cell proliferation in the absence of TT. Moreover, gamma irradiation was not required for effective presentation of TT by non-T cells (data not shown). When TT was used at 0.5 μg/ml, generating T cell proliferation with a stimulation index of 2 compared with the AMLR, that low level response was abrogated by the CI. These results suggest that caspase enzymes and apoptosis may play important roles in the induction of the AMLR, and perhaps in responses to low concentrations of Ag, although not being required for Ag-induced T cell proliferation stimulated by optimal concentrations of soluble Ag.

DC mediate the augmented AMLR induced by apoptotic non-T cells

One mechanism that could account for the described observations would involve the ingestion of apoptotic debris by viable APC remaining among the gamma-irradiated or staurosporine-treated non-T cells, with subsequent presentation of components of self-Ag to autologous T cells. To investigate a role for uptake of cellular debris in AMLR activation, non-T cells were preincubated with Ab to the αβ2 integrin, present on monocytes and macrophages, Ab to the αβ3 integrin, expressed on DC, or isotype control Ab. The non-T cells were then gamma irradiated, the Abs were washed away, and the non-T cells were used in AMLR cultures. As demonstrated in Fig. 8, the Ab specific for αβ3, but not the anti-αβ2 or control Abs, inhibited induction of T cell proliferation in the AMLR. To directly test the role of DC in the presentation of apoptotic material to autologous T cells, macrophage-derived DCs were generated in vitro with GM-CSF and IL-4. At 24 h after the initiation of the DC induction cultures, autologous non-T cells that had been maintained at 37°C either untreated or after gamma irradiation with 1000 rad were added to the differentiating and maturing DCs to permit uptake of those cells. After 6–7 days of culture, the macrophage-derived DC were CD14 negative and expressed the typical phenotype of DC, with CD83, HLA-DR, and αβ5 integrin present on a high proportion of the cell population (Fig. 9A). DC derived in the presence of gamma-irradiated apoptotic non-T cells were superior to DC exposed to nonirradiated non-T cells or DC alone in their capacity to stimulate proliferation of autologous T cells, even when used at a 0.2:1 stimulator-responder cell ratio, conditions in which the number of fresh gamma-irradiated non-T cells is not sufficient to stimulate T cell proliferation (Fig. 9B and C). Moreover, 10 μg/ml anti-αβ5, but not anti-αβ2, Ab, when added to differentiating DC together with gamma-irradiated autologous non-T cells, abrogated the increased capacity of these DC to stimulate autologous T cells. Taken together, these data, generated in an entirely autologous system, support an important role for DC in the uptake and presentation of caspase-modified self-Ags to T cells.

Discussion

The data reported here elucidate the requirements for activation of T cells by autologous non-T cells in the in vitro AMLR and have implications for understanding similar cellular interactions in vivo. We demonstrated that induction of autologous T cell proliferation requires apoptotic events among the non-T stimulator cell population. Both gamma irradiation and staurosporine effectively induced both non-T cell apoptosis and augmented AMLR stimulatory capacity, and the dose response for these two effects was similar. Doses of 800–1000 rad gamma irradiation were optimal for inducing both apoptosis and T cell activation, with higher doses of irradiation proving less efficacious. As expected, caspases were required for non-T cell apoptosis, but remarkably, induction of autologous T cell proliferation was also dependent on non-T cell caspase activity. In addition, T cell proliferation induced by low concentrations of the soluble Ag TT was also sensitive to treatment of Ag-pulsed non-T cells with CI. Although the CI used in this study are only relatively selective for members of the inducer and effector caspase families, the capacity of Z-IETD.fmk and Z-DEVD.fmk to inhibit the induction of T cell proliferation by autologous non-T cells suggests a role for the effector caspases, caspase-3, -6, and -7, in this function (38, 39). An inhibitor of caspase-1 had no effect on apoptosis or AMLR. Multiple caspases are involved in the cellular pathways that mediate apoptosis triggered by various stimuli, including death receptors, gamma irradiation, and chemical apoptosis, such as that induced by staurosporine (39). These experiments were not designed to definitively identify the caspases that are required for AMLR stimulation, but the data strongly support a role for activation of at least some of the effector caspases in modifying self-components among the non-T preparation, and possibly foreign soluble Ags, such that they acquire the capacity to induce autologous T cell proliferation.

Recent publications have focused on potential activities for caspases beyond their well-documented role in mediating programmed cell death. Caspase activity in T cells was implicated in the proliferative response of those cells to mitogens and anti-CD3 mAbs (36, 37). In those experiments, T and non-T cell populations were not compared for effects of CI, and in one of the studies (36), the inhibitors were routinely used at 100 μM. The data presented here indicate that a broad spectrum CI, Z-VAD.fmk, inhibits non-T cell-stimulatory capacity at concentrations as low as 6.25 μM, and more selective CI used at the relatively low 25 μM dose also act directly on the non-T cell population. These CI had at most a modest direct effect on the capacity of T cells to proliferate in response to autologous non-T cells, mitogens, or optimal concentrations of soluble Ag. There are persuasive data in the literature...
indicating that caspase activation within T cells is associated with T lymphocyte activation (36, 37, 40), but we contend that those caspases are not required for T cell proliferation, and in the AMLR the major site of caspase action contributing to T cell proliferation is in the non-T stimulator population.

These experiments do not define the stimulatory epitopes recognized by T cells in the AMLR, but the clear link of non-T cell apoptosis and caspase activity to T cell proliferation suggests that cellular components present in the non-T cell fraction are enzymatically modified such that innocuous cells are rendered stimulatory to cocultured T cells. Enzymatic cleavage of a large number of self-proteins is mediated by caspases, as well as by granzymes, in the setting of apoptosis (26, 27). PARP cleavage was documented in our experiments, but many other proteins are likely to be fragmented, potentially exposing previously cryptic epitopes. Other modifications might also be mediated by caspase activity, resulting in induction of T cell activation. Apoptosis might modulate self-Ags by increasing APC acidity or prolonging residence of self-proteins in the Ag-loading compartments (41–43). Release of reactive oxygen species in the course of apoptosis might biochemically modify cell proteins and support a form of polyclonal T cell activation that was first reported several decades ago using sodium periodate (44–45). Newly phosphorylated proteins might be recognized, or exposure of normally cryptic membrane lipids on apoptotic cells might provide a stimulus for T cell activation based on presentation of lipid moieties on nonclassical MHC molecules (44, 46).

Because the AMLR has been documented to be predominantly an MHC class II-restricted response of CD4+ T cells (17, 18, 23), it seems most likely that the relevant caspase-mediated modification would involve generation of MHC class II-associated peptides. T cells reactive with those peptides and capable of a low magnitude proliferative response are proposed to be present in the AMLR culture. An additional interpretation of these data is that caspase-dependent generation of apoptotic cells induces maturation or altered function of APC, such as DC. Previous reports showed induction of MHC class II and CD86 expression on DC incubated with apoptotic cells at a 5:1 ratio or with CD40 ligand+ apoptotic cells (33–34). However, other studies indicate that ingestion of apoptotic cells by DC inhibits the maturation of those cells (47, 48). In our experiments, MHC class II expression on non-T cells was not increased by irradiation-induced apoptosis, and the apoptotic cells were unlikely to have achieved a 5:1 ratio with DC or to have been enriched in CD40 ligand (because they were non-T cells).

A role for DC in the induction of autologous T cell proliferation is supported by the data presented here, along with substantial data in the literature (49–53). Recent work has characterized the role of immature DC in phagocytosing and processing components of apoptotic cells. Once matured, the DC present Ags derived from the apoptotic cells to CD8+ T cells, inducing activation, proliferation, and effector function, a phenomenon termed cross-priming (54–57). DC use CD36, αβ integrin, and perhaps other cell surface
receptors to bind and internalize apoptotic cells, and peptides derived from those cells are then presumably expressed with MHC molecules on the DC surface for presentation to T cells (58–60). However, in contrast to the present report, in those studies the source of apoptotic cells was not autologous to the responding T cell. An important role for DC in the uptake and presentation of autologous apoptotic material to T cells is demonstrated by our data and is supported by studies in the murine and rat systems (52, 61). An Ab that blocked the DC surface integrin, αβ2, implicated in the uptake by DC of apoptotic cellular debris (52, 59), strongly inhibited AMLR stimulation. In contrast, an Ab that blocked αβ3 integrin, typically expressed on monocyte-macrophages, had no effect on AMLR stimulation. The capacity of DC to present autologous apoptotic material to T cells was directly demonstrated using macrophage-derived DC matured in the presence of gamma-irradiated non-T cells. These results suggest a scenario in which DC engulf autologous cells undergoing caspase-mediated apoptosis and present peptides derived from that cell debris or modified by the actions of caspases to autoreactive T cells. We propose that the AMLR reflects such a scenario and that a similar set of events may routinely occur in vivo.

Abundant data from earlier studies indicate that the CD4+ T cells activated in the AMLR generate suppressor activity, equivalent to that of the T-regulatory cells that have recently garnered attention (2, 19, 23, 62). Additional study will be required to relate the T cells activated by caspase-modified autologous non-T cells to the proposed phenotype of T-regulatory cells. If apoptotic cells provide a stimulus for activation of autologous T cells, including T-regulatory cells, a question arises regarding the fate of T cells that interact with the abundant caspase-cleaved self-proteins encountered in the thymus during T cell repertoire development. Although it is likely that many high affinity self-peptide reactive thymocytes are deleted, some thymic CD4+ T cells may be sensitized to peptides derived from caspase-cleaved self-proteins and survive to become part of the peripheral T cell compartment. Once in the periphery, those cells would have the capacity to become activated, undergo proliferation, and differentiate to become regulatory/suppressor cells when re-exposed to their cognate peptide, stimulated to respond to autologous mixed lymphocyte culture. J. Immunol. 123:1889.

In summary, these experiments identify an essential role for caspase activity and apoptosis in the capacity of DC to induce the proliferation of autologous T cells. The activation and low magnitude expansion of AMLR T cells may reflect a mechanism designed to maintain immune homeostasis in a microenvironment in which modified-self frequently arises.

References


