Rescuing Melanoma Epitope-Specific Cytolytic T Lymphocytes from Activation-Induced Cell Death, by SP600125, an Inhibitor of JNK: Implications in Cancer Immunotherapy

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Rescuing Melanoma Epitope-Specific Cytolytic T Lymphocytes from Activation-Induced Cell Death, by SP600125, an Inhibitor of JNK: Implications in Cancer Immunotherapy

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Activation-induced cell death (AICD) as well as programmed cell death (PCD) serve to control the expansion of activated T cells to limit untoward side effects of continued effector responses by T cells and to maintain homeostasis. AICD of T cells in tumor immunotherapy can be counterproductive particularly if the activated T cells undergo apoptotic death after the very first secondary encounter of the specific epitope. We examined the extent to which tumor epitope-specific CTLs that are activated and expanded in an in vitro-matured dendritic cell-based primary stimulation protocol undergo AICD following their first secondary encounter of the cognate epitope. Using the MART-127–35 epitope as a prototype vaccine epitope, we also examined whether these CTLs could be rescued from AICD. Our results demonstrate that a substantial fraction of MART-127–35 epitope-specific primary CTLs undergo AICD upon the very first secondary encounter of the cognate epitope. The AICD in these CTLs is neither caspase dependent nor is it triggered by the extrinsic death signaling pathways (Fas, TNFR, etc.). These CTLs, interestingly, could be rescued from AICD by the JNK inhibitor, SP600125. We also found that SP600125 interferes with their IFN-γ response but does not block their cytolytic function. The rescued CTLs, however, regain their capacity to synthesize IFN-γ if continued in culture without the inhibitor. These observations have implications in tumor immunotherapy and in further studies for regulation of AICD in CTLs.


Programmed cell death (PCD) and activation-induced cell death (AICD) in T cells are important physiologic processes that prevent untoward side effects of a continued and uncontrolled T cell-mediated effector response as well as maintain homeostasis (1–3). Both processes involve apoptotic deletion of T cells. PCD entails the deletion of the expanded T cell population usually during the contraction phase of the response. AICD, in contrast, involves the apoptotic deletion of a significant fraction of the activated population after an effector response. In AICD, the effector function and the death are, paradoxically, triggered by TCR-driven signaling (i.e., activation induced). In any event, both processes (i.e., PCD and AICD) are designed to serve useful purposes by limiting the expansion of activated T cells. AICD in activated T cells in tumor immunotherapy, however, can be counterproductive particularly if the activated T cells undergo apoptotic death after the very first encounter of the specific epitope.

Lately, much interest has been generated in cancer vaccine therapy with specific peptides, proprotein Ags, DNA, etc. Most of these immunogens are “self” Ag (4), yet many cancer patients as well as normal healthy hosts harbor precursor CTLs for such “self” epitopes. Ex vivo stimulation of T cells as well as in vivo immunization with such self peptides or tumor-associated Ags (TAA) lead to the activation and expansion of the Ag-specific CTLs (5, 6). These TAA-specific T cells are also susceptible to AICD. To our knowledge, neither the extent of AICD in these self-but-TAA reactive primary CTLs nor the feasibility of rescuing them from AICD has been carefully examined. We have recently shown that Melan-A/MART-127–35 epitope-specific CTLs expanded in an in vitro dendritic cell (DC)-based stimulation protocol undergo apoptotic death on repetitive stimulation by immature as well as by fully activated DCs (7). Using the Melan-A/MART-127–35 epitope (8, 9) as a prototype self but melanoma-associated Ag, we studied the extent of AICD in MART-127–35 epitope-specific primary CTLs and examined whether it could be prevented. In this study, we show that a large fraction of MART-127–35 epitope-specific CTLs indeed undergo AICD upon the very first secondary encounter of the cognate epitope. The AICD in these CTLs is neither caspase dependent nor is it triggered by the engagement of extrinsic death receptors. We also show that the JNK inhibitor, SP600125, can rescue a significant fraction of them from death. In the process of rescuing, the JNK inhibitor interferes with their capacity to produce IFN-γ but does not interfere with their cytolytic function. Of further interest, the rescued MART-127–35 epitope-specific CTLs, when continued in culture in IL-15 and without the inhibitor, regain capacity to synthesize IFN-γ. These observations, therefore, have implications in cancer immunotherapeutic strategies and in further studies of AICD in CTLs.

Materials and Methods

Study population

The study population consisted of HLA-A2-positive melanoma patients or healthy donors. The participants were included in this study with informed consent.
Culture medium and reagents

The MART-127–35 peptide (AAAGIGILTV) and MAGE-3271–279 (FLWGPRALV) were purchased from Multiple Peptide Systems (San Diego, CA) while β2-microglobulin was purchased from Sigma-Aldrich (St. Louis, MO). Culture medium consisted of IMDM (Invitrogen Life Technologies, Grand Island, NY) supplemented with 10% FBS (Gemini Bioproducts, Calabasas, CA), 0.55 mM t-arginine, 0.24 mM t-asparagine (both from Invitrogen Life Technologies), 1.5 mM t-glutamine (Sigma-Aldrich), 50 U/ml penicillin, and 50 μg/ml streptomycin (both from Abbott Laboratories, North Chicago, IL). This will be referred to as complete media (CM). The TAP-deficient line, T2, was a gift of P. Cresswell (Yale University, New Haven, CT). Recombinant human GM-CSF was purchased from Immunex (Seattle, WA). Recombinant human IL-12, IL-18, rhIFN-γ was purchased from R&D Systems (Minneapolis, MN). LPS from Escherichia coli 055:B5 was purchased from Sigma-Aldrich. An Annexin V kit to track the early apoptotic cells for exposure of phosphatidylserine was purchased from BD Pharmingen (San Jose, CA). MART-127–35 (EAGIGILTV) tetramer labeled with PE with and without FITC-labeled anti-CD8 was purchased from R&D Systems.

Generation of DCs from peripheral blood monocyte

The procedure for generating myeloid DCs from peripheral blood monocyte was followed. Briefly, circulating monocyte were isolated by 2 h adherence of Ficoll-Hypaque density gradient-cut PBMC as previously described (7). The adherent cells were cultured in CM with 1000 U/ml GM-CSF and 500 U/ml IL-4 for 3–5 days to obtain a population of immature DCs. Maturation of immature DCs was done by first priming in IFN-γ (1000 U/ml) for 2 h and then priming in the presence of 100 ng/ml LPS.

IFN-γ response assay

IFN-γ response assay for the effector cells has been described previously (10). Briefly, the effector cells were cocultured with the peptide-pulsed (1 μg/ml) T2 cells. After 4–16 h, culture supernatants were harvested and IFN-γ was measured by ELISA as per manufacturer’s protocol (R&D Systems).

FACS analysis

The procedure for phenotypic analyses and for determining the number of epitope-specific cells with tetramer staining by flow cytometry has been described (7). To determine tetramer and annexin V-positive cells, the effector cells were stained with CD8, MART-127–35/HLA-A2 tetramer, and annexin V. The stained cells were then analyzed for single-positive vs double-positive populations in flow cytometry using a FACSCalibur and CellQuest software (BD Biosciences).

Microcytotoxicity assay

The chromium release microcytotoxicity assay has been previously described (11).

Activation of CD8+ T cells by DC-based presentation of epitopes, in vitro

The basic procedure for peptide-loaded DC-based in vitro activation and expansion of epitope-specific CD8+ T cells has been described (7). Briefly, Ficoll-Hypaque gradient separated blood mononuclear cells were purified for CD8+ T cells ( Routinely exceeding 90%) by a Dynal magnetic bead isolation kit (Dynal, Oslo, Norway) and cocultured with autologous DCs pulsed with relevant peptides (100 μg/ml) and 5 μg/ml β2-microglobulin at a CD8+ T cell to DC ratio of 100. For our current purpose, because IL-2 has been known to facilitate apoptosis in activated T cells (12), the cocultures were conducted in the presence of rhIL-15 (10 ng/ml). Before setting up cocultures, the DCs were irradiated to 3000 rad. The activated CTLs were also maintained in culture in IL-15.

Assay for AICD induction

To test whether or not the epitope-specific primary CTLs undergo AICD, the activated CTLs were exposed to peptide (1 μg/ml) loaded T2 cells (E:T = 100) at different time points of the cultures. Thereafter, the evidence of apoptosis was determined by flow cytometry with triple color staining (CD8, MART-127–35/HLA-A2 tetramer, and annexin V) at different time points (4–18 h). Experiments were conducted in triplicate wells and significance was calculated by one-way ANOVA using Sigma Stat statistical software (Chicago, IL).

To evaluate the effect of various agents in modulating AICD, the CTLs were preincubated with various compounds at optimal concentration for 45 min at 37°C and then exposed to T2 cells alone or loaded with peptide. The optimal dose used in the experiments shown in this paper was determined by using these compounds at different concentrations in the preliminary experiments (data not shown).

Results

We selected donors (healthy donors as well as melanoma patients) who harbor MART-1-27–35 epitope-specific CTLs in relatively high frequencies and whose CTL precursors could be easily activated (7). Because IL-2 has been implicated in apoptosis in activated T cells (12), we looked for an alternative cytokine support in the in vitro CTL generation protocol. We found that rhIL-15 supports the activation and expansion of the epitope-specific CTLs in the mature DC-based epitope presentation system. IL-15 not only supports the primary activation process, but also supports survival of the activated T cells in an Ag-independent manner (13). IL-15 receptor is not down-regulated after T cell activation (14) and IL-15 has an important role in T cell memory generation, as such, in T cell survival (15). Therefore, we conducted all our experiments in the presence of IL-15. The MART-1-27–35 epitope-specific CTLs were readily activated and expanded when they were stimulated by peptide-pulsed matured DCs in the presence of IL-15, in vitro. Fig. 1A shows an example of the expansion of the MART-1-27-35 epitope-specific CTLs—derived from a normal healthy individual and a melanoma patient. The expanded MART-1-27–35 epitope-specific cells also exhibited IFN-γ response in an epitope-specific manner (Fig. 1B).

The fate of the MART-1-27–35 epitope-specific CTLs upon secondary encounter of the cognate epitope (i.e., whether following effector function, they survive or die?) was then examined. The CTLs and the peptide-pulsed T2 cells were cocultured in CM without any cytokine and 4 h later the evidence of early death was examined by annexin V staining. As shown in Fig. 2, although the CTLs were fully functional in IFN-γ assay (Fig. 2A), a large fraction of these activated CTLs became annexin V positive at 4 h (Fig. 2B). When the cocultures were continued in IL-15 for 5 days and the number of epitope-specific CTLs was counted, only 20–30% of the starting population could be recovered from the coculture of the CTLs with the cognate target. An example for loss of the epitope-specific CTLs following effector function is shown in Fig. 2C. Thus, ~50% of the Ag-specific population showed early evidence of death at 4 h (Fig. 2B) and a much larger fraction eventually died following secondary encounter of the cognate epitope for the very first time.

We then examined the mechanism of AICD on these CTLs and also examined whether these CTLs could be rescued from AICD by interfering with the death signaling pathway(s). We examined the effect of interfering with the external death signal receptors (such as Fas, TNFR, TRAIL, etc.) on AICD. We also examined whether any of the MAPK inhibitors could block the AICD in these CTLs because MAPKs have been implicated in the negative selection of developing thymocytes (16, 17). As shown in Fig. 3A, the death of these CTLs was not caspase dependent, as the pan
caspase inhibitor, z-VAD-fmk did not prevent apoptosis. The apoptosis was not also affected by the blockade of the common extrinsic death signal receptors such as Fas, TNFR, or TRAIL. However, we found that while the p38 and ERK inhibitors had no effect, the JNK inhibitor, SP600125, rescued a significant fraction of the CTLs from AICD. Interestingly, SP600125 also inhibited the IFN-γ response by these CTLs (Fig. 3B) but did not affect their cytotoxic function (Fig. 3C) suggesting that the cytolytic machinery and the IFN-γ response pathways are differently regulated in these CTLs. Induction of AICD upon secondary exposure to the cognate epitope in the primary CTLs and the protective effect of SP600125 from AICD were observed in CTLs generated from two
normal donors and two melanoma patients (collective data not shown).

The caspase-independent death in these primary CTLs upon TCR engagement was confirmed as poly(ADP-ribose) polymerase (PARP) was found to be uncleaved (Fig. 4A). Of note, PARP was cleaved in staurosporine-treated Jurkat cells and z-VAD-fmk blocked the PARP cleavage. We also confirmed that SP600125 abrogates c-jun activation (Fig. 4B).

We examined the status of the mitochondrial membrane potential in the CTLs during AICD. As shown in Fig. 5A, the CTLs surprisingly exhibited hyperpolarization of the mitochondrial transmembrane potential (Δψm) (18, 19) while the apoptotic Jurkat

![FIGURE 3](image1)

FIGURE 3. Effect of external death receptor blockade and MAPK inhibitors on AICD in the epitope-specific CTLs upon secondary encounter of the cognate epitope. The CTLs were preincubated at optimal concentration for 45 min at 37°C with pan caspase inhibitor -z-VAD-fmk (100 μM); human Fas/Fc chimera, human TNF-R1/Fc chimera, human TRAIL-R1/Fc chimera, human TRAIL-R1I/Fc chimera, and human IFN-γRI/Fc chimeric proteins (10 μg/ml); p38 kinase inhibitor SB203580, JNK inhibitor SP600125 JNK, ERK inhibitor PD98059 (25 μM). Then the pretreated as well as untreated CTLs were incubated with T2 cells either alone or loaded with peptide. Four hours after secondary exposure, cells were stained for determining A, the number of tetramer+ cells of the CD8 T cells (top panels) and the number of tetramer+/annexin V− and tetramer+/annexin V+ populations (bottom panels). The numbers on the top margin of the bottom panels represent tetramer+/annexin− from the mean ± SE of three replicate samples while superscript letters a, b, c, and d indicate that the increase in the number of tetramer+/annexin V− population in a, when compared with that in b, c, and d, was significant with p < 0.001 (one-way ANOVA). B, Effect of the MAPK inhibitors on IFN-γ response by the effector cells. (M3 = MAGE-3271–279, M1 = MART-127–33). The reduction in IFN-γ synthesis in a, when compared with b, c, and d, was significant at p ≤ 0.001 by one-way ANOVA. M3 = MAGE-3271–279, M1 = MART-127–33. C, Effect of the MAPK inhibitor on cytotoxic response by the effector cells. (M3 = MAGE-3271–279, M1 = MART-127–33). The difference in the percent-specific lysis in the MART-1 peptide-loaded T2 cells was significant (*, p ≤ 0.001 by Student’s t test) only when compared with that of MAGE-3271–279 peptide-loaded T2 cells at all E:T ratios (ET). A represents one of four separate experiments while B and C represent one of two separate experiments.

![FIGURE 4](image2)

FIGURE 4. Evidence for a caspase-independent pathway for apoptosis in MART-1-specific primary CTLs. A, The CTLs were preincubated at optimal concentration for 45 min at 37°C with p38 kinase inhibitor SB203580 and JNK inhibitor SP600125 (25 μM). The pretreated as well as untreated CTLs were then incubated with T2 cells either alone or loaded with peptide. Six hours after secondary exposure cells were used for Western blot analysis for PARP cleavage (top panel). Jurkat T cells either preincubated with pan caspase inhibitor -z-VAD-fmk (100 μM) or untreated were exposed with staurosporine (1 μM) to establish the PARP cleavage alongside. B, Jurkat T cells were stimulated with PMA (10 ng/ml)/ionomycin (0.5 μM) for the indicated time both in the presence and absence of JNK inhibitor SP600125 JNK (25 μM). Immunoprecipitation was done to ascertain the blockade of c-jun phosphorylation/activation in the presence of SP600125, as seen in the blot.
cells exhibited hypopolarization of the mitochondrial membrane. Hyperpolarization has been associated with the apoptotic process in peripheral blood lymphocytes and has also been shown to occur independently from activation of caspases (20, 21). Further, hyperpolarization of mitochondrial membrane potential has been shown to be a reversible stage in the mitochondrial death decision process while hypopolarization usually represents a point of irreversible commitment to death (18, 19). Interestingly, SP600125 (JNK inhibitor) as well as SB203580 (p38 inhibitor) decreased the level of hyperpolarization induced by the restimulation with the cognate peptide (data not shown) suggesting that SP600125-mediated rescue is not specifically mediated through the modulation of mitochondrial membrane potential.

The status of Bcl family pro- and antiapoptotic proteins and costimulation through certain TCRs (CD28, 4-1BB, OX-40, etc.) influence the survival of activated T cells (22). As such, we examined the expression levels of the anti- and proapoptotic Bcl family proteins and these receptors in the CTLs in a condition that induces AICD. As shown in Fig. 5B, while Bcl-2 and Bcl-xL were nonselectively up-regulated by SB203580 and SP600125, only phosphorylated Bcl-2 and Mcl-1 were selectively up-regulated by the JNK inhibitor SP600125. Bim—a pro-apoptotic protein—was markedly up-regulated (or released from sequestered sites) by the CTLs undergoing AICD. Of interest, the CTLs up-regulated 4-1BB (10- to 20-fold increase of the geometric mean intensity of fluorescence) and CD25 (3- to 6-fold increase of the geometric mean intensity of fluorescence) upon secondary encounter of the Ag (Fig. 5C).

Finally, we examined the functional status of SP600125-rescued CTLs upon continuous culture. After overnight exposure of the CTLs to the cognate epitope in the presence or absence of SP600125, the CTLs were washed and then maintained in continuous culture in IL-15 containing CM without the inhibitor. Five days after, the number of viable MART-127–35 tetramer-positive cells were determined and their function was tested in the IFN-γ response assay. Fig. 6A shows that only a small fraction of the starting population could be recovered from the cognate epitope-exposed culture in the absence of the JNK inhibitor. A larger fraction of the starting epitope-specific population was recovered from the coculture that was started in the presence of the JNK inhibitor, SP600125. Remarkably, the rescued CTLs regained their capacity to synthesize IFN-γ (Fig. 6B).
in apoptosis and has also been shown to interfere with IFN-γ response but not with cytotoxicity in CTLs (32, 33). A connection between 4-1BB-mediated signaling and activation of the JNK pathway has also been suggested (34). Second, the MART-127–35 epitope-specific CTLs generated in the in vitro DC-based epitope presentation protocol in the presence of IL-15 and maintained in IL-15 were just as susceptible to AICD as the CTLs that were generated and maintained in IL-2 (collective data not shown). Third, the results clearly reveal that SP600125 is capable of protecting these CTLs from AICD (Fig. 3). Because SP600125 functions as an inhibitor of the JNK, the data suggest a role for JNK in the apoptotic process. Fourth, while SP600125 negatively regulates the IFN-γ responsiveness of the CTLs, it does not affect their cytotoxic function, suggesting that the two functional pathways in CTLs are differently regulated. Finally, and most importantly, the rescued CTLs regained IFN-γ responsiveness in continued culture after the inhibitor being washed off.

The JNK is a member of the MAPK family of signaling proteins. JNK has been associated with a variety of biological processes—most notably in inflammation and transformation (35). All three MAPKs (i.e., p38, JNK, and ERK) have been found to have different roles in T cell biology such as in proliferation, effector function, survival as well as death (16, 17, 36–40). Different isoforms of JNK have been found to have divergent roles in CD4+ and CD8+ T cells (17). For example, JNK1 is needed for the expression of IL-2Rs (CD25) upon activation and for proliferation. As such, jnk-1-null mice exhibit marked reduction in the expansion of Ag-driven CD8+ T cells (39), JNK2 down-regulates IL-2 production in CD8+ T cells (39, 40). JNK has also been implicated in negative selection of thymocytes (17, 40). Both JNK1 and JNK2 have been found to have stage-dependent roles in T cell development. For example, while JNK1 has been associated with anti-CD3-induced apoptosis of double-positive thymocytes, jnk2-null double-positive thymocytes are resistant to anti-CD3-induced apoptosis although they are sensitive to apoptosis induced by dexamethasone, anti-Fas Ab, or UV radiation (38, 39). Thus, immature thymocytes seem to need one form of JNK or another for undergoing receptor-driven apoptosis but mature T cells need
factors such as AP-1 and other proteins some of which are associated with apoptosis. Given that death in CD8+ T cells is turning out to be mostly driven by the internal pathway of apoptosis and results from mitochondrial dysfunction, the release of reactive oxygen intermediates, and stress (29, 30), and as JNK can play a role in stress-induced activation of the cytochrome c-mediated death pathway (41), a role for JNK in AICD in Ag-specific CD8+ CTLs can be envisioned.

As mentioned earlier, Bcl family pro- and antiapoptotic proteins play an important role in the survival or death of cells (22). Our observation on the modulation of some of these proteins, therefore, is of interest. The JNK inhibitor, SP600125, increased the level of pBcl-2 and Mcl-1. The effect of phosphorylated Bcl-2 on apoptosis of T cells remains an unsettled issue (42, 43). However, Mcl-1 is an important Bcl family member with a positive effect on T cell survival (44). It is therefore tempting to suggest that the JNK inhibitor SP600125 prevents AICD in these CTLs by up-regulating Mcl-1. Admittedly, more work will be needed to establish this issue.

A role for JNK in AICD in CTLs in an Ag-specific manner in our study has been based from the observation of the effect of SP600125 on AICD in these cells (Fig. 3). SP600125 has been extensively used as a JNK inhibitor in numerous studies although it is not absolutely specific for JNK (35). The possibility exists that the rescuing effect of SP600125 could have resulted from its effect on some other molecule(s). More work will be needed to firmly establish a role for JNK in AICD in Ag-specific CTLs and to “connect” JNK with the upstream and downstream events in the apoptotic processes in primary CTLs. Meanwhile, considering that the antitumor effects of the CTLs—induced by active vaccination or adoptively transferred—can be vastly enhanced by preventing their “premature” death and as JNK inhibitors have generated much interest in inflammatory diseases and have also entered into clinical trials (35), our observations have implications in active as well as adoptive immunotherapeutic strategies for cancer and in further studies of the regulation of survival and death in CTLs.

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References


