Dendritic Cells Prolong Tumor-specific T-Cell Survival and Effector Function after Interaction with Tumor Targets

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Abstract

Tumor specific CTLs are susceptible to tumor-mediated activated induced cell death (AICD) after target engagement. The presence of dendritic cells (DCs) at the site of tumors correlates with an improved prognosis in patients with a variety of histological tumor types. We examined whether DCs can modify the survival of tumor specific CTLs during encounter with tumor targets. HLA-A2+ gp100-specific CD8+ CTLs were used as effectors against gp100+, A2+ melanoma FEM-X, and Mel526 (A2−) targets as well as the melanoma target Mel397. Cytolytic assays and [3H]DNA fragmentation (JAM) assays were used to evaluate CTL specificity and tumor-mediated AICD, respectively. A functional assay, ARK (activity of rescued killer), was developed to measure cytolytic activity of surviving CTLs after a 12-h coinoculation with tumor. In JAM assays, the CTLs proved more susceptible to apoptosis when exposed to the relevant tumors FEM-X and Mel526 than the irrelevant Mel397 (37 and 23% versus 3%; P < .001). The addition of human A2+ monocyte-derived immature DCs significantly (P < 0.001) limited this tumor-induced death of CTLs. In ARK assays, the presence of DCs decreased tumor-mediated suppression of CTLs, with increases in cytolytic function of CTLs reaching up to 2-fold. These findings suggest that DCs may play an important role during the effector phase of the immune response by enhancing the survival and function of CTLs in the tumor microenvironment.

Introduction

An immune response to tumor has been extensively studied and clearly demonstrated in both animal models and in humans. In recent years, studies of tumor immunity in murine models have clarified the critical role of innate and adaptive antitumor immune responses. Encouraging results in these studies have led to promising immunotherapeutic strategies for the treatment of cancer in humans. Although many immunologically based approaches, including the use of cytokines, tumor antigens/adjuvants, and genetically modified cells have been applied in human clinical trials to augment specific and nonspecific antitumor responses, these approaches have been generally unsuccessful (1–4). Although antigen-specific T cells can clearly be generated in vitro (5) and in vivo (6) against identified tumor-associated antigens, clinical responses to cancer using available immunotherapy regimens still remain frustratingly infrequent.

The variability and limited success of such immunotherapeutic approaches for the treatment of patients with cancer may in part be attributable to the “immunosuppressive” nature of the tumor microenvironment. Although tumor-induced immunosuppression has been evaluated in the past, the fundamental mechanisms of tumor escape from immune recognition are only now accessible for study and identification. Tumors can inhibit NK3 (7) or CTL (8) function, induce macrophage (9, 10) or DC dysfunction (11–13), as well as induce NK, T cell, and DC apoptosis (14, 15). As others have shown previously (16), in this study we demonstrate that tumor-specific CTLs are themselves susceptible to tumor target-induced immune elimination, probably through AICD after engagement of their specific T-cell receptor. Because a primary objective for many immunotherapeutic approaches for the treatment of cancer is to elicit specific T-cell responses and immune memory to tumor antigens, designing strategies to protect these immune effectors becomes a potentially critical aspect to allow for the development of more effective immunotherapies.

Infiltration of DCs into primary tumor lesions has been associated with significantly enhanced patient survival and reduced incidence of metastatic disease in virtually every cancer carefully examined (17–22). In contrast, a comparatively poor clinical prognosis has been observed for patients having tumor lesions that exhibit a sparse infiltration, with metastatic lesions shown frequently to be deficient in DCs. DCs play a critical role in the induction of antigen-specific immune responses by acquiring antigen at the site of inflammation, protecting it from degradation, and processing and presenting the antigen (23). DCs provide the necessary costimulatory signals (24) to specifically activate naive and memory T cells in secondary lymph node sites and may modify the local tumor microenvironment by sustaining T-cell survival and limiting angiogenesis. One can assume that the more DCs available to potentially acquire antigen at the site of the tumor, the better the chances are that a number of DCs would survive to effectively present antigen to T cells in the lymph node or at the tumor site. This in itself may be a major factor contributing to the improved prognosis associated with tumor infiltration of DCs.

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3 The abbreviations used are: NK, natural killer; DC, dendritic cell; AICD, activated induced cell death; rhIL, recombinant human interleukin; GM-CSF, granulocyte/macrophage-colony stimulating factor; ARK, activity of rescued killer.
We hypothesized that within the tumor microenvironment, DCs function not only to acquire antigen but also to provide critical signals (25–29) in the form of cytokines and/or costimulatory molecules necessary for prolonging the survival and function of immune effector cells, which may otherwise undergo AICD. In this study, we show that in vitro, the presence of DCs can indeed promote CTL survival in the presence of melanoma targets. We also show that the cytolytic function of the CTLs, which diminishes after interaction with the relevant melanoma tumor target, is sustained in the presence of immature DCs.

Materials and Methods

Tumor Cell Lines. The human melanoma FEM-X (A2+) and the transformed human B-cell lymphoma Daudi cell lines were kindly provided by Dr. Theresa Whiteside (University of Pittsburgh Cancer Institute, Pittsburgh, PA). The melanoma cell lines MEL526 (A2+) and MEL397 (A2–) were generously provided by Dr. Steven Rosenberg (National Cancer Institute, Surgery Branch, Bethesda, MD). These cell lines were all cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and maintained in a humidified incubator with 5% CO2 at 37°C. All cultures were monitored daily and used for experiments during the log phase of their cell proliferation. All cell culture medium and reagents mentioned above were obtained from Life Technologies, Inc. (Rockville, MD). Cell lines were also determined to be free of Mycoplasma contamination prior to and after use in these studies by per- forming Mycoplasma detection assays (Gen-Probe, Inc., San Diego, CA).

B7.1-neo-expressing Tumor Targets. The human melanoma FEM-X was transduced using a retroviral supernatant containing viral vectors expressing either the human B7.1 gene tagged with a neomycin-resistant selectable gene or the neomycin-resistant gene alone. The Human Gene Therapy Institute Vector Core of the University of Pittsburgh supplied viral vec- tors. FEM-X cells were harvested for experiments during the log phase of their cell proliferation. All cell culture medium and reagents mentioned above were obtained from Life Technologies, Inc. (Rockville, MD). Cell lines were also determined to be free of Mycoplasma contamination prior to and after use in these studies by performing Mycoplasma detection assays (Gen-Probe, Inc., San Diego, CA).

Malignoma Antigen-specific CTLs. The HLA class I A2-restricted melanoma antigen gp100 (209–217, IT- DQVPFSV)-specific CD8+ CTLs were generously provided by Drs. Steven Rosenberg and John Wunderlich (National Cancer Institute, Surgery Branch, Bethesda, MD). These antigen-specific T cells were expanded in AIM-V medium containing 5% heat-inactivated human serum (Atlanta Biologicals, Norcross, GA) supplemented with rhIL-2 (1000 IU/ml). The IL-2 was withdrawn from the CTL cultures 24 h prior to their testing.

Human Monocyte-derived DCs. PBMCs were obtained after Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient separation of normal healthy donor leukopheresis products or whole blood buffy coat preparations supplied by the Central Blood Bank of Pittsburgh (Pittsburgh, PA). PBMCs were placed in six-well tissue culture plates (Costar, Corning, NY) in AIM-V medium (Life Technologies) at a concentration of 1 X 10^7 cells/ml and placed for 2 h in a humidified incubator with 5% CO2 at 37°C. After removal of nonadherent cells, adherent monocytes were cultured for 6–7 days in AIM-V containing 5% heat-inactivated human AB serum (Atlanta Biologicals, Norcross, GA), rhGM-CSF (1000 IU/ml), and rhIL-4 (1000 IU/ml). Only loosely adherent and suspended cells were harvested for experimental use. DCs were further purified using the NycoPrep (Nycomed Pharma, Oslo, Norway) osmotic density gradient separation technique as they described. The DCs resulting from this culture method are largely of an immature phenotype (CD83–, CD80 low, CD86 moderate). Fig. 1 presents a FACScan analysis of a variety of cell surface molecules of DCs at various stages of DC maturation. The immature DC culture demonstrates a mixed phenotype with increased expression of CD86, CD40, CD80, CD54 (data not shown), and HLA-DR. CD83 remains negative with only dim expression appearing in some cultures. Also not expressed on immature DCs is CD14, commonly expressed on macrophages, and CD25 (data not shown), which appears on fully mature DCs. Immature DCs were used in our study because they represent the final stage of maturation one would expect of those that infiltrate tumor sites.

Antibodies and Flow Cytometry. Phenotypic expression of cell surface antigens was determined after flow cy- tometric analysis using a FACScan (Becton Dickinson, San Jose, CA). Data were analyzed using either the Lysis II software (Becton Dickinson) or the WinMDI Version 2.8 Software (Joseph Trotter, Scripps Research Institute, La Jolla, CA). Fluorescent (FITC and PE) labeled monoclonal antibodies (mouse antihuman CD3, CD4, CD8, CD56, CD19, CD80, CD86, CD14, CD54, CD25, CD28, and HLA-DR) and fluorescent (FITC and PE) labeled mouse IgG1 and IgG2a isotype controls used to characterize CTL and DC populations were obtained from PharMingen (San Diego, CA). The FITC-labeled antihuman CD40 was purchased from Ancell (Bayport, MN). PE- and FITC-labeled mouse antihuman CD83 was obtained from Coulter-Immunotech (Miami, FL). The following is the general procedure used for cell surface staining. In polystyrene tubes, a total of 5 X 10^6 cells were suspended in 100 µl of cold staining buffer consisting of 1 X PBS (Life Technologies) containing 0.1% BSA and 0.1% sodium azide. Antibodies were added for 20 min at 4°C, followed by two washes with staining buffer. Cells were fixed in 2% paraformaldehyde, and data were acquired immediately.

Cytokines. The Chiron Corp. (Emeryville, CA) generously donated the rhIL-2, and Schering Plough (Kenilworth, NJ) kindly provided the rhGM-CSF and rhIL-4.

ELISA Assays for IFN-γ. All ELISAs were performed with reagents and procedures described in the product literature inserts supplied by Endogen (Woburn, MA). For ELISA, high binding, 96-well, flat-bottomed plates (Costar) were used. Briefly, the ELISA plates were coated with anti-IFN monochlo- nal antibody (M-700A-E). Nonspecific binding sites were blocked with BSA protein, followed by several washes. After this, culture supernatants were added to the wells and incubated overnight. After several washes, biotin-labeled anti-IFN mono-
Fig. 1  “Immature” DCs used in this study as defined by cell surface molecule expression using FACScan analysis. The DCs we used in this study were the “immature” monocyte-derived DCs, shown on the second row of the panel, and were cultured for 7 days in medium supplemented with rhIL-4 (1000 IU/ml) and GM-CSF (1000 IU/ml). The immature DC cultures are very heterogeneous and phenotypically differ from macrophages, primarily in their loss of CD14 expression and an increase in expression of a variety of costimulatory molecules including CD86, CD80, and CD40. Once DCs “mature” after activation (in this case, the cells are activated with IL-1β, 50 ng/ml), the phenotype of the cultures becomes very homogeneous, with a high level expression of costimulatory molecules CD86, CD80, and CD40, as shown on the bottom row of the panel. The high expression level of CD83 and CD25 (data not shown) are distinguishing phenotypic characteristics of the “mature” DCs.

DNA Fragmentation Assay. To measure tumor induced CTL apoptosis, 8 h [3H]thymidine DNA fragmentation (JAM) assays were performed with minor alterations from those described previously (30) to optimize our system. CTLs were labeled with [3H]thymidine (NEN Life Technologies) at 5 μCi/ml/10^6 cells 18 h prior to the assay in the absence of rhIL-2. The CTLs were then washed free of excess [3H]thymidine and adjusted to a final concentration of 6 × 10^5 cells/ml. In round-bottomed, 96-well plates, relevant (A2+ gpl00-expressing melanoma) and irrelevant (A2- melanoma) tumor lines are plated at 6 × 10^4 cells/ml (6 × 10^5/100 μl) and serially diluted for a final volume of 100 μl/well. When DCs are added to the assays, serial dilutions of DCs are first performed in polypropylene tubes, and 50 μl of the cell suspensions are subsequently added to the appropriate wells at various concentrations ranging from 1 × 10^3 to 1 × 10^4 cells/well. The [3H]thymidine-labeled CTLs were then added to each well at a constant number of 3 × 10^4 cells/well (50 μl). An assay incubation time of 8 h is used, at which time the cells are lysed and harvested onto glass fiber filter mats (Wallac, Turku, Finland) using a TOMTEC (Wallac) plate harvester. The filter mats were placed in liquid scintillation sample bags (Wallac) with scintillation fluid, and the nuclear bound radioactive [3H]thymidine was measured using a Beta-plate beta counter (Wallac) and recorded as cpm. Data are expressed as a percentage of DNA fragmentation based on the percentage decrease in cpm as compared with untreated controls using the following formula:

% DNA fragmentation = \( \frac{\text{Control cpm} - \text{sample cpm}}{\text{Control cpm}} \times 100 \)
CTL Survival/Rescue Assay (ARK). To demonstrate the presence of functional CTLs after CTL-tumor interaction, an assay was developed to measure function based on CTL cytolytic activity. The ARK assay is performed by coincubating FEM-X with the CTLs in 96-well, round-bottomed plates at tumor:CTL ratios of 3:1 and 1:1 (1 × 10^4 CTL/well) in the presence of absence of HLA class I (A2+) single allele-matched DCs (DC numbers range from 1 × 10^3 to 3 × 10^4 cells/well). HLA-A2+ EBV-BLCL are used as controls for the DCs in this particular assay system. The initial tumor challenge was followed 12 h later by a second challenge with relevant 51Cr-labeled FEM-X tumor targets using 5000 targets/well. Just prior to adding the 51Cr-labeled target cells, the assay wells were mixed using a multi-channel pipettor to disassociate and resuspend the cells that settled at the bottom of the wells. Control wells containing only target cells are also prepared to determine the amount of spontaneous and maximum release of 51Cr (see "Cytotoxicity Assays"). Irrelevant cold target cells (either Daudi or normal fibroblasts) are added to the CTL control wells as a nonspecific blocker just prior to addition of 51Cr-labeled tumor targets to account for possible nonspecific inhibition of lytic activity attributable to the resulting cellular debris occurring from the initial CTL-tumor interaction. After 4 h, the assay supernatants were harvested, and the radioactivity released from the 51Cr-labeled tumor cells was measured by gamma counter (see "Cytotoxicity Assays" for harvesting procedure). Data were recorded as the percentage of lysis and the percentage of retained activity as compared with the non-tumor-treated CTLs.

Statistical Analysis. Statistical analysis for our results was performed using standard paired, one-tailed Student’s t tests using the Microsoft Excel software (version 4.0).

Results

CTLs That Recognize and Lyse Tumor Targets Are Also More Susceptible to Tumor-induced Apoptosis. Cytotoxicity assays were performed to confirm the antigen specificity of CTLs. In 4-h 51Cr-release assays, the CTLs specifically kill the two MHC class I (A2+) gp100-expressing melanoma cell lines FEM-X and MEL526, whereas no significant lysis occurs with the irrelevant MHC class I (A2−) melanoma cell line MEL397 or the B-cell lymphoma cell line Daudi (Fig. 2A). At an E:T cell ratio of 10:1, 57 ± 3.6% and 50.8 ± 1.8% lysis was shown in the relevant targets FEM-X and MEL526, respectively. Relatively high amounts of IFN-γ could was also detected by ELISA when the CTLs were exposed to the relevant FEM-X and MEL-526 but not the irrelevant MEL397 or Daudi (data not shown).

In an 8-h JAM assay, the relevant melanoma tumor cells (FEM-X and MEL526), which were effectively lysed by CTLs in 51Cr-release assays, were also the most potent inducers of CTL apoptosis, whereas the irrelevant melanoma MEL397 showed little if any affect on survival of the CTLs (Fig. 2B). At a 20:1 tumor:CTL ratio, 37 ± 3.7% and 23 ± 2.3% DNA fragmentation occurred in the CTLs when exposed to FEM-X and MEL526, respectively, whereas only 3 ± 2.9% of DNA fragmentation was recorded with exposure to MEL397. On the basis of these results, the FEM-X cells were used for most of our experiments because of their greater capacity to induce CTL apoptosis.

DCs Protect Specific CTLs from Tumor-mediated AICD But Induce Apoptosis in CTLs in the Absence of Tumor Antigen. Immature DCs were added in JAM assays measuring CTL survival in the presence of the relevant tumor, FEM-X. CTLs were labeled for 18 h with [3H]thymidine and plated in round-bottomed, 96-well plates at a concentration of 3 × 10^4 cells/well. FEM-X cells are added to the wells (6 × 10^5 cells/well) to achieve a tumor:CTL ratio of 20:1. The DCs were then titrated into the assays. The impact of DCs on the survival of CTLs was dependent on the presence of tumor. As shown in Fig. 3, when CTLs were treated with FEM-X in the absence of DCs, a 19.7% loss of the CTL DNA content occurred within 8 h as compared with the baseline loss of DNA content of CTLs in the absence of tumor. When DCs were added at concentrations of 3 × 10^3 to 1 × 10^4 cells/well, CTL survival could be maintained in the presence of tumor. In fact, the percentage of CTL DNA fragmentation observed in some experiments was

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Fig. 2 Specific CTLs that lyse relevant tumor targets are susceptible to tumor target-induced apoptosis. A, melanoma gp100 (209-217)-specific CTLs (MHC class I, A2+) specifically recognizes and lysed relevant MHC class I, A2+ gp100-expressing melanoma targets FEM-X and MEL526 in a 4-h 51Cr release assay. No significant lysis occurs with the irrelevant tumor controls, the A2− melanoma MEL397, and the B-cell lymphoma Daudi. B, in 8-h DNA fragmentation (JAM) assays, the CTLs were labeled with [3H]thymidine and served as the target for relevant and irrelevant tumor cells. At tumor:CTL ratios ranging from 10:1 to 40:1, the relevant A2+ melanoma tumor cells FEM-X and MEL526 induced significantly more (P < 0.001) DNA fragmentation of CTLs than does the irrelevant A2− MEL397. This type of tumor-mediated killing requires specific cognate interaction between tumor and effector cells and is an example of so-called AICD. Bar, SD.
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remaining constant at 1 × 10^5 cells/well. At this cell number, tumor. In an 8-h DNA fragmentation (JAM) assay, [3H]thymidine-labeled FEM-X. When the CTLs were pretreated with an equal number of FEM-X (tumor:CTL ratio, 1:1), the remaining cytotoxic activity of the CTLs was inhibited in the absence of any relevant tumor, the DCs were also capable of inducing CTL apoptosis (I), supporting some of our previously reported studies. These data are representative of three separate experiments, all showing similar results. Bars, SD.

<0, indicating a survival greater than the control non-tumor-treated CTLs. These CTLs may undergo some degree of spontaneous apoptosis in the absence of cytokines or other DC-derived survival factors. Surprisingly, CTLs underwent apoptosis in the presence of immature DCs when tumor cells were not added. This DC-mediated apoptosis increases in a dose-dependent fashion and is more apparent when the number of DCs added is greater than 3 × 10^5 cells/well (data not shown). This finding supports some of our earlier observations in which we found that immature DCs are uniquely capable of inducing apoptosis in activated CTLs when compared with macrophages while losing this ability upon maturation and/or antigen presentation (31).

**DCs Maintain CTL Function after Encounter with Tumor Targets (ARK Assay).** To determine whether the presence of DCs could maintain CTL function after interactions with their relevant target tumor cells, an assay was developed to measure the degree of tumor-induced suppression of CTLs based on the remaining lytic activity of surviving CTLs. As described earlier, CTLs were treated for 12 h with FEM-X at a tumor:CTL ratio ranging from 3:1 to 1:1 (these ratios were determined optimal for our studies) with the CTL cell number remaining constant at 1 × 10^5 cells/well. At this cell number, control untreated CTLs induced a 62.9% lysis of the 51Cr-labeled FEM-X. When the CTLs were pretreated with an equal number of FEM-X (tumor:CTL ratio, 1:1), the remaining cytolytic activity dropped to 18.1%. The lytic activity of the CTLs was shown to be significantly (P < .001) higher, with 33.4% lysis occurring when 3 × 10^6 DCs were included with tumor (Fig. 4A). When recorded as the percentage of baseline lytic activity retained as compared with the non-tumor-treated CTLs containing irrelevant Daudi cells as cold target inhibitors, the tumor-treated CTLs only retained 28.8 ± 1.4% of their baseline lytic activity, whereas 53.1 ± 6.6% of the CTL lytic function was maintained when DCs were present (Fig. 4B). Although the degree of tumor-induced AICD was enhanced when the tumor cell number was increased 3-fold (tumor:CTLs, 3:1), similar findings were observed, with 24.4 ± 0.8% of the baseline CTL lytic activity being maintained in wells containing DCs whereas only 12.1 ± 0.6% was shown in the absence of DCs (Fig. 4B). These values correspond to >2-fold increases in retained CTL cytolytic function and decreases in tumor-mediated CTL suppression. EBV-BLCL controls showed no effect on CTL activity, and DCs alone did not show significant lytic potential.

**DCs Enhance Survival of CTLs Independently of B7.1/CD28 Interaction.** Many attempts were made to impede the ability of the DCs to protect CTLs from tumor target-induced death by blocking B7.1/CD28 interactions. In both JAM and ARK assays, CTLA-4 immunoglobulin was added to competitively bind to B7.1. The ability of the CTLA-4 immunoglobulin to block B7.1 costimulation of T cells was first confirmed by using it in a standard mixed lymphocyte reaction proliferation assay. The addition of CTLA-4 immunoglobulin to the JAM and ARK assays showed no effect on CTL survival (data not shown). Retrovirally transfected B7.1-expressing FEM-X cells were also tested in both JAM (data not shown) and ARK assays (Fig. 5) to determine whether the target cells would be less effective inducers of CTL death. Again, expression of B7.1 did not alter CTL survival. Also, flow cytometric analysis of the CTLs used in our study revealed that they in fact did not express CD28, the costimulatory receptor for B7.1. Thus, in this study, DCs appear to mediate CTL survival through a B7.1/CD28-independent pathway.

**Discussion**

Adoptive transfer of melanoma-specific CTLs in conjunction with systemic administration of rIL-2 in melanoma patients revealed objective responses in ~30% of patients, with complete regression of disease sustained for several years realized in ~10% of the patients treated with rIL-2 alone (32). The therapeutic potential for such approaches is clear but limited by cost and our incomplete understanding of the relevant mechanisms allowing for the generation and maintenance of an effective immune response. Although eliciting specific T-cell responses to tumor antigens remains a major goal of tumor immunology, recent studies show that tumors can “counterattack” by inducing dysfunction and/or apoptosis in these effector cells, limiting the effectiveness of the immune response. Designing ways to enhance the survival and function of tumorspecific T cells becomes an issue worthy of great consideration (33–35).

Tumor-mediated suppression and destruction of activated immune effector cells have not been adequately dealt with in previous clinical studies. Sustaining a strong and effective immune response to cancer likely requires functional, activated DCs and a continued survival or expansion of effector cells capable of mediating their direct effects at the site of the tumor. During the chronic inflammatory response to cancer there may be inadequate numbers of DCs capable of presenting tumor antigens, costimulatory molecules, and cytokines necessary to prolong the survival and function of effector T cells and other.
DCs maintain the cytolytic function of CTLs after exposure to relevant melanoma target. The CTLs are plated in 96-well, round-bottomed plates at a concentration of 1 × 10^5 cells/well and are mixed with tumor cells at ratios of 1:1 or 3:1, with or without the addition of DCs (3 × 10^4 cells/well). Twelve h later, 51Cr-labeled FEM-X was added to measure the remaining lytic activity of the tumor-treated CTLs as compared with the control non-tumor-treated cells. A, the control CTLs (non-tumor-treated) induce 62.9% lysis of the 51Cr-labeled FEM-X, whereas the lytic potential drops to 18.1% when CTLs are pretreated with tumor at a 1:1 ratio. When DCs are present in the tumor-treated wells, a significantly (P < 0.001) greater amount of lytic activity is retained, with a value of 33.4% lysis being recorded. B, when recorded as percentage of CTL lytic activity retained, an enhanced degree of tumor-induced suppression of CTLs is shown with an increased numbers of tumor cells. The addition of DCs to the mix consistently shows the same trend, with the CTLs retaining a greater percentage of baseline cytolytic function. EBV-BLCL controls showed no significant enhancement of CTL activity. Bars, SD.

Fig. 4 DCs maintain the cytolytic function of CTLs after exposure to relevant melanoma target. The CTLs are plated in 96-well, round-bottomed plates at a concentration of 1 × 10^5 cells/well and are mixed with tumor cells at ratios of 1:1 or 3:1, with or without the addition of DCs (3 × 10^4 cells/well). Twelve h later, 51Cr-labeled FEM-X was added to measure the remaining lytic activity of the tumor-treated CTLs as compared with the control non-tumor-treated cells. A, the control CTLs (non-tumor-treated) induce 62.9% lysis of the 51Cr-labeled FEM-X, whereas the lytic potential drops to 18.1% when CTLs are pretreated with tumor at a 1:1 ratio. When DCs are present in the tumor-treated wells, a significantly (P < 0.001) greater amount of lytic activity is retained, with a value of 33.4% lysis being recorded. B, when recorded as percentage of CTL lytic activity retained, an enhanced degree of tumor-induced suppression of CTLs is shown with an increased numbers of tumor cells. The addition of DCs to the mix consistently shows the same trend, with the CTLs retaining a greater percentage of baseline cytolytic function. EBV-BLCL controls showed no significant enhancement of CTL activity. Bars, SD.

Fig. 5 Tumor targets expressing B7.1 show no effect on CTL survival (ARK assay). Relevant melanoma tumor targets were retrovirally transduced and selected to express high levels of B7.1. In ARK assays, CTLs were cocultured for 12 h with either the B7.1-expressing tumor targets or the parental and neo-transduced controls. Upon a second challenge with 51Cr-labeled parental target, the remaining lytic activity of the CTLs was measured. No significant difference in CTL survival or retained lytic activity was found as a result of target B7.1 expression. Bars, SD.

These reagents are extremely difficult. Because the specific arrangement of the T-cell receptor of the CTL is so that it recognizes only the gp100 peptide in the context of the HLA-A2 molecule, we feel that limiting the selection criteria to this HLA-A2 haplotype was a quite reasonable alternative. Although differences in the origin of these cells would not exist in vivo, we feel that they have little impact on the in vitro data generated in this study. Nevertheless, it would be worthwhile to translate these findings to future in vivo studies to clarify these issues.

Krummel et al. (36) describes similar finding to ours showing that B7.1, a costimulatory molecule found on DCs, can amplify the lytic potential of effector T cells, enhancing the duration of their activity after encounter with target antigen-expressing cells. Our study contrasts with theirs in two major ways. Their study was carried out in a murine model using an ovalbumin-based peptide system and did not include professional antigen-presenting cells in their assays. Also, unlike their study, B7.1 does not seem to be involved as a mechanism responsible for the enhanced survival and function of the tumor-specific CTLs we evaluated. EBV-BLCL used as a control in our assays also expresses high levels of B7.1 but failed to protect CTLs from AICD. We also tested melanoma cell lines that were retrovirally transduced and selected to express high levels of B7.1. In both JAM (data not shown) and ARK assays, the differences in CTL survival and function when using these B7.1-expressing tumor lines as compared with their parental counterparts were insignificant. We were also unable to block the DC-mediated protection of CTLs with the addition of CTLA4-immunoglobulin, which could competitively bind and block the B7.1 molecule on the surface of the DCs (data not shown). Finally, the cultured CTLs used in our experiments do not express CD28, the costimulatory receptor for B7.1. Thus, B7.1 costimulation is not involved as a mechanism responsible for the enhanced survival and function when using these B7.1-expressing tumor lines as compared with their parental counterparts.
however, supply other survival factors for effector cells that are CD28 independent.

Until recently, most of the focus in DCs and tumor immunology has been on the cellular and molecular mechanisms of lymphocyte activation, proliferation, and effector function. During the last several years, the role of apoptosis as a consequence of lymphocyte regulation and function has been found to be an important aspect of tumor response. Apoptosis is indeed a natural mechanism needed to eliminate unnecessary or dysfunctional T cells. The DC-mediated apoptosis of activated CTLs, which we have demonstrated to occur in the absence of relevant tumor target, may be an example of this natural process. This would be important to avoid damage of healthy tissue that occurs in cases of autoimmunity (37). In our studies, it appears that DC involvement in both CTL activation as well as survival depends also on the presence of antigen. The antigen becomes an important requirement for the DCs to maintain a specific immune response. Therefore, the DC regulation of T cells during the inflammatory response becomes, as it should be, an antigen-driven system (38).

Tumors modify and dysregulate the general homeostasis of the immune response. They try to be capable of modifying immunity before adequately being eliminated by causing immunosuppression through the release of cytokines (39), such as IL-10 and transforming growth factor-β, or by causing a premature apoptotic death of specific CTLs through AICD, as well as indirectly through the dysfunction (40) and/or apoptotic death of DCs. AICD involves cognate antigen recognition in activated immune cells (41, 42) and is also a potent negative feedback mechanism that insures removal of activated lymphocytes to avoid unwanted tissue injury. This death of effector cells is presumably "premature." Curtailing this phenomenon in the tumor microenvironment could ensure the future success of tumor immunotherapy trials.

In Figs. 2 and 3, we show that the same A2+ gp100-expressing melanoma tumor targets that are specifically lysed by CTLs are also the most potent inducers of CTL death, as measured by JAM assay. When exposing CTLs to such a relatively high number (20×) of tumor cells, nonspecific toxic factors, such as IL-10 and transforming growth factor-β, or the deprivation of basic nutrients from the culture medium may of course contribute to the death of the CTLs. Although we do not attempt to define the exact mechanism of this tumor-induced death of CTLs, it appears to be specific because of the fact that the irrelevant melanoma cell line showed little killing in 8 h as compared with the relevant melanoma targets. Therefore, this is probably an example of AICD. Curiously, the amount of DNA loss from CTLs measured in the JAM assays typically only reached a plateau between 20 and 30%, even if the number of tumor cells was increased. One explanation for this is that the T cells that are being killed may be only those effectors that are specifically activated by the tumor targets. This may reflect only 20–30% of the CTL culture. Another possibility is that [3H]thymidine released from the dying CTLs could be reincorporated into the healthy dividing tumor cells. If this would happen, the cpm values would remain higher and would not completely reflect the total amount of CTL killing. The ARK assay offers another way to gauge the viability of CTLs as a result of the three-cell mix by measuring the lytic function of the surviving CTLs.

Impediments to enhancing the effectiveness of such biological therapy strategies may be related to our incomplete understanding of the intimate and dynamic interactions that take place between effector lymphocytes and antigen-presenting cells, including DCs, especially within the tumor microenvironment (35). We have described previously murine tumor-induced apoptosis in DCs as an immune escape mechanism, noting that the interaction CD40 on the DCs with its ligand CD154 (CD40L) can protect DCs from apoptosis via IL-12-dependent and independent pathways (43). Interestingly, this suggests that T cells can also protect DCs from tumor-mediated suppression. CD154 expression is primarily a surface molecule found on activated CD4+ T cells. Kalinski et al. (44) suggests the idea that DCs can be polarized to elaborate various cytokine production profiles, allowing the immune response to skew toward either a T helper 1 or T helper 2 pathway in response to signaling factors encountered by the DCs at the site of inflammation. Of particular interest is the fact that IFN-γ can modify DCs and thereby promote a T helper 1 immune response in part by greatly augmenting their IL-12 production upon CD40 ligation. IL-12 itself has anti-apoptotic effects, promoting lymphocyte as well as DC survival, and may play a critical role in the enhanced survival of effector cells at tumor sites. Within the tumor microenvironment, IFN-γ arising from either CTL or NK cell interactions with tumor antigen could then modify the effectiveness and function of residual DCs. It may be the effector cell that provides the DC with the nominal "danger" (45) and costimulatory signals at the site of inflammation, allowing DCs to then provide additional survival factors for the effector cells.

Sustaining an effective immune response to cancer requires effective interactions in the tumor microenvironment between DCs, T cells, the tumor, and potentially other cells. The DCs function by acquiring and processing antigen that they obtain at the site of inflammation, acting as professional antigen-presenting cells once migrating to the lymph node, and induce the specific activation and proliferation of naïve T cells. They also provide survival factors that enhance the effectiveness and durability of these immune cells at the effector phase of the response at tumor sites. T cells, in turn, enhance the function and survival of the DCs (35, 46) by providing antigen for DC uptake through the elimination of target cells, costimulatory signals such as CD154 (CD40L), and cytokine priming signals such as IFN-γ. The tumor itself in many ways plays the most critical role for the response, disrupting the dialogue between the T cells and the DCs by inducing cell death and/or dysfunction, while indirectly shutting the immune response down with its absence. Therefore, using DCs at tumor sites as a supplemental addition to current and future immunotherapeutic designs, as well as to those approaches that have shown some degree of success in the past, may offer the necessary means to enhance and sustain immune responses to cancer resulting in more effective treatments.

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References


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