Roles of Idiotype-Specific T Cells in Myeloma Cell Growth and Survival: Th1 and CTL Cells Are Tumoricidal while Th2 Cells Promote Tumor Growth

Sungyoul Hong, Jianfei Qian, Jing Yang, Haiyan Li, Larry W. Kwak, and Qing Yi

Department of Lymphoma and Myeloma, Division of Cancer Medicine, and Center for Cancer Immunology Research, University of Texas M. D. Anderson Cancer Center, Houston, Texas

Abstract

Idiotype (Id) protein, secreted by myeloma cells, is a tumor-specific antigen. Id-based immunotherapy has been explored in patients with myeloma, and results were disappointing. Although previous studies have shown that Id-specific CTLs are able to lyse myeloma cells, it is unclear whether other types of Id-specific T cells, such as type-1 T-helper (Th1) and type-2 T-helper (Th2) cells, are also able to suppress or kill myeloma cells. Using a 5T murine myeloma model, we generated T-cell clones of different subsets and examined their function in the context of myeloma cells. Id-specific CTLs specifically lysed myeloma cells via MHC class I, perforin, and Fas ligand (FasL), and Th1, but not Th2, cells lysed the myeloma cells by FasL–Fas interaction. CTL and Th1 cells also suppressed the growth and function of myeloma cells, whereas Th2 cells promoted the proliferation and enhanced the secretion of Id protein and cytokines by myeloma cells. CTL and Th1, but not Th2, cells were able to eradicate established myeloma in vivo after adoptive transfer. These results show that Id-specific CTL and Th1 are promising effector cells, whereas Th2 provide no protection and may even promote tumor progression in vivo. [Cancer Res 2008;68(20):8456–64]

Introduction

Multiple myeloma (MM) is a B-cell malignancy, characterized by an accumulation of malignant plasma cells within the bone marrow. Myeloma cells secrete a monoclonal immunoglobulin (idiotype; Id) and induce skeletal destruction and hypercalcemia. Despite the progress in therapy of the disease, MM still remains an incurable malignancy (1, 2). Therefore, there is a great need for new treatments to stabilize or eradicate minimal residual tumors achieved after high-dose chemotherapy supported by autologous stem cell transplantation.

Id protein secreted by myeloma cells is a tumor-specific antigen because of the unique antigenic structure in its variable regions. Id-based immunotherapy has been explored in patients with MM and other B-cell tumors (3). As Id-based immunotherapies activate different subsets of Id-specific T cells (4) and T cells are potent effectors and critical components of antitumor immunity, many investigators have examined the roles of Id-specific T cells in these malignancies. Early studies showed not only the presence of low frequencies of naturally occurring Id-specific T cells in patients with monoclonal gammapathy of undetermined significance (MGUS) and MM stage I (5, 6) but also Id-specific type-1 helper T cells (Th1) in MGUS and MM stage I and type-2 helper T cells (Th2) in MM stages II and III (7). In addition, the presence of MHC class II–restricted, Id-specific CD4+ T cells and MHC class I–restricted Id-specific CD8+ T cells has been reported in unimmunized patients with MGUS or MM (8). These results indicate that these naturally occurring T cells are unable to suppress or eradicate myeloma cells in vivo due to inadequate numbers and functional suppression (9).

Although more recent studies have shown that Id-specific CD8+ CTLs, which were generated by using Id-pulsed dendritic cells (DC) were able to lyse primary myeloma cells from patients (10, 11), it is still unclear whether other types of Id-specific T cells, such as CD4+ Th1 and Th2 cells, are able to suppress or kill myeloma tumor cells. As Id-based immunotherapy may activate all T-cell subsets in patients (4), it is important to understand the functions of these T cells in the context of myeloma cells. In this study, we used the 5TGM1 myeloma murine model originally derived from 5T33 myeloma cells (12–14) to generate Id-specific T-cell subsets in C57BL/KaLwRij mice and explored the functional roles and antimyeloma immune responses of Id-specific T-cell subsets on the myeloma tumor cells in vitro and in vivo.

Materials and Methods

Mice and cell lines. Male C57BL/KaLwRij mice were purchased from Harlan CPB. This study was approved by the Institutional Animal Care and Use Committee of the University of Texas M. D. Anderson Cancer Center. The 5TGM1 myeloma murine cell line was cultured in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), 100 units/mL penicillin-streptomycin, and 2 mmol/L L-glutamine (both from Invitrogen). The B16 melanoma cell line, originated from C57BL/6 mice, was purchased from American Type Culture Collection and cultured in IMDM.

Preparation of Id protein. The 5TGM1 myeloma cells were cultured in A1-V serum-free medium and mouse IgG2b Id protein, secreted by the 5TGM1 myeloma cells, was purified from cell culture supernatant using Hi-Trap Protein A affinity chromatography (GE Healthcare), as described previously (15). Id protein and keyhole limpet hemocyanin (KLH; EMD Biosciences) conjugate was made using 0.1% glutaraldehyde (Sigma) as described previously (16) to enhance the immunogenicity of the Id protein.

Generation of dendritic cells. DCs were generated from bone marrow stem cells of mice as described previously (17, 18). Briefly, bone marrow mononuclear cells were cultured at a density of 2 × 10^6 cells/mL in RPMI 1640 with 20 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF; R&D Systems) at 37 °C in 5% CO2. At day 4, medium was replaced with fresh medium containing GM-CSF (10 ng/mL), and at day 8, immature DCs were pulsed for 8 h with Id-KLH proteins at a concentration of 50 μg/mL, followed by addition of tumor necrosis factor-α (TNF-α; 10 ng/mL)
and interleukin-1β (IL-1β; 10 ng/mL; both from R&D Systems) for 48 h to induce DC maturation. Mature DCs were collected and used to immunize mice.

**Generation of Id-specific T-cell clones.** Mice were immunized by three weekly s.c. injections of Id-KLH–pulsed mature DCs (10⁶ DCs per injection per mouse). After each immunization, GM-CSF (200 ng/d) was injected s.c. adjacent to the immunization sites for three consecutive days (4). One week after the final immunization, T cells were isolated from the spleens of immunized mice and cultured with 50 μg/mL Id protein in RPMI 1640 containing recombinant human IL-2 (10 units/mL; Roche Diagnostic) and IL-15 (5 ng/mL; R&D Systems). At day 7, T cells were collected and seeded into 96-well U-bottomed tissue culture plates (Corning, Inc.) at a concentration of one cell per well by a limiting dilution in RPMI 1640 containing recombinant human IL-2 (20 units/mL) and IL-15 (10 ng/mL) and subjected to functional tests.

**Immunophenotyping.** Phycoerythrin (PE)–conjugated or FITC-conjugated monoclonal antibodies (mAb) against CD11c, CD40, CD80, CD86, and MHC class II for DCs or CD4, CD8, CD69, and Fas ligand (FasL; all from BD Biosciences) for T-cell clones were added to cells, incubated for 30 min at 4°C, washed twice, and analyzed using a FACSCalibur flow cytometer (BD Biosciences). Intracellular perforin staining was performed using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instruction.

**Detection of cytokine production.** ELISA was used to measure secreted cytokines, such as IFN-γ, TNF-α, IL-2, IL-4, or IL-5 from T-cell clones. T-cell clones were incubated with irradiated (30 Gy) mature DCs pulsed with Id or irrelevant IgG2b protein, and unpulsed DCs were used as control. Supernatants were collected, and the amounts of cytokines were quantified using commercially available ELISA kits (R&D Systems).

**Proliferation assay.** T-cell clones (5 × 10⁴/100 μL/well) were seeded into 96-well U-bottomed culture plates. Various numbers of irradiated (30 Gy) mature DCs pulsed with Id or irrelevant mouse IgG2b protein were added and cultured for 4 d at 37°C in 5% CO₂. Cells were pulsed with 0.5 μCi/well [³H]thymidine (GE Healthcare) and harvested 18 h later. Radioactivity was measured using a β-liquid scintillation analyzer. Unpulsed DCs were used as control. Results are shown as mean count per minute.

In some experiments, T-cell clones were prelabeled with 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) for 10 min at 37°C. After washing, labeled T-cell clones were seeded and cocultured with irradiated mature DCs pulsed with Id or irrelevant mouse IgG2b protein for 5 d. Unpulsed DCs were used as control. After that, cells were incubated with PE-conjugated CD8 or CD4 mAb for 30 min, washed, and ready for analysis.

**Figure 1.** Generation and characterization of Id-specific T-cell clones. A, Id-specific T-cell clones of different subsets were generated and selected based on Id-induced IFN-γ and IL-4 secretion using ELISA assay. B, flow cytometry analysis showing the expression of CD8⁺ or CD4⁺ T-cell surface marker and CD69 activation marker on three T-cell clones. C, cytokine secretion pattern of three T-cell clones. T-cell clones were stimulated with irradiated DC pulsed with Id protein or irrelevant mouse IgG2b protein. Cytokines in cell culture media were quantified by ELISA. Representative results of three independent experiments.
Cytotoxicity assay. The standard 51Cr-release assay was performed to measure the cytotoxicity of the T-cell clones against 5TGM1 myeloma cells or DCs pulsed with Id protein, as described previously (19). B16 melanoma cells or unpulsed DCs were used as control target cells. CD8+ or CD4+ T cells purified from spleens of naive mice using anti-CD8 or CD4 mAb-coated magnetic beads (Miltenyi Biotec) were used as controls for effectors cells. Target cells were labeled with 51Cr-sodium chromate (GE Healthcare) for 1 h and incubated with various numbers of T cells in 96-well U-bottomed culture plates. After 4 h, 50% of the supernatants were collected, and radioactivity was measured by a γ-counter. Results are shown as mean percentage of 51Cr release, calculated as follows: \[
\frac{\text{sample counts} / \text{C}_{0} \text{spontaneous counts}}{\text{maximum counts} / \text{C}_{0} \text{spontaneous counts}} \times 100.
\]

In some experiments, 5TGM1 myeloma cells were prelabeled with PKH26 red fluorescent using cell linker kits (Sigma) and cocultured with T-cell clones. 5TGM1 myeloma cells cultured without T cells were used as negative control. CD8+ or CD4+ T cells isolated from splenocytes of naive mice served as controls for effector T cells. After 18 h, FITC-conjugated Annexin V (BD Biosciences) was added to the cells, incubated for 15 min, and analyzed using a flow cytometer.

Inhibition of T-cell–mediated cytotoxicity. To determine whether the cytotoxic activity of the T cells was restricted or mediated by MHC molecules, perforin, or FasL, mAbs against MHC class I, MHC class II, or FasL were used to block the cytotoxicity of the T-cell clones against 5TGM1 myeloma cells. CTL or Th1 clones were cocultured with 5TGM1 myeloma cells in the presence or absence of 20 μg/mL mAbs against MHC class I or MHC class II (both from BD Biosciences) or FasL (R&D Systems). Isotypic control IgG (Jackson ImmunoResearch) was used as control. In addition, concanamycin A (CMA; Sigma), an inhibitor of vacuolar type H⁺-ATPase, was used as selective inhibitor of perforin-mediated cytotoxicity (20). Effector T-cell clones were pretreated with 100 nmol/L of CMA for 2 h and assayed for cytotoxicity in the presence of the reagent.

T-cell inhibition assay. The suppressive activity of T-cell clones on myeloma cell growth and secretion of Id protein or cytokines, such as vascular endothelial growth factor (VEGF) was performed by using [3H]thymidine incorporation and ELISA assays. 5TGM1 myeloma cells were seeded at a density of 1 x 10⁴/100 μL/well into 96-well U-bottomed tissue culture plates. Irradiated (30 Gy) T-cell clones were added at a density of 5 x 10⁴/100 μL/well to the plates and cocultured with the myeloma cells for 3 d at 37°C in 5% CO₂. Cells were pulsed with 0.5 μCi/well [3H]thymidine.
and harvested 18 h later. Radioactivity was measured using a β-liquid scintillation analyzer.

In some experiments, supernatants from T-cell clones cocultured with 5TGM1 myeloma cells were collected. Secreted IgG2b Id protein or VEGF from 5TGM1 myeloma cells were measured by ELISA assay as described previously (21).

**In vivo adoptive transfer of T-cell clones.** Mice were challenged i.v. with \(2 \times 10^6\) 5TGM1 myeloma cells. One week later, when myeloma growth was established, \(2 \times 10^6\) T-cell clones were injected i.v. into tumor-bearing mice. Control mice received an injection of PBS or the same number of splenocytes from naive mice. Tumor burden was monitored by measuring serum IgG2b Id protein by ELISA assay. Mice were euthanized when moribund or when hind-leg paralysis developed.

**Statistical analysis.** Student’s *t* test was used to compare various experimental groups. *P* < 0.05 was considered statistically significant. Survival was evaluated from the day of tumor injection until death, and Kaplan-Meier test used to compare mouse survival between the groups. All data are shown as mean and SD.

**Results**

**Generation of Id-specific T-cell clones.** To obtain Id-specific T-cell clones, immature DCs generated from C57BL/KaLwRij mouse bone marrow stem cells were pulsed with purified Id-KLH conjugate, matured with TNF-α and IL-1β, and injected into mice. One week after the third immunization, mice were sacrificed and splenocytes were restimulated *in vitro* with Id protein for 1 week. Using limiting dilution assay, we generated Id-specific T-cell clones that secreted high levels of IFN-γ or IL-4 (Fig. 1A), which were expanded for further functional studies. T-cell clones of different subsets were identified based on their expression of CD8+ or CD4+ T-cell surface markers and secretion of cytokines, such as IFN-γ, TNF-α, IL-2, IL-4, or IL-5 in response to Id protein stimulation.

Figure 1 shows three representative Id-specific T-cell clones of CD8+ CTL and CD4+ Th1 and Th2 cells. The phenotype of the T cells is shown in Fig. 1B and pattern of cytokine secretion in Fig. 1C. Clone 2 expressed surface CD8 and CD69 (an activation marker), secreted IFN-γ and TNF-α, but not IL-2, IL-4, or IL-5, and thus may be CTL T-cell clone. Clone 8 expressed CD4 and CD69, secreted high levels of IFN-γ and IL-2 and a low level of TNF-α, but not IL-4 or IL-5, and could be a Th1 T-cell clone. Clone 16 also expressed surface CD4 and CD69, secreted IL-4 and IL-5, but not IFN-γ, TNF-α, or IL-2, and may thus be a Th2 T-cell clone. The same pattern of surface markers and cytokine secretion was also
seen with other T-cell clones (data not shown). These results show that CD8+ Id-specific T-cell clones of the CTLs and Id-specific CD4+ clones of Th1 and Th2 cells were generated.

**Proliferative responses of the T-cell clones.** To examine the functional properties of these T-cell clones, we first examined T-cell proliferative response induced by Id protein using [3H]thymidine incorporation assay. Findings from clones 2, 8, and 16 are shown as representative results in the following studies. As shown in Fig. 2A, all of the T-cell clones significantly proliferated in response to syngeneic DCs pulsed with Id protein (P < 0.01, compared with unpulsed or irrelevant mouse IgG2b-pulsed DCs). Similar results were also obtained with CFSE dilution assay to measure T-cell proliferation (Fig. 2B). Cultures of the CTL, Th1, and Th2 clones with DCs pulsed with Id protein resulted in high percentages of dividing T cells (60–82%), whereas the percentages of proliferating T cells cultured with unpulsed or irrelevant mouse IgG2b-pulsed DCs were low (6–10%). These T-cell clones did not respond to DCs pulsed with KLH (data not shown). These results were confirmed with other T-cell clones of CTL, Th1, and Th2 cells (data not shown). These results further confirm the specificity of the T cells for Id protein.

**Cytotoxic activity of the T-cell clones against myeloma cells.** Next, we evaluated the cytolytic activity of these T-cell clones against myeloma cells. Both the standard 51Cr-release assay and Annexin V–binding assay were used, and the target cells were 5TGM1 myeloma cells and Id-pulsed DCs. As shown in Fig. 3A, the CTL and Th1 clones efficiently and specifically lysed 5TGM1 myeloma cells and Id-pulsed DCs, although the CTL clone displayed stronger cytotoxic activity compared with the Th1 cells. No killing was observed against B16 melanoma cells, unpulsed DCs, or DCs pulsed with KLH. In contrast, the Th2 clone was not cytolytic to the target cells, and neither were isolated CD8+ or CD4+ T cells from spleens of naive mice.

We obtained similar results by using Annexin V–binding assay. As shown in Fig. 3B, coculture of the CTL and Th1 clones with PKH26-prelabeled 5TGM1 myeloma cells (to identify and gate on myeloma cells) resulted in high percentages of apoptotic (Annexin V+) 5TGM1 cells (P < 0.01, compared with 5TGM1 alone
or 5TGM1 cocultured with naive CD8+ and CD4+ T cells). Coculture with the Th2 clones or with purified CD4+ or CD8+ T cells from naive mice did not increase the percentages of apoptotic 5TGM1 cells. Figure 3C shows the pooled data of T cell–induced apoptosis in the myeloma cells. These results were confirmed with other T-cell clones of CTL, Th1, and Th2 cells (data not shown). These findings indicate that both Id-specific CTL and Th1, but not Th2, cells are efficient killer cells against myeloma cells, and the T cells recognized Id epitopes naturally processed by and presented on 5TGM1 myeloma cells.

**MHC restriction of T cell–mediated cytotoxic activity.** To elucidate the mechanism underlying T cell–mediated cytocotoxicity against the tumor cells, flow cytometry analysis was used to examine the expression of perforin and Fasl. by the T-cell clones. As shown in Fig. 4A, the CTL clone expressed both perforin and Fasl., whereas the Th1 cells expressed Fasl., but not perforin. In contrast, the Th2 cells did not express either perforin or Fasl. Interestingly, 5TGM1 myeloma cells expressed Fas molecules (Fig. 4B).

To determine whether the cytotoxic activity of the CTL or Th1 clones was restricted by MHC molecules and mediated by perforin or Fasl., we evaluated the inhibition of cytotoxicity using anti–MHC class I mAb, anti–MHC class II mAb, or mAb against Fasl., or CMA, an inhibitor of perforin-mediated cytotoxicity. As shown in Fig. 4C, mAbs against MHC class I, but not class II, CMA, and mAb against Fasl., although to a lesser extent, significantly inhibited cytotoxic activity against 5TGM1 myeloma cells (P < 0.01 and P < 0.05, compared with isotype IgG control). These findings indicate that myeloma cells naturally process and present MHC class I–restricted Id epitopes to CD8+ T cells.

Surprisingly, our studies showed that mAbs against Fasl., but not MHC class I or II, significantly inhibited Th1-mediated cytotoxic activity (Fig. 4C; P < 0.01, compared with isotype IgG control). We then examined the surface expression of MHC classes I and II by 5TGM1 cells and showed that the myeloma cells express MHC class I (data not shown), but not class II, molecules (Fig. 4D, surface). However, intracellular MHC class II molecules could be detected in the myeloma cells (Fig. 4D, cytoplasm). Treatment of 5TGM1 cells with IFN-γ (500 units/mL for 48 hours) did not induce surface expression of MHC class II molecules. Taken together, these findings suggest that, although myeloma cells do not express MHC class II molecules, Id-specific CD4+ Th1 cells can still recognize and kill the tumor cells by Fas–FasL interaction.

**Suppressive activity of the T-cell clones against myeloma cells.** Next, we examined the suppressive activity of the T-cell clones in the growth and function of myeloma cells. As shown in Fig. 5A, 5TGM1 myeloma cells cocultured with irradiated CTL or Th1 cells showed significantly inhibited proliferative response (P < 0.01, compared with 5TGM1 alone). In contrast, 5TGM1 myeloma cells cocultured with irradiated Th2 cells showed significantly enhanced proliferative response (P < 0.05, compared with 5TGM1 alone). No changes were observed in cell proliferative response of 5TGM1 cells when cocultured with irradiated CD4+ or CD8+ T cells from naive mice.

We also examined whether the T cells could regulate the secretion of Id protein and cytokine VEGF by the myeloma cells. As shown in Fig. 5B, 5TGM1 myeloma cells cocultured with irradiated CTL or Th1 cells secreted significantly lower levels of VEGF (P < 0.01 and P < 0.05, compared with 5TGM1 alone), whereas coculture with irradiated Th2 cells slightly, but significantly, up-regulated the secretion of VEGF by the tumor cells (P < 0.05, compared with 5TGM1 alone). The same results were also observed with the secretion of IgG2b Id protein by the myeloma cells (P < 0.01 and P < 0.05, compared with 5TGM1 alone; Fig. 5C). No changes were observed in cell secretion of these factors when cocultured with irradiated CD4+ or CD8+ T cells from naive mice. T cells alone did not produce VEGF or Id protein (data not shown). These results were confirmed with other T-cell clones of CTL, Th1, and Th2 cells (data not shown). These results indicate that Id-specific CTL and Th1 cells could suppress the function of myeloma cells, whereas Id-specific Th2 cells promote the proliferation and enhance the function of myeloma cells in vitro.

**In vivo effect of the T-cell clones on myeloma cell growth.** To evaluate the in vivo effects of the T-cell clones on myeloma growth and survival, adoptive transfer experiments were performed. Mice were first injected with 5TGM1 cells to establish myeloma, and 1 week later, a small number of the T cells, 2 × 106 cells per mouse, were injected i.v. into myeloma-bearing mice. No additional T cells were given, and mice were followed for disease progression until death (euthanasia). As shown in Fig. 6A, myeloma-bearing mice

![Figure 5. Suppressive activity of the T-cell clones on myeloma cells. A. proliferation of 5TGM1 myeloma cells measured by [3H]thymidine incorporation assay. B, concentrations of secreted VEGF by 5TGM1 myeloma cells. C, concentrations of IgG2b Id protein secreted by 5TGM1 myeloma cells. Irradiated T cells were cocultured with 5TGM1 myeloma cells for 3 d. 5TGM1 myeloma cells cultured without T-cell clones (5TGM1) or cocultured with CD8+ and CD4+ T cells from splenocytes of naive mice were used as controls. T cell/myeloma cell ratio of 5:1 was used. Representative results of three independent experiments. *, P < 0.05; **, P < 0.01 compared with 5TGM1 cells alone.](http://www.aacrjournals.org/.../cancerres.aacrjournals.org)
Figure 6. In vivo therapeutic effect of the T-cell clones. C57BL/KaLwRij mice (five per group) were challenged i.v. with $2 \times 10^5$ 5TGM1 myeloma cells, and one week later, $2 \times 10^5$ T-cell clones were adoptively transferred into tumor-bearing mice by an i.v. injection. PBS and splenocytes from naive mice served as controls. Serum samples were collected weekly, and tumor burden was monitored by measuring circulating IgG2b Id protein. A, concentrations of serum IgG2b Id protein in mice receiving PBS or different T cells as indicated. B, survival data of mice receiving PBS or different T cells. Arrows, injection of T cells. Representative results of one of two experiments performed.
receiving an injection of PBS or splenocytes from naive mice all developed myeloma, whereas four of five mice receiving Id-specific CTLs \((P < 0.01, \text{ compared with mice receiving PBS or splenocytes)}\) and three of five mice receiving Th1 cells \((P < 0.05, \text{ compared with mice receiving CTLs or Th1 cells)}\). Mouse survival data are shown in Fig. 6b; mice receiving PBS, splenocytes of naive mice, or the Th2 cells all died within 52 days after tumor injection, whereas 80% and 60% of mice receiving CTLs and Th1 cells, respectively, survived without detectable tumors \((P < 0.01, \text{ compared with mice receiving PBS or splenocytes)}\; \text{Kaplan-Meier test).} \) The difference between mice receiving CTLs and Th1 cells was not statistically significant. These results show that Id-specific CTLs and Th1 cells, but not Th2 cells, could efficiently eradicate established myeloma \textit{in vivo}.

**Discussion**

The 5TGM1 murine myeloma model \((22)\) was used in this study to generate Id-specific T-cell subsets and examine their role in myeloma \textit{in vitro} and \textit{in vivo} because this model manifests similarly to human myeloma, including monoclonal gammopathy, marrow replacement, osteolytic bone lesions, and hypercalcemia \((23)\). Mature DCs pulsed with Id proteins were used as immunogen to stimulate induction of Id-specific T-cell responses in mice before spleens were taken to propagate the T cells \textit{in vitro}. Vaccination with Id-pulsed DCs has been used for immunotherapy in MM \((24–26)\). Recent studies have shown that Id-based DC vaccines were able to protect mice from developing myeloma and were therapeutic against established myeloma in the murine model by inducing Id-specific CTL, Th1, and Th2 cell responses. By using \textit{in vivo} immunization with Id-pulsed DCs, we successfully generated Id-specific T-cell subsets from the mice. Based on the surface CD4 and CD8 expression and pattern of cytokine secretion, we obtained several clones of three T-cell subsets. We chose three clones corresponding to CTL, Th1, and Th2 cells for functional analyses. These T cells expressed activation marker CD69 and responded by proliferation and secretion of cytokines specifically to Id protein, but not to mouse IgG2b or KLH, indicating that, indeed, these T cells were Id-specific. We anticipate that it may be more difficult to generate functional Id-specific CTL and Th1 cells from tumor-bearing mice, because the frequencies of these T cells in the mice may be lower due to the presence of tumor cells and regulatory T cells \((4)\).

We examined \textit{in vitro} cytolytic activity of the T-cell subsets against target cells, including 5TGM1 myeloma cells and Id-pulsed DCs. As expected, Id-specific CTLs effectively lysed these cells, but not unpulsed DCs and irrelevant tumor cells, such as B16 melanoma cells. CD8+ T cells from naive mice could not kill 5TGM1 myeloma cells. In addition, mAbs against MHC class I or FasL, or CMA significantly inhibited CTL-mediated cytotoxic activity against 5TGM1 myeloma cells. Studies have shown that CTLs usually kill their target cells via the mechanisms of the pore-forming perforin and FasL-Fas interaction \((27, 28)\). In this study, our findings confirm that cytotoxic activity of our CTLs was restricted by MHC class I molecules and mediated by the perforin pathway and FasL-Fas interaction. We also examined the ability of the CTLs in inhibiting the growth of myeloma cells and showed that the CTLs significantly suppressed tumor cell proliferation and secretion of Id protein and VEGF. Furthermore, adoptive transfer experiments showed that 80% of mice receiving a small number \((2 \times 10^6 \text{ per mouse)}\) of CTLs survived of myeloma without increase in serum IgG2b Id protein. Thus, our findings provide strong evidence to support that Id-specific CTLs are potent effector cells for immunotherapy of MM.

In this study, we also show that Id-specific Th1 cells had strong cytolytic and suppressive activities against myeloma cells \textit{in vitro}. Furthermore, 60% of mice receiving Th1 cells survived of myeloma without detectable tumor burden after adoptive transfer in our murine myeloma model. CTLs have been considered to be the most important T cells in immunotherapy for tumor due to the fact that the cells can directly kill tumor via MHC class I loaded with tumor-derived peptides. However, the tumor killing effects of Th1 cells are less well defined. A number of studies have reported that CD4+ T cells could directly recognize tumor cells expressing MHC class II molecules through antigen presentation and kill the tumor cells \((29–31)\). However, myeloma cells express almost no MHC class II molecules \((32, 33)\), which is consistent with our findings with 5TGM1 cells. Hence, our findings suggest that Id-epitope presentation via MHC class II molecules to Id-specific CD4+ T cells was not an important mechanism for Th1-mediated cytotoxicity against MHC class II–negative myeloma cells. Indeed, Id-specific Th1 cell recognition and killing of MHC class II–negative B-cell tumors has been reported previously \((34, 35)\). It has been shown that CD4+ Th1 cells can directly induce tumor apoptosis by FasL-Fas interaction and also indirectly inhibit tumor growth by destroying tumor angiogenesis through IFN-γ \((36, 37)\).

In this study, we show that 5TGM1 myeloma cells expressed Fas molecules, and mAbs against FasL significantly inhibited Th1-mediated cytotoxic activity. Thus, Id-specific Th1 cells are able to recognize and kill the myeloma cells via FasL-Fas interaction. These results indicate that Id-specific Th1 cells are also potent effector cells for immunotherapy in MM.

Th2 cells are known to promote the recruitment of tumoricidal eosinophils and macrophages into the tumor microenvironment and promote antitumor immune response \((38)\). However, it is also recognized that Th2 cell–derived cytokines, such as IL-4 and IL-10, inhibit cell-mediated immunity \((39)\), and elimination of CD4+ T cells, especially Th2 cells, enhances antitumor effect in mouse melanoma \((40)\). Th2 responses may subvert Th1 cell–mediated immunity and provide a microenvironment to promote disease progression in patients with renal cell carcinoma or melanoma \((41)\). Nevertheless, the direct effects of tumor-specific Th2 cells on tumor cells, such as myeloma cells, are not well studied. We show that Id-specific Th2 cells did not have cytolytic activity against the target cells, including 5TGM1 myeloma cells. Furthermore, Id-specific Th2 cells promoted the proliferation and secretion of Id and VEGF by myeloma cells \textit{in vitro} and, after adoptive transfer to tumor-bearing mice, displayed no tumor protection \textit{in vivo}. These results are in a disagreement with an early study showing that murine Id-specific Th2 were suppressive to a B-cell tumor \((42)\). Considering the facts that MM is a B-cell malignancy and T cell/B cell interaction is crucial for regulating B-cell function, our findings suggest that Id-specific Th2 cells may positively regulate myeloma cell growth and survival \textit{in vivo}. Therefore, it will be unbeneificial to induce a Th2 response in patients after Id-based immunotherapy.

In conclusion, our study shows that Id-specific CTLs recognized Id peptides naturally presented by myeloma cells in the context of surface MHC class I molecules, and Id-specific CTL and Th1, but
not Th2, cells specifically and effectively lysed myeloma cells and Id-pulsed DCs. In addition, CTL and Th1 cells displayed significantly suppressive activity in the growth and function of myeloma cells, whereas Th2 cells enhanced the proliferation and cytokine secretion of myeloma cells. To further examine the functional roles of the T-cell subsets on myeloma cells in vivo where tumor cells are protected by the bone marrow microenvironment, adoptive transfer experiments were performed. As shown by the results, a small number of Id-specific CTL and Th1 cells was able to eradicate established myeloma and cured most of myeloma-bearing mice, whereas all mice receiving Th2 cells died of myeloma. Thus, our study indicates that Id-specific CTL and Th1 responses are beneficial and will lead to tumor eradication after immunotherapy in MM. In contrast, a Th2 response provides no protection and may even promote tumor progression in vivo. Although our results were obtained from 5TGM1 myeloma cells in a mouse model, we anticipate that similar functional roles of Id-specific T-cell subsets may be observed in other B-cell tumors as well.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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