Induction of Unresponsiveness Limits Tumor Protection by Adoptively Transferred MDM2-Specific Cytotoxic T Lymphocytes

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ABSTRACT

There is evidence showing that high avidity CTLs can be more effective than low avidity CTLs for adoptive tumor immunotherapy. Because many T cell–recognized tumor antigens are nonmutated self-proteins, tolerance mechanisms are likely to render high avidity T cells unresponsive or cause T cell elimination by clonal deletion. We recently used the allo-restricted strategy to circumvent immunologic tolerance to a ubiquitously expressed tumor-associated protein, MDM2, and raised high avidity CTLs in humans and in mice. In this study, we investigated whether high avidity MDM2-specific CTLs can mediate tumor protection without causing damage to normal tissues in mice. Although the CTLs prolonged survival of tumor-bearing mice without causing damage to normal tissues, tumor protection was incomplete. We show that tumor growth occurred despite the continued presence of MDM2-specific CTLs and the continued susceptibility of tumor cells to CTL killing. However, analysis of the CTLs revealed that they had been rendered unresponsive in vivo because they did not produce interferon γ in response to antigen-specific stimulation. These experiments suggest that induction of unresponsiveness may be an important mechanism limiting the efficacy of adoptive CTL therapy.

INTRODUCTION

To date, the majority of CTL-recognized tumor-associated antigens (TAAs) that have been identified are expressed by a limited set of tumors. The most desirable targets for CTL-based immunotherapy would be TAAs that are expressed in a broad range of malignancies. Furthermore, it would be desirable to select target proteins that are required for the maintenance of the malignant phenotype, thereby preventing the possibility of tumor escape by down-modulation of the target antigen.

The murine double-minute 2 (Mdm2) oncogene functions by inactivating the p53 tumor suppressor protein (1–3). Because MDM2 is overexpressed in many malignancies and has been shown to contribute to the process of malignant transformation, it represents an attractive target antigen for CTL-based immunotherapy. Like most TAAs, MDM2 also is expressed in normal tissues. High avidity T cells specific for MDM2 consequently are likely to be subject to mechanisms of central and/or peripheral tolerance induction, resulting in low avidity T cell responses that may be unable to effectively control tumor growth in vivo (4, 5). In the recent past, a number of strategies have been used to circumvent tolerance to obtain CTLs with improved avidity (6–10) and tumor protective efficacy (6, 7).

We have used the allo-restricted strategy (11) to bypass self-tolerance to MDM2 and raised high avidity allo-MHC–restricted CTLs specific for peptide epitopes of human and murine MDM2 (6, 10, 11). A murine MDM2-derived peptide, pMDM100, that is naturally presented on H2-Kb MHC class I molecules has been identified, and high avidity allo-restricted CTL clones have been isolated from splenic T cells of H2b BALB/c mice.

In this study, we have explored whether high avidity MDM2-specific CTLs can mediate tumor protection without causing damage to normal tissues. CTLs were transferred into H2ab F1 and H2b RAG-2(−/−) mice previously challenged with the H2b tumor cells MBL-2 or C205. We have examined the effect of the CTLs on tumor growth, the possible emergence of tumor escape variants, and the risk of CTL-mediated damage to normal tissues. Furthermore, we analyzed whether the injected CTLs were able to persist in tumor-challenged mice and retain the ability to develop effector function on antigen encounter.

MATERIALS AND METHODS

Animals. BALB/c (H2b) mice and (BALB/c × C57BL/6) F1 (H2ab) mice were supplied by Harlan (Indianapolis, IN). H2b RAG-2(−/−) mice were supplied from the breeding colony of Imperial College London, Hammersmith Hospital Campus.

Peptides. The synthetic peptide pMDM100 (ProImmune, Oxford, United Kingdom) corresponds to amino acids 100 to 107 (YAMiYRNL) of the murine MDM2 protein. Synthetic peptide pSV9 (ProImmune) corresponds to the amino acids (FAPGNYPAL) of the Sendai virus. In a previous study, we have shown that pMDM100 and pSV9 bind efficiently to H2-Kb MHC class I molecules (12). Peptides were dissolved in PBS to give a concentration of 2 mmol/L and stored at −20°C.

Tumor Cell Lines. MBL-2 cells (H2b) were derived from a Moloney murine leukemia virus–induced T-cell lymphoma (13). C205 (H2b) is a methylicholanthrene-induced fibrosarcoma (14). RMA-S cells (H2b) were derived from RMA cells, a Rauscher virus–induced C57BL/6N T-cell lymphoma, by mutagenesis (15). RMA-S cells are TAP deficient because of a point mutation in the TAP2 gene and express low levels of MHC class I molecules compared with intact RMA cells (12). H2 up-regulation was induced in tumor cell lines by incubating cells for 3 days in the presence of 400 units/mL recombinant human interferon γ (IFN-γ; Roche, Basel, Switzerland).

CTL Clone. Allo-MHC–restricted CTL clones were generated by in vitro stimulation of naïve BALB/c splenocytes with pMDM100-coated TAP-deficient Kb–expressing stimulator cells, followed by limiting dilution cloning (6). The high avidity pMDM100-specific CTL clone, 3Fb, was used in all of the experiments. This CTL clone uses a Vα5/Vβ7 T-cell receptor (TCR) and recognizes RMA-S target cells coated with low concentrations of pMDM100 peptide and H2b tumor cells expressing MDM2 endogenously. These CTLs were maintained in vitro by restimulation every 2 weeks on 24-well plates. Each well contained 1 × 105 pMDM100-specific CTLs, 1 × 103 irradiated (80 Gy) stimulator cells (RMA-S loaded with 10 μmol/L pMDM100 peptide), and 2 × 105 irradiated BALB/c splenocytes as feeders in 2 mL complete RPMI 1640 medium (Life Technologies, Inc., Rockville, MD) enriched with 10% FCS (Biowest, Nuaillé, France) and 50 μmol/L 2-mercaptoethanol (Sigma, St. Louis, MO). Cultures were supplemented with 10 units/mL recombinant human interleukin 2 (IL-2).

CTL Assays. Cytotoxic activity was determined in 4-hour 51Cr release assays against the tumor cell lines, concanavalin A (ConA)–activated spleno-
cytes, and RMA-S cells coated with pMDM100 peptides or MHC class I-binding control peptides as described previously (16).

Adoptive Transfer and Tumor Treatment. Experiments in the (BALB/c × C57BL/6) F1 (H2<sup>ab</sup>) mice and the H2<sup>ab</sup> RAG-2<sup>−/−</sup> mice were carried out identically, with the exception that (C57BL/6 × BALB/c) F1 mice received total body irradiation of 6 Gy at day −1 to provide space for the injected CTLs. On day 0, mice were injected intraperitoneally with tumor cells, followed by two intraperitoneal injections of CTLs at a dose of 2 × 10<sup>6</sup> after 6 hours and 5 days. All of the mice also were injected intraperitoneally with 1 × 10<sup>6</sup> units IL-2 (Chiron, Emeryville, CA) daily from day 0 until day 6. Tumor burden and health deterioration were monitored daily, and mice were sacrificed when they reached a lethal tumor burden according to United Kingdom home office regulations.

Tumor Cell Reisolation. MBL-2 tumor cells were reisolated from the ascites of tumor-challenged mice by peritoneal lavage. The tumor cells were washed and cultured in vitro under the same conditions as the parental MBL-2 tumor cells. For reisolation of C205 tumor cells, tumor tissue was removed from solid tumors in the peritoneal cavity of C205 tumor-challenged mice that had been sacrificed because of high tumor burden. The tumor tissue was cut into smaller pieces, macerated, washed, and cultured in vitro under the same conditions as the parental C205 tumor cells.

Histology. Samples of skin, liver, gut, spleen, and kidney from treated and untreated tumor-challenged H2<sup>ab</sup> F1 and H2<sup>ab</sup> RAG-2<sup>−/−</sup> mice were fixed in 10% neutral buffered formalin, processed routinely, and stained with H&E. Samples were evaluated by light microscopy using a light microscope (Nikon, Tokyo, Japan).

Flow Cytometry. Cell surface expression levels of H-2K<sup>b</sup> on tumor cell lines were determined using an antihouse-H-2K<sup>b</sup> FITC monoclonal antibody (mAb). To analyze in vivo CTL survival, treated and untreated control mice were sacrificed, and spleen and lymph nodes (inguinal and lumbar) were harvested. Single cell suspensions were obtained by macerating tissues through a 40-mm nylon cell strainer (BD Biosciences, San Jose, CA). To identify injected CTLs, samples were stained with rat–antimouse CD8<sup>+</sup> allophycocyanin mAb, rat–antimouse TCR Vβ7 phycoerythrin mAb, and antimouse H-2K<sup>b</sup> FITC mAb. Propidium iodide staining cells were excluded from analysis. All of the antibodies were purchased from BD Biosciences. Samples were analyzed using a FACScalibur flow cytometer and CELLQuest software (BD Biosciences).

Intracellular Interferon γ Assay. The functional activity of the CTLs was measured by enumerating IFN-γ–producing T cells. Splenocytes from CTL-treated and untreated control mice were stimulated ex vivo for 6 hours with RMA-S cells coated with 100 μmol/L pMDM100 or PSV9, or with 25 ng/mL phorbol myristate acetate and 50 μg/mL ionomycin. During the last 4 hours of stimulation, Brefeldin A (Sigma) was added at a concentration of 10 μg/mL to block cytokine secretion. The cells subsequently were washed and permeabilized with permeabilizing solution (BD Biosciences). Staining with rat–antimouse–IFN-γ FITC mAb then was carried out. A specificity control was performed using the appropriate isotype mAb.

RESULTS

Characterization of MBL-2 and C205 Tumor Cells. Flow cytometric analysis was used to determine MHC class I expression on MBL-2 and C205 tumor cells. The level of H-2K<sup>b</sup> MHC class I expression on the MBL-2 cells was high (Fig. 1A), whereas C205 expressed low levels but responded to IFN-γ treatment by up-regulation of K<sup>b</sup> (Fig. 1B). The MBL-2 tumor cells were efficiently killed regardless of whether the tumor cells had been induced previously with IFN-γ (Fig. 2A). There was no significant lysis of untreated C205 tumor cells, whereas IFN-γ treatment resulted in efficient recognition and CTL lysis (Fig. 2B). The increase in killing of the C205 tumor cells following IFN-γ induction correlated with the increased expression level of H2-K<sup>b</sup> class I molecules following IFN-γ induction (Fig. 1B). Control experiments showed that the CTLs specifically killed pMDM100-coated RMA-S target cells and did not kill ConA-activated normal cells unless they were coated with pMDM100 peptide (Fig. 2E). Collectively, these data indicated that the CTLs displayed effective killing activity against H2<sup>b</sup> tumor cells but not ConA-blistered control cells.

High Avidity pMDM100-Specific CTLs Delay the Development of a Lethal Tumor Burden in Mice. In vivo tumor protection experiments were carried out with the MBL-2 and C205 tumor cell lines to determine the level of tumor protection and the autoimmune risk of adoptive immunotherapy with high avidity pMDM100-specific CTLs. H2<sup>ab</sup> F1 mice were challenged intraperitoneally with a lethal dose of MBL-2 or C205, followed by two intraperitoneal doses of CTLs after 6 hours and 5 days and daily doses of 10<sup>5</sup> units of IL-2 for 7 days. Treatment of MBL-2 tumor-bearing mice led to a statistically significant delay in development of a lethal tumor burden (P = 0.0016; Fig. 3A). There was disease-free survival in one of the five mice that received CTLs. Inhibition of tumor growth by the CTLs also was observed in C205 tumor-challenged mice (Fig. 3B). All of the untreated treated and untreated control mice were sacrificed ex vivo for 6 hours with RMA-S cells coated with 100 μmol/L pMDM100 or PSV9, or with 25 ng/mL phorbol myristate acetate and 50 μg/mL ionomycin. During the last 4 hours of stimulation, Brefeldin A (Sigma) was added at a concentration of 10 μg/mL to block cytokine secretion. The cells subsequently were washed and permeabilized with permeabilizing solution (BD Biosciences). Staining with rat–antimouse–IFN-γ FITC mAb then was carried out. A specificity control was performed using the appropriate isotype mAb.

Statistical analysis of survival data by log rank analysis was carried out using JMP version 5 software (SAS Institute Inc., Cary, NC).

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treated mice died of ascitic tumor growth by day 22, whereas mice treated with CTLs showed a significant delay in development of lethal tumor burden (P = 0.044). Two of the mice treated with CTLs did not develop ascites but eventually developed solid intraperitoneal tumors.

The same in vivo tumor challenge experiments also were carried out in H2b T cell–deficient RAG-2(−/−) mice to determine whether CTLs could mediate protection in the absence of host T lymphocytes. As with the tumor challenge experiments in the H2ab F1 mice, treatment of H2b RAG-2(−/−) bearing MBL-2 and C205 tumors led to a delay in the development of a lethal tumor burden. However, this delay was only statistically significant in the case of the MBL-2 (P < 0.05) and not the C205 tumor-challenged mice (P > 0.05; Fig. 3C and D).

Transfected pMDM100-Specific CTLs Do Not Mediate Damage to Normal Tissues. Histologic analysis of liver, skin, gut, spleen, and kidney from treated and untreated tumor-challenged H2ab F1 and H2b RAG-2(−/−) mice revealed that these tissues showed similar histology (data not shown). Furthermore, none of the mice exhibited any acute side effects after CTL injection. Collectively, this indicates that the injected CTLs did not cause damage to normal tissues expressing MDM2.

CTL Unresponsiveness Correlates with Limited Tumor Protection. To determine whether the tumors growing in the treated mice were CTL escape variants, tumor cells were isolated from mice that had reached a lethal tumor burden. The expression level of H2-Kb MHC class I molecules on the reisolated and parental tumor cells before and after IFN-γ induction was assessed by flow cytometry. This showed that the H2-Kb class I expression levels were similar on the reisolated and parental tumor cells (Fig. 1C and D). The reisolated tumor cells also were killed by the CTLs, as shown in a 51Cr release assay (Fig. 2C and D).

The pMDM100-specific CTL clone expresses a Vα5/Vβ7 TCR and is CD8+ (Fig. 4A). To determine whether apoptosis of injected CTLs accounted for the lack of lasting tumor protection, flow cytometric analysis using Vβ7 and CD8 antibodies was performed in RAG-2(−/−) and F1 mice at time points when animals had developed a lethal tumor burden. Cell suspensions prepared from lymph nodes and spleens of CTL-treated RAG-2(−/−) mice were stained with Vβ7 and CD8 antibodies, and flow cytometric analysis revealed that the injected CTLs persisted in these mice (Fig. 4C). The injected CTLs were detected in all four of the CTL-treated RAG-2(−/−) mice analyzed, with the percentage of CD8+Vβ7+ cells ranging from 1.24% to 11.62%. No CD8+Vβ7+ cells were detectable in tumor-challenged RAG-2(−/−) control mice that were not injected with CTLs (Fig. 4B). Splenocytes and lymph node cells of CTL-treated H2ab F1 mice were stained with anti-H2-Kb antibodies in addition to the Vβ7 and CD8 antibodies. Because the adoptively transferred CTLs were of H2b origin, they should be distinguishable from endogenous F1 lymphocytes by their CD8+, Kb−, Vβ7+ phenotype. Flow cytometric analysis showed that a population of CD8+Kb− cells was detectable in CTL-treated F1 mice (Fig. 4E) but not in F1 tumor-challenged control mice that were not injected with CTLs (Fig. 4D). Gating of the CD8+Kb− cells revealed that they were Vβ7+ (Fig. 4F). The injected CTLs were detected in all five of the CTL-treated F1 mice analyzed, with the percentage of CD8+Kb− cells ranging from 0.18% to 0.35%. Collectively, these data indicated that tumor growth in RAG-2(−/−) and F1 mice occurred despite the continued persistence of the injected CTLs.

We subsequently went on to explore the functionality of the CTLs. Splenocytes were stimulated ex vivo with pMDM100-coated RMA-S cells, followed by intracellular cytokine staining to assess IFN-γ production. Strikingly, we found that none of the pMDM100-specific CTLs (Vβ7+CD8+) from the three CTL-treated RAG-2(−/−) mice analyzed produced IFN-γ in response to stimulation with pMDM100-coated RMA-S cells (Fig. 5A and B) shows the analysis of two mice). In contrast, when resting CTLs that had been maintained in vitro were mixed with splenocytes of tumor-bearing control mice, the majority of the CTLs produced IFN-γ in response to stimulation with pMDM100-coated RMA-S cells (Fig. 5C). Analysis of splenocytes isolated from four CTL-treated H2ab F1 mice that had reached a lethal tumor burden revealed that the CTLs from these mice also were unresponsive and did not produce IFN-γ in response to antigen-specific stimulation (Fig. 5D). The failure of CTLs to respond was not because of difficulties in detecting IFN-γ following transfer in vivo because ex vivo analysis of CTLs 4 days after injection into mice that received daily doses of IL-2 revealed peptide-specific IFN-γ production (Fig. 5E). Furthermore, phorbol myristate acetate and ionomycin stimulation of CTLs from tumor-bearing mice revealed IFN-γ production, indicating that the antigen-specific CTL unresponsiveness involved TCR proximal signaling events (Fig. 5E).
We observed that MDM2-specific CTLs delayed the development of a lethal tumor burden in tumor-challenged mice. The data indicated that protection was more efficient in H2<sup>ab</sup> F1 mice compared with H2<sup>b</sup> RAG-2<sup>–/–</sup> mice. A possible explanation is that F1 mice, but not RAG-2<sup>–/–</sup> mice, were preconditioned with 6 Gy nonmyeloablative irradiation to create a lymphopenic environment facilitating proliferation and engraftment of the injected CTLs. However, the improved efficacy of CTLs in the F1 mice is unlikely to be associated with the creation of a lymphopenic environment because the RAG-2<sup>–/–</sup> mice also are lymphopenic and support homeostatic expansion of adoptively transferred T cells (24). A more likely explanation is that following total body irradiation, the documented release of lipopolysaccharide and cytokines such as tumor necrosis factor α and IL-1β (25) produced an inflammatory milieu that improved the efficacy of T cell treatment. A role for preconditioning in adoptive immunotherapy is supported by murine and human studies showing improved efficacy after nonmyeloablative chemotherapy or irradiation in combination with adoptive T cell transfer (18, 26).

Generating T cell responses against MDM2, as is the case for other TAAs, carries with it the risk of causing damage to normal tissues (27, 28). However, histologic analysis revealed that the transferred high avidity MDM2-specific CTLs did not damage normal tissues. This suggests that the interaction of the CTLs with normal tissues expressing MDM2 could be classified as a “neutral” event for the tissue. Considering that high avidity CTLs were used in this study, the results suggest that the risk of autoimmune attack of normal tissues is small, which is in line with results obtained after adoptive transfer of high avidity CTLs specific for p53 (7).

Although we observed a prolongation in the survival of CTL-treated mice ultimately, in all but one mouse, there was not long-term protection. One potential explanation for this is that the transferred pMDM100-specific CTLs may have selected for tumor escape variants, which are able to escape CTL-mediated killing. However, analysis of C205 and MBL-2 tumor cells isolated from mice that had reached a lethal tumor burden suggests that it is unlikely that the selection of tumor escape variants could have accounted for the lack of long-term tumor protection.

The clonal deletion of the transferred pMDM100-specific CTLs could account for the observed lack of long-term tumor protection in mice, as described for peripheral T cells with specificity for self-antigen (29, 30). Although analysis of lymph node cells and splenocytes from mice that were still present, they had been rendered unresponsive because they were unable to produce IFN-γ in response to antigen-specific stimulation. Thus, it seems likely that the failure of the transferred pMDM100-specific CTLs to mediate long-term tumor protection in mice was a result of the CTLs being rendered unresponsive, possibly because of long-term antigen exposure, as described in models of self-tolerance (31, 32). It will be interesting to determine whether it was because of long-term antigen presentation by tumor cells or normal tissues. Although the MDM2-specific CTLs used in this study did not display in vitro killing activity against nonmalignant normal cells and did not attack normal tissues in vivo, it is possible that in vivo antigen presentation is sufficient to induce functional tolerance in the injected CTLs. This is supported by preliminary experiments showing that transfer of CTLs into tumor-free mice can result in the induction of CTL tolerance. The notion that normal tissues may render MDM2-specific CTLs tolerant is supported by recent observations with TCR transgenic mice expressing the TCR genes isolated from the high avidity CTLs used in this study. Despite the results described here showing that MDM2 expression in normal tissues may render MDM2-specific CTLs tolerant, it is also possible that long-term tumor protection could be achieved by adoptive transfer of MDM2-specific CTLs in combination with adoptive T cell transfer.

Collectively, these data illustrate that although the CTLs could be detected in tumor-challenged mice, they were unresponsive to antigen-specific stimulation and did not display effector function, as measured by IFN-γ production. We were unable to assess cytotoxicity because the CTL number was too small for killing experiments and in vitro expansion of CTLs failed because of the overgrowth of tumor cells.

DISCUSSION

The adoptive transfer of antigen-specific T cells can result in regression of established tumors in patients and murine models (17–22). Most of the adoptive T cell therapy models in mice have used CTLs directed against artificial model antigens that are not present in normal tissues. Relatively few studies have documented successful adoptive immunotherapy with T cells specific for tumor-associated antigens (7, 23).
tissues was insufficient to trigger CTL attack, it might be sufficient to switch off the effector function of the adoptively transferred CTLs. Thus, the interaction of CTLs with normal tissues appears to be a neutral event for the tissue but a tolerizing event for the CTLs.

There are a number of strategies that may prevent the development of unresponsiveness in transferred tumor-reactive CTLs and therefore merit additional investigation. The prolonged administration of T-cell growth factors, such as IL-2 or IL-15, may help prevent the development of “unresponsiveness.” The addition of exogenous IL-2 is known to prevent “classical” CD4+ T cell anergy (33). We administered IL-2 to mice for 7 days following treatment with CTLs, but the continued administration of IL-2 might improve the efficacy of the CTL treatment by preventing the development of unresponsiveness. Furthermore, the critical role of CD4+ helper T cells in tumor-protective T cell immunity is becoming increasingly recognized (34), with help for CTL priming and effector function being a part of their functional activities (35). It also recently has been shown that vaccination after T cell transfer can greatly improve the efficacy of adoptive T cell immunotherapy (23). Therefore, the cotransfer of CD4+ helper T cells with specificity for MDM2, together with the administration of MDM2-specific CD8+ T cells, followed by vaccination with activated peptide-coated dendritic cells, may help to prevent the induction of CD8+ T cell unresponsiveness. Finally, the selection of target antigens that are found in a variety of human malignancies but with a more restricted pattern of expression in normal tissues would be desirable to reduce the probability of tolerance induction of adoptively transferred CTLs.

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REFERENCES


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