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Adoptive T Cell Immunotherapy of Human Uveal Melanoma Targeting gp100

Roger P. M. Sutmuller,† Luc R. H. M. Schurmans,* Leonie M. van Duivenvoorde,* John A. Tine,‡ Ellen I. H. van der Voort,* René E. M. Toes,* Cornelis J. M. Melief,* Martine J. Jager,† and Rienk Offringa 2*

HLA-A*0201-restricted CTL against human gp100 were isolated from HLA-A*0201/Kb (A2/Kb)-transgenic mice immunized with recombinant canarypox virus (ALVAC-gp100). These CTL strongly responded to the gp100154–162 epitope, in the context of both the chimeric A2/Kb and the wild-type HLA-A*0201—molecule, and efficiently lysed human HLA-A*0201+, gp100+ melanoma cells in vitro. The capacity of the CTL to eradicate these tumors in vivo was analyzed in A2/Kb-transgenic transgenic mice that had received a tumorigenic dose of human uveal melanoma cells in the anterior chamber of the eye. This immune-privileged site offered the unique opportunity to graft xenogeneic tumors into immunocompetent A2/Kb-transgenic mice, a host in which they otherwise would not grow. Importantly, systemic (i.v.) administration of the A2/Kb-transgenic gp100154–162-specific CTL resulted in rapid elimination of the intraocular uveal melanomas, indicating that anti-tumor CTL are capable of homing to the eye and exerting their tumoricidal effector function. Flow cytometry analysis of ocular cell suspensions with HLA-A*0201-gp100154–162 tetrameric complexes confirmed the homing of adoptively transferred CTL. Therefore, the immune-privileged state of the eye permitted the outgrowth of xenogeneic uveal melanoma cells, but did not protect these tumors against adoptive immunotherapy with highly potent anti-tumor CTL. These data constitute the first direct indication that immunotherapy of human uveal melanoma may be feasible. The Journal of Immunology, 2000, 165: 7308–7315.

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*Department of Immunohematology and Bloodbank, Leiden University Medical Center, The Netherlands; † Department of Ophthalmology, Leiden University Medical Center, The Netherlands; and ‡Virugenetics Corporation, Troy, NY 12180

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Adaptive immunotherapy experiments
A modified quantitative technique for deposition of a definitive number of tumor cells into the anterior chamber of the mouse eye was used (28). Mice were deeply anesthetized with a mixture (ratio 1:1) of xylozine (Rompun 2%; Bayer, Leverkusen, Germany) and ketamine hydrochloride (Aescotek, Aesculaap BV, Boxtel, The Netherlands) given i.p. The eye was viewed through the low-power (8×) of a dissecting microscope, and a sterile 30-gauge needle was used to puncture the cornea at the comosecular junction, parallel and anterior to the iris. A glass micropipet (~80 μm in diameter) was fitted into a sterile infant-feeding tube, which was mounted onto a sterile 0.1 Hamilton syringe (Hamilton, Whittier, CA). The pipette, loaded with OMM-1 cell suspension (7.5 × 10^6/ml), was introduced through the puncture site of the cornea, and 4 μl of the OMM-1 cell suspension was delivered into the anterior chamber. The eyes were examined three times per week with a dissecting microscope to observe and document tumor growth. Subcutaneous induction of tumors, as well as i.v. administration of CTL clones, was performed as described previously (28, 29). Tumor-challenged mice were assigned randomly to treatment protocols with relevant CTL or control CTL. All CTL treatments were performed in combination with a s.c. injection of 10^7 Cetus units of IL-2 in an IFA depot. Histological analysis of eyes of treated and control mice was performed as described previously (28).

Flow cytometry of tumor-infiltrating lymphocytes (TIL)
Eyes were mashed through a nylon filter to obtain single-cell suspensions, passed over a Ficoll gradient, washed, and triple stained with propidium iodide (1 μg/ml), anti-mCD8-FITC (1:200 dilution, PharMingen, San Diego, CA), and HLA-A*0201-allophycocyanin tetrameric complexes harboring the gp100(154–162) peptide (1:10 dilution; a kind gift from Dr. H. Spits, The Netherlands Cancer Institute, The Netherlands). Analysis by flow cytometry (FACSscilbur; Becton Dickinson, Mountain View, CA) of propidium iodide-negative cells was performed immediately after staining.

Results
Induction of HLA-restricted, human gp100-specific immunity in HLA-A*0201/Kb-transgenic mice
Human gp100-specific CTL immunity was induced in HLA-A*0201/Kb (A2/Kb)-transgenic mice through immunization with recombinant canarypox viruses (ALVAC) that encode the three known HLA-A*0201-restricted epitopes gp100(154–162) (22), gp100(209–217) (23), and gp100(204–212) (29). The full-length human gp100 protein is known to harbor, in addition to the three epitopes of interest, at least one and possibly more epitopes that are immunogenic in the context of murine class I MHC (31), while the A2/Kb-transgenic mice coexpress the H-2D^b and K^b molecules (13). To skew the CTL response of the mice toward the HLA-restricted epitopes of interest, we immunized not only with ALVAC-gp100, encoding the full-length human gp100 Ag, but also with multiepitope ALVAC, encoding synthetic polypeptides that comprise a string-of-beads arrangement of the three epitopes (see Materials and Methods for details). Similar multiepitope vaccines have previously been employed successfully by several laboratories for the induction of effective anti-tumor CTL immunity in mice (e.g., Refs. 27 and 32).

Mice received three subsequent i.v. doses of the same ALVAC vaccine, after which the splenocytes were restimulated in vitro in the presence of cells positive for the three human gp100 epitopes and HLA-A*0201. We submitted the splenocytes to three sequential rounds of stimulation with different stimulator cells in the following order: A2/Kb-transgenic LPS blasts loaded with the three gp100 peptides (see Materials and Methods for details), an A2/Kb-transfectant of the gp100-positive human melanoma cell line Mel397, and A2/Kb-transgenic mouse embryo cells transfected with the genes for human gp100 and murine B7.1 (M1B-gp100). After this three-step restimulation protocol, the T cell cultures were tested for their reactivity against a panel of target cells in a TNF-α release assay. The T cell cultures responded against human and murine cell lines expressing both human gp100 and
A2/Kb, but not against the controls (Fig. 1A). Parallel splenocyte cultures from mice immunized with control ALVAC did not exhibit specific responses to these targets (not shown). Reactivity was also observed against human HLA-A*0201-positive, gp100-negative osteosarcoma cells (SAOS) that were infected with ALVAC-gp100 (Fig. 1A). This suggested that the responder cells were capable of recognizing their target not only in the context of the chimeric A2/Kb molecule, but also in the context of the wild-type HLA-A*0201 (see below). Monitoring of reactivity against peptide-loaded targets revealed that the CTL cultures were exclusively directed against the gp100154–162 epitope (Fig. 1B). This pattern of reactivity was also observed in cytolytic assays (not shown).

A2/Kb-transgenic CTL clones efficiently lyse human melanoma cells in vitro

After initial analysis of the polyclonal T cell cultures, CTL clones were isolated through limiting dilution using BLM-gp100 tumor cells (HLA-A*0201) as stimulator cells. These clones showed a reactivity pattern identical with that of the bulk cultures from which they were derived, in that they recognized human and murine cells that presented the gp100154–162 epitope in the context of the A2/Kb molecule (Fig. 2). Importantly, the CTL were also capable of lysing gp100-positive human melanoma cells expressing the wild-type HLA-A*0201 restriction element (Fig. 2C). Because the murine CD8 molecule on these CTL cannot efficiently associate with the α3 domain of the HLA-A*0201 molecule (13), this interaction is apparently not required, implying that the TCRs of these CTL exhibit high affinity for their target Ag. This notion was confirmed by measuring the reactivity of the A2/Kb-transgenic CTL clones against titered amounts of exogenously loaded gp100154–162 peptide. The CTL exhibited efficient lysis of peptide-loaded target cells, reaching 50% of their maximal lytic activity at peptide concentrations of 10 ng/ml (Fig. 3A). This sensitivity, which is similar to that found for TIL1200 cells, a human TIL line recognizing the same epitope (22), is commonly found for CTL that are capable of responding to physiological quantities of naturally processed Ag (33).

The specificity of the A2/Kb-transgenic CTL was compared in more detail to that of the TIL1200 cells by testing the reactivity of these responders to a series of variants of the gp100154–162 peptide in which residues at the different positions were replaced by alanine. This revealed that, at least for the A2/Kb-transgenic CTL clone tested, the fine specificity with respect to the gp100154–162 epitope was very similar, if not identical, to that of the human-derived TIL1200 cells (Fig. 3B, and Ref. 22). Taken together, our data show that immunization of A2/Kb-transgenic mice with recombinant ALVAC vaccines encoding human gp100 CTL epitopes can elicit potent HLA-A*0201-restricted CTL immunity against the gp100154–162 epitope.

Gp100-specific CTLs control the growth of intraocular human uveal melanomas in A2/Kb-transgenic mice

At present, no effective treatment is available for metastatic uveal human melanoma (15). In contrast, a majority of the human uveal melanomas tested were found to express both gp100 and class I HLA molecules (16–18, 34). Furthermore, it has been shown that uveal melanoma cells can be lysed by tyrosinase and MAGE-specific CTL clones in vitro (35). Therefore, we set out to analyze the sensitivity of human uveal melanoma cells for our A2/Kb-transgenic gp100-specific CTL in vitro as well as for adoptive immunotherapy with these CTL in vivo. We employed the human uveal melanoma cell line OMM-1, which is positive for both HLA-A*0201 and gp100 (17). In accordance with the expression of these Ags, OMM-1 was shown to be an excellent target for lysis by A2/Kb-transgenic gp100154–162-specific CTL in vitro (Fig. 4). We subsequently performed adoptive transfer experiments in A2/Kb mice that were challenged with an intraocular dose of this human uveal melanoma cell line. We have recently found that highly immunogenic adenovirus type 5 (Ad5)-transformed cells, which otherwise fail to grow in syngeneic immunocompetent mice (29), do form tumors when inoculated in the anterior chamber of the eye.
(28). This observation supports the notion that the eye is an immune-privileged site and suggests that this setting may also permit the outgrowth of xenogeneic tumors in immunocompetent mice. Indeed, injection of OMM-1 in the anterior chamber of the eye of A2/Kb-transgenic mice resulted in outgrowth of these tumor cells (Figs. 5, A–C, and 6), whereas s.c. injection of up to 10^7 OMM-1 cells failed to induce tumors (not shown). Tumor take was 100% when 3 x 10^3 OMM-1 cells were applied. Although in the majority of mice the OMM-1 tumor regressed spontaneously at around day 20 after tumor inoculation, similar to what was previously reported for intracocular Ad5-transformed tumors (28), this setting still allowed us to study the prevention of ocular human uveal melanoma engraftment. Importantly, adoptive transfer of the A2/Kb-transgenic, gp100 154–162-specific CTL in combination with IL-2 prevented the outgrowth of OMM-1 in the eyes of these mice (Fig. 6, A–G). Treatment with a control CTL and IL-2 (Fig. 6, H–N) did not restrict the growth of this tumor. Treatment with IL-2 alone did not differ significantly from treatment with control CTL and IL-2 (not shown). The tumoricidal efficacy of the gp100-specific CTL indicated that, despite the immune privilege of the eye, the CTL were capable of homing to the tumor site and exerting their Ag-specific effector function. The eradication of the tumor by the CTL was not accompanied by any sign of immunopathological damage to the eye (Fig. 5, D–F). To confirm the homing of the adoptively transferred CTL toward the anterior chamber of the eye, we analyzed ocular single-cell suspensions for the presence of gp100 154–162-specific CTL. Mice challenged with OMM-1 in the anterior chamber of the left eye were treated with 10^7 gp100 154–162-specific CTL and IL-2. As shown in Fig. 7, gp100 154–162-specific CTL can be detected in the tumor-bearing eye after 24 h, whereas no CTL are detectable in the tumor-free eye of the same animal. This result directly shows that the CTL indeed are capable of homing toward OMM-1 tumors in the anterior chamber of the eye.

In conclusion, A2/Kb-transgenic mice can be employed to raise highly effective HLA-restricted anti-melanoma CTL that are capable of eradicating human melanoma cells in vitro as well as in vivo. Furthermore, our data demonstrate that the anterior chamber of the eye can be employed as a unique location permitting the outgrowth of, and the testing of immunotherapeutic approaches against, human tumors in an HLA-transgenic host. Finally, the efficient eradication of human uveal melanoma cells in their physiologic context by systemically administered CTL constitutes the first indication that adoptive immunotherapy of such tumors may be feasible.

Discussion

The present manuscript describes the isolation from A2/Kb mice of HLA-A*0201-restricted gp100 CTL that are capable of efficiently eliminating human melanoma cells in vitro and in vivo. An advantage of using A2/Kb mice instead of mice expressing the wild-type HLA-A*0201 molecule is that the H-2Kb-derived α3 domain permits efficient interaction of HLA-restricted murine CTL with their target through their CD8 receptor (13). However, a drawback may be that the HLA-restricted CTL generated in A2/Kb-transgenic mice, especially in mice that have been immunized with considerable quantities of synthetic peptides, do not exhibit very high affinity for their target epitope and therefore depend for their reactivity on the interaction with CD8. The consequence of this CD8 dependency would be that such CTL, although capable of recognizing their target epitope in the context of the A2/Kb molecule, would fail to respond to this epitope in the context of the physiologically relevant HLA-A*0201 molecule (36). To prevent the induction of CTL of insufficient affinity, we avoided immunization with synthetic peptides and instead immunized with recombinant canarypox viruses (ALVAC) encoding these epitopes. To ensure induction of HLA-A*0201-restricted gp100-specific CTL instead of CTL directed against epitopes restricted by the endogenous H-2Dα and Kβ molecules, we immunized not only with ALVAC-gp100, encoding the full-length human gp100 Ag, but also with multiepitope ALVAC encoding synthetic polypeptides, which comprises a string-of-beads arrangement of the three gp100 epitopes. In all cases, CTL specific for the gp100 154–162 peptide were obtained, suggesting that this epitope is the immunodominant HLA-A*0201-restricted gp100 epitope in A2/Kb mice. This can most readily be explained by the fact that the gp100 154–162 and gp100 280–286 epitopes, in contrast to the gp100 209–217 epitope, are not conserved between mice and humans (10), whereas the
gp100<sub>154–162</sub> peptide exhibits the strongest binding to HLA-A<sup>0201</sup> (37).

Our adoptive immunotherapy experiments in A2/K<sup>b</sup> mice challenged by intraocular injection with human uveal melanoma cells show that systemic (i.v.) administration of the A2/K<sup>b</sup>-transgenic anti-gp100 CTL can completely control the outgrowth of these tumors. Moreover, we demonstrated that these CTL can be detected in eyes bearing an OMM-1 tumor in the anterior chamber. As noted before, mice receiving control treatment do exhibit spontaneous regression of established intraocular tumors (Ref. 28, and this paper). Additional studies have indicated that this phenomenon largely depends on the activity of endogenous CD<sup>4+</sup> T cells (L.R.H.M.S. and R.E.M.T., unpublished observations). Despite spontaneous regression, a sufficient time-window is available for evaluation of the tumoricidal efficacy of adoptively transferred CTL. In two previous papers, HLA-A<sup>0201</sup>-restricted CTL isolated from HLA-transgenic mice were shown to partially control the outgrowth of human tumors in SCID mice (11, 12).

**FIGURE 3.** Sensitivity and fine-specificity of gp100-specific A2/K<sup>b</sup>-transgenic CTL. The cytolytic activity of A2/K<sup>b</sup>-transgenic CTL (clone 8J) was determined against Jurkat-A2/K<sup>b</sup> cells loaded with the indicated concentrations of the wild-type gp100<sup>154–162</sup> epitope (A) or variant peptides (10 µg/ml) of the gp100<sup>154–162</sup> epitope in which the indicated residues were replaced by alanine (B).

**FIGURE 4.** Human uveal melanoma cells are efficiently lysed in vitro by A2/K<sup>b</sup>-transgenic CTL. The cytolytic activity of a representative A2/K<sup>b</sup>-transgenic CTL (clone 8J) was determined against three different human uveal melanoma cell lines: OMM-1 (gp100<sup>+</sup>, HLA-A*0201<sup>+</sup>), OCM-3 (gp100<sup>+</sup>, HLA-A*0201<sup>+</sup>), and 92–1 (gp100<sup>-</sup>, HLA-A*0201<sup>-</sup>).

**FIGURE 5.** Growth of human uveal melanoma cells in the eye of A2/K<sup>b</sup>-transgenic mice. Macroscopic view of OMM-1 tumor growth in the eye of mock-treated mice (A) and mice treated with A2/K<sup>b</sup>-transgenic anti-gp100<sup>154–162</sup> CTL (D). Microscopic analysis shows location of the tumor in the anterior chamber of the eye in control mice (B and C), and complete eradication in the absence of immunopathological damage in mice treated with gp100<sup>154–162</sup>-specific CTL (E and F).
Effective adoptive immunotherapy of intraocular tumors consisting of human uveal melanoma cells. A2/Kb-transgenic mice received an intraocular injection of 3 × 10^6 OMM-1 human uveal melanoma cells. One day later, mice (seven per group) were treated by adoptive transfer with either A2/Kb-transgenic gp100<sup>154–162</sup>-specific CTL (clone 8J, panels A–G) or Ad5-specific CTL (clone 100B6, panels H–N), both in combination with a s.c. depot of rIL-2 in IFA. Graphs show growth of tumors in individual mice as expressed by the percentage of the iris that was covered by the tumor (see example in Fig. 5A).

FIGURE 6.

Studies with xenogeneic models in which in vivo efficacy of HLA-restricted anti-tumor CTL was demonstrated involved either peritumoral injection of effector cells (e.g., Refs. 38–40) or injection of a mixture of tumor and effector cells in a Winn-type assay (e.g., Refs. 41–43). In these latter experiments, homing of the effector cells to the tumor site is not a prerequisite. Notably, the requirement of homing involves not only the ability of the CTL to travel through the periphery of the host and track down their target, but also the capacity of a sufficient proportion of these cells to survive this journey as well as the power of this fraction to launch a ferocious tumoricidal attack. This implies that adoptive immunotherapy involving systemic injection of CTL into mice bearing xenogeneic tumors requires high-quality CTL.

In addition to the adoptive transfer experiments in A2/K<sup>b</sup>-transgenic mice, we performed parallel experiments in C57BL/6 (A2/K<sup>b</sup>-negative) nude mice. As mentioned above, immunodeficient mice such as nude or SCID mice are commonly used as hosts in xenogeneic tumor models. However, in the latter setting, our gp100-specific A2/K<sup>b</sup>-transgenic CTL failed to eradicate the intraocular OMM-1 tumors (data not shown). This suggests that expression of the A2/K<sup>b</sup> molecule in the host is essential for the in vivo efficacy of the A2/K<sup>b</sup>-transgenic CTL. The mechanistic aspects of this phenomenon lie outside the scope of the present study. Importantly, we demonstrate that the in vivo efficacy of the A2/K<sup>b</sup>-transgenic CTL against a xenogeneic tumor can be tested in an immunocompetent syngeneic host by engrafting the xenogeneic human tumor in the anterior chamber of the eye. Because we and others have shown that intraocular engraftment enables the in vivo growth of tumors that otherwise fail to grow (28, 44), this experimental setting will most likely be applicable for analyzing the efficacy of adoptive immunotherapy with HLA-transgenic T cells against a variety of human tumors.

Metastatic uveal melanoma constitutes a formidable therapeutic challenge. At first sight, these tumors seem an equally poor target for immunotherapeutic approaches. They arise in an immune-privileged site that can sustain the growth of foreign tissues and in which different mechanisms for suppression of immune responses are operational (19, 20, 45, 46). Furthermore, the microenvironment of the eye was shown to endow human uveal melanoma cells with lymphocyte-inhibitory properties (21). Another compelling observation is that lack of expression of class I HLA-Ags on human uveal melanoma was found to be correlated with a better, rather than poor, patient survival (34). This suggests that NK cells have a protective role in the development of metastatic disease, whereas, in contrast to what has been observed for skin melanoma (47–49), CTL-mediated immunity does not impose a selective pressure for loss of HLA-expression by these tumors. Paradoxically, work with animal models for intraocular tumors has demonstrated that adoptively transferred T cells can in principle be effective against such tumors (28, 50). In the latter study, we demonstrated that adoptive transfer of human Ad5-specific CTL resulted in rapid and complete eradication of intraocular tumors of Ad5-transformed cells. An explanation for this paradox could be that these murine studies made use of model tumors that had not originally developed in the eye and that therefore lacked certain features typical of uveal melanomas. Importantly, our present work in A2/K<sup>b</sup> mice demonstrates that adoptive immunotherapy is effective not only against such model tumors but also against the intraocular engraftment of human uveal melanoma. This is in accordance with the fact that OMM-1 and many human uveal melanomas express class I HLA as well as a variety of melanoma Ags (16–18, 34), and that such cells are excellent targets for lysis by tumor-specific CTL in vitro (as shown by this study and previous studies (35)). Although we showed that adoptively transferred CTL eliminated the intraocular tumor in the absence of detectable damage to the eye, it should be noted that the CTL used so far were directed against foreign Ags. The gp100<sup>154–162</sup> epitope is not conserved between mice and humans (10). Therefore, it is important to test the effect of CTL against autologous Ags, especially epitopes that are also expressed in the eye. An exquisite opportunity to address this issue is offered by the recent observation that potent anti-tumor CTL immunity against the gp100<sup>154–162</sup> epitope, which is conserved between mice and humans, can be raised through immunization with recombinant virus encoding a variant of this peptide exhibiting improved binding to HLA-A*0201 (10). Although in these experiments, involving vaccination of A2/K<sup>b</sup> mice
suspensions and tumors. Therefore, we evaluated whether vaccination of A2\(/^\text{Kb}\) mice where they exert their tumoricidal effects. The prime question will pertain to the possibility of targeting the MAGE-2 gene product. Int. J. Cancer 75:125.


