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Anti-CD40 Conditioning Enhances the T<sub>CD8</sub> Response to a Highly Tolerogenic Epitope and Subsequent Immunotherapy of Simian Virus 40 T Antigen-Induced Pancreatic Tumors<sup>1</sup>

Pavel Otahal, Barbara B. Knowles, Satvir S. Tevethia, and Todd D. Schell

Rapid loss of adoptively transferred tumor-specific CD<sup>8</sup> T cells (T<sub>CD8</sub>) following Ag recognition in the periphery and their limited accumulation within the tumor stroma reduces the effectiveness of T cell-based immunotherapy. To better understand the role of T<sub>CD8</sub> in the control of autochthonous tumors, we have used mice of the RIP1-Tag4 lineage that develop pancreatic β cell tumors due to expression of the SV40 large T Ag from the rat insulin promoter. We previously showed that the kinetics of functional T<sub>CD8</sub> tolerance varies toward two distinct epitopes derived from T Ag. Epitope I (206SAINNYAQKL215)-specific T<sub>CD8</sub> tumors due to expression of the SV40 large T Ag from the rat insulin promoter. We previously showed that the kinetics of functional T<sub>CD8</sub> tolerance varies toward two distinct epitopes derived from T Ag. Epitope I (206SAINNYAQKL215)-specific T<sub>CD8</sub> are rapidly deleted whereas T<sub>CD8</sub> targeting epitope IV (404VYDFLKC<sub>411</sub>) persist over the lifetime of tumor-bearing animals. In this report, we show that the conditioning of tumor-bearing RIP1-Tag4 mice with agonistic anti-CD40 Ab induces extensive expansion of naive epitope I-specific TCR transgenic (TCR-I) T cells in this tolerogenic environment and delays their loss from the host. In addition, functional TCR-I T cells intensively infiltrate pancreatic tumors, resulting in increased survival of RIP1-Tag4 mice. These results suggest that a similar approach could effectively enhance T cell-based immunotherapies to cancer when targeting other highly tolerogenic epitopes. The Journal of Immunology, 2007, 179: 6686–6695.

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4 Abbreviations used in this paper: T<sub>CD8</sub>, CD8<sup>+</sup> T cell; DAPI, 4',6'-diamidino-2-phenylindole; Dbl/Fnu, H-2Dβ/Fnu NP epitope; Dbl/F, H-2D<sup>b</sup>/T Ag epitope I; Flu, influenza virus; GP33, glycoprotein 33; NP, nucleoprotein; PD-L1, programmed death ligand 1; RT4, RIP1-Tag4; TCR-I, TCR transgenic T<sub>CD8</sub> specific for T Ag epitope I.

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mice, leading to the development of islet hyperplasia by three months of age. Macroscopic insulinsomas appear at 5 to 6 mo of age. The average life span of RT4 mice is 200 days and all mice eventually succumb to tumors due to hypoglycemia (25). Thus, RT4 mice provide a more suitable model to test the ability of anti-CD40 conditioning to modulate peripheral tolerance of epitope I-specific T<sub>CD8</sub> and the resulting effect on tumor progression.

The immune response to T Ag in mice of the C57BL/6 background is characterized by a hierarchical T<sub>CD8</sub> response to multiple epitopes (26). The H-2K<sup>b</sup>-restricted response to epitope IV (T Ag 404–411) is the most dominant, followed by responses to the H-2D<sup>β</sup>-restricted T Ag epitopes I (T Ag 206–215; SAINNYAQKL) and II/III (T Ag 223–231) (27, 28). A fourth epitope, designated epitope V, is immunorecessive and does not induce T<sub>CD8</sub> unless the three dominant epitopes are inactivated (29). In previous studies using T Ag transgenic mice, we demonstrated that epitope I-specific T<sub>CD8</sub> are eliminated from the peripheral T cell repertoire more rapidly than T<sub>CD8</sub> specific for epitope IV (8, 9). In addition, immunization toward epitope IV can prevent the development of insulinsomas in RT4 mice, although this approach fails to mediate the regression of established tumors (9). In contrast, the ability to target epitope I is hampered by the rapid onset of peripheral tolerance for both the endogenous T cell population and adoptively transferred T cells (5, 9). The adoptive transfer of TCR transgenic T<sub>CD8</sub> specific for epitope I (TCR-I) results in their initial proliferation in RT4 mice but not their accumulation in either the lymphoid tissues or the tumor (9). These cells disappear from RT4 mice within 10 days following adoptive transfer unless the mice eventually succumb to tumors due to hypoglycemia (25). Thus, RT4 mice provide a more suitable model to test the ability of anti-CD40 conditioning to modulate peripheral tolerance of epitope I-specific T<sub>CD8</sub> and the resulting effect on tumor progression.

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The goal of the current study was to determine the extent to which TCR-I T cell survival could be enhanced in RT4 mice and whether these cells accumulate in the tumor, resulting in control of tumor progression. We find that rapid deletion of TCR-I T cells in RT4 mice can be delayed by the coadministration of agonistic anti-CD40 Ab. These TCR-I T cells proliferate extensively in RT4 mice after treatment with anti-CD40, acquire effector function, and intensively infiltrate pancreatic tumors, correlating with an increased lifespan for RT4 mice. These results demonstrate that therapeutic effects can be achieved even with T<sub>CD8</sub> that are highly susceptible to peripheral tolerance.

**Materials and Methods**

**Mice**

C57BL/6J (H2<sup>b</sup>), mice were purchased from The Jackson Laboratory and maintained at the animal facility of the Milton S. Hershey Medical Center (Hershey, PA) and used between 8 and 16 wk of age. TCR-I mice that were first labeled with 5-6-CFSE (Molecular Probes) for 10 min at 37°C and washed three times with cell medium. Peptide-coated targets were then labeled with different concentrations (5 μM) of CFSE<sup>W87</sup> (1:1 with 10<sup>3</sup> CFSE<sup>W67</sup> for 1 h at room temperature). In situ staining of tissues with MHC tetramers was performed using a FACScan flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star). Production and characterization of the H-2D<sup>d</sup>/T Ag epitope I (Db/I), H-2D<sup>d</sup>/T Ag epitope V (Db/V), H-2K<sup>b</sup>/T Ag epitope IV (Kb/IV), and H-2D<sup>b</sup>/Flu NP epitope (366–374 (Db/Flu) tetramers was previously described (28). The following Abs were purchased from BD Pharmingen: PE-labeled and PE-Cy5-labeled rat anti-CD8α (clone 53-6-7), FITC-labeled rat anti-mouse CD4 (clone RM4–5), PE-labeled rat anti-CD44 (clone H129.19), PE-labeled rat anti-mouse CD69 (clone H1.2F3), and FITC-labeled rat anti-mouse IFN-γ (clone XMG1.2). The percentage of CD8<sup>+</sup> T cells that stained positive with a T Ag-specific tetramer was determined by subtracting the percentage of cells that stained positive with the control Db/Fu tetramer from the tetramer stained with a T Ag-specific tetramer. Immunization toward epitope IV can prevent the development of insulinsomas in RT4 mice, although this approach fails to mediate the regression of established tumors (9). In contrast, the ability to target epitope I is hampered by the rapid onset of peripheral tolerance for both the endogenous T cell population and adoptively transferred T cells (5, 9).

**Adoptive transfers, immunizations, Ab treatment, and blood glucose measurement**

Mice received the adoptive transfer of sex-matched RBC-depleted lymphocytes derived from spleens and lymph nodes of TCR-I transgenic mice. Recipients were injected i.v. in the tail vein with a cell suspension containing 1–10<sup>6</sup> clonotypic TCR-I T cells, as determined by tetramer staining, in 0.2 ml HBSS. One day before and one day after adoptive transfer the mice received 100 μg (i.p. in 0.2 ml of PBS) of the purified anti-CD40 Ab FGK45 (32) or control rat IgG (Sigma-Aldrich). Immunization of mice with T Ag-expressing cell lines was conducted by the injection of 3–10<sup>5</sup> cells (i.p. in 0.5 ml of HBSS). Glucose levels from tail vein-derived peripheral blood were measured by using an Elite glucometer (Bayer).

**In vivo cytotoxicity and proliferation assay**

Sex-matched C57BL/6J splenocytes were incubated in the presence of the indicated peptides (1 μM) in RPMI 1640 with 10% FBS at 37°C for 90 min and washed three times with cell medium. Peptide-coated targets were then labeled with different concentrations (5 μM CFSE<sup>W87</sup> and 0.5 μM CFSE<sup>W67</sup>) of 5- and 6-CESE (Molecular Probes) for 10 min at 37°C in PBS with 0.1% BSA, washed two times, and, mixed together at a 1:1 ratio, and then 4–10<sup>5</sup> cells were injected into the tail vein (0.2 ml in HBSS). Elimination of CFSE-labeled target cells was assessed after 16 h. The in vivo proliferation assay was performed by i.v. injection of RBC-depleted lymphocytes from TCR-I mice that were first labeled with 5 μM-CFSE for 10 min at 37°C. After washing cells three times with PBS, cells were resuspended in HBSS and injected i.v. at a dose of 5 × 10<sup>5</sup> clonotypic TCR-I T cells per mouse. The proliferation of injected cells was determined 3 days later by staining the cell suspension prepared from spleen and cervical, and pancreatic lymph nodes with anti-CD8 Ab and Db/Fu tetramer. The extent of proliferation was determined by measuring the mean fluorescence intensity of CFSE dye in CD8<sup>+</sup> Db/Fu tetramer<sup>+</sup> cells.

**In situ tetramer staining and tumor histology**

In situ staining of tissues with MHC tetramers was performed using a modification of a previously described method (33). Native tumor samples were cut into 3- to 4-mm pieces and stained overnight at 4°C in PBS and 2% FBS containing PE-labeled tetramers (1/10). After washing with PBS for 2 h at 4°C, the tissues were fixed with 4% paraformaldehyde for 1 h at room temperature and snap frozen. Cryostat-cut, 10-μm-thick sections were exposed for 1 h at room temperature to rat anti-CD8 Ab (1 μg/ml) and rabbit anti-PE serum (Biomeda) diluted 1/1000 in PBS with 2% normal goat serum. After washing with PBS, slides were then incubated with Alexa Fluor 555 goat anti-rabbit and Alexa Fluor 647 goat anti-rat Abs (Molecular Probes) diluted 1/500 in PBS and 2% goat serum containing 4′,6′-diamidino-2-phenylindole (DAPI; 1 μg/ml) for 1 h at room temperature.
To demonstrate the specificity of the staining, we analyzed sections prepared from spleens of TCR-I transgenic mice and B6 mice (see Fig. 3C). The Db/I tetramer bound specifically to the TCR-I in the TCR-I mice, whereas no tetramer staining was detected in the control B6 mice.

Fluorescent staining to detect the presence of GFP<sup>+</sup> TCR-I T cells in the tumors was performed on 10-μm-thick frozen sections prepared from paraformaldehyde-fixed tumors. Sections were then incubated with rat anti-CD8 Ab (1 μg/ml) and guinea pig anti-insulin Ab (DakoCytomation) in PBS with 2% goat serum for 1 h at room temperature. After washing with PBS, the primary Abs were detected with Alexa Fluor 647 goat anti-rat Ab and Rhodamine Red-X donkey anti-guinea pig Ab (Jackson ImmunoResearch Laboratories) diluted in PBS and 2% goat serum containing DAPI (1 μg/ml). For simultaneous detection of insulin and T Ag expression, paraformaldehyde-fixed tumors were embedded in OCT medium and snap frozen in liquid nitrogen. Sections cut in a cryostat were then treated with 2 M HCl for 1 h at 37°C followed by incubation with mouse Ig-blocking solution (Vector Laboratories) for 1 h at room temperature. Sections were then stained with guinea pig anti-insulin and mouse anti-T Ag 901 mAb (34) for 45 min at room temperature in PBS, 2% normal goat serum, and 0.1% Nonidet-P-40. After washing, the primary Abs were visualized with Rhodamine Red-X donkey anti-guinea pig Ab and Cy5-labeled goat anti-mouse Ab. After washing with PBS, all stained sections were then fixed with 4% paraformaldehyde for 10 min, mounted in Aqua-Poly/Mount (Polysciences), and examined with a Leica AOBS confocal microscope using a ×40 immersion objective (Leica Microsystems). Images obtained by sequential scanning were processed and overlaid in Adobe Photoshop (Adobe Systems).

Statistical analysis
Kaplan-Meier survival plots were constructed with Prism software (GraphPad Software) and statistical significance was determined by the log rank posttest.

Results
Conditioning RT4 mice with activating anti-CD40 FGK45 Ab increases accumulation of TCR-I T cells following recognition of endogenous T Ag

In a previous study (9), we found that TCR-I T cells are initially activated and induced to proliferate following transfer into RT4 mice but are rapidly deleted. In the present study, we addressed whether administering the anti-CD40 agonistic Ab, FGK45 (19), could rescue TCR-I T cells from deletion. This approach has been shown to overcome the development of tolerance in several experimental models (5, 6, 13) and is indicated as being mediated via activation of host APCs (35). Thus, we adaptively transferred TCR-I T cells (CD45.1 positive) into 5-wk-, 3-mo-, and 6-mo-old RT4 mice and control B6 mice. One day before and one day after adoptive transfer, the mice were treated with FGK45 or control rat IgG. Ten days after T cell transfer, the splenic T cells were stained with anti-CD8, anti-CD45.1, and Db/I tetramer to determine the total number of TCR-I T cells per spleen. The results in Fig. 1A show that treatment of RT4 mice with FGK45 resulted in the extensive accumulation of transferred TCR-I T cells compared to treatment with the control Ab. Accumulation reached similar levels in all groups of RT4 mice, suggesting that it was independent of the age of the RT4 mice. We observed 4.2-, 7.2- and 5.9-fold increases in TCR-I T cell accumulation when transferred into 5-wk-, 3-mo-, and 6-mo old RT4 mice, respectively, compared with the levels achieved in Ag-free B6 mice. Control IgG-treated RT4 mice at all three ages had reduced levels of TCR-I T cells compared with B6 mice by 10 days after adoptive transfer, indicative of T cell deletion in the absence of anti-CD40 conditioning.

The enhanced TCR-I T cell accumulation observed following the conditioning of RT4 mice with FGK45 could be explained by the more rapid proliferation of TCR-I T cells. To address this possibility, CFSE-labeled TCR-I T cells were transferred into 3-mo-old RT4 mice with or without administration of FGK45, and the extent of proliferation was examined 3 days after adoptive transfer. The data in Fig. 1B show that the administration of FGK45 results in a robust accumulation of TCR-I T cells in just 3 days, representing 30% of CD8<sup>+</sup> T cells, and that all of these cells had undergone multiple divisions (mean fluorescence intensity of 348). In contrast, there was no increase in TCR-I T cells in mice that received the control IgG at this time point, and the detectable cells had undergone fewer divisions within the same time period (mean fluorescence intensity of 452). These data indicate that all of the TCR-I T cells were activated in the absence of FGK45, but the T cells that had undergone more than seven divisions, resulting in loss of CFSE fluorescence, failed to accumulate in mice given the control Ab. This result could be explained by either slower proliferation or the abortive death of TCR-I T cells after four to five divisions in the absence of FGK45 conditioning. However, no difference in the percentage of apoptotic TCR-I T cells was detected among cells recovered from FGK45- or rat IgG-treated RT4 mice 3 days after adoptive
transfer (data not shown). Taken together, these data suggest that FGK45 conditioning results in accelerated proliferation of TCR-I T cells following recognition of the endogenous T Ag, with ~10-fold expansion in 3 days.

**TCR-I T cells infiltrate tumors in RT4 mice after administration of FGK45**

Because conditioning RT4 mice with FGK45 induces an extensive accumulation of TCR-I T cells in the spleen, we determined whether these T cells migrated into tumors in RT4 mice. TCR-I T cells were transferred into tumor-bearing, 6-mo-old RT4 mice that were also given FGK45 or control rat IgG. Ten days later, pancreatic β cell tumors of ~5–8 mm in diameter were excised, cut into pieces and enzymatically digested to yield cell suspensions that could be analyzed for the presence of TCR-I cells by tetramer staining using flow cytometry (Fig. 2A). RT4 mice treated with control rat IgG+ TCR-I T cells or with FGK45 without adoptive transfer of TCR-I T cells contained few total TCD8+ T cells in their tumors (Fig. 2A, left panels). In comparison, RT4 mice that received TCR-I T cells and FGK45 contained a significant number of tumor-infiltrating TCD8+ with ~20% of total cells representing epitope I-specific T cells.

To assess whether TCR-I T cells that accumulated in response to conditioning with FGK45 had gained effector functions in tumor-bearing RT4 mice, an in vivo killing assay was used. We found that peptide I-pulsed targets were efficiently eliminated in FGK45-treated RT4 mice but not in RT4 mice receiving control rat IgG (Fig. 2B). To specifically test the function of the tumor-infiltrating TCR-I cells, IFN-γ production was assessed by intracellular cytokine staining. To differentiate the donor lymphocytes from the host TCD8, we used CD45.1+ TCR-I T cells. At 10 days after adoptive transfer, epoIpe I-specific TCD8 were detected by both MHC tetramer staining (Fig. 2C) and intracellular cytokine staining (Fig. 2D) in parallel samples. The data show that the Db/I tetramer+ cell detec-
ted in the spleens and pancreatic tumors of RT4 mice were of donor origin, as shown by CD45.1 expression, whereas the Db/I tetramer negative fraction contained only host-derived cells (Fig. 2C, histograms). In addition, epoIpe I-specific IFN-γ production was detected in both the spleens and tumors of these mice, demonstrating the presence of functional epitope I-specific T cells within the tumor. We found that the frequency of splenic TCD8 that produced IFN-γ was approximately half that detected by Db/I tetramer staining but represented only a quarter of the Db/I tetramer+ cells detected in the tumor (Fig. 2D).

Thus, while functional TCR-I TCD8 were detected in both the spleen and the tumor of RT4 mice, not all epitope I-specific TCD8 were able to produce IFN-γ after their recovery at this time point.

Previous studies in a related line of T Ag transgenic mice that develop insulinomas revealed that activated T lymphocytes accumulate at the periphery of T Ag-expressing islets unless a secondary trigger is also provided, such as CpG or irradiation, resulting in T cell infiltration into the tumor stroma (36, 37). Recent clinical observations also indicate that CD8+ T cell infiltration of tumors correlates with a positive prognosis (38, 39). To determine whether TCR-I T cells accumulate within the tumor stroma after administration of FGK45, GFP+ TCR-I T cells were transferred into 6-mo-old RT4 mice that were also given FGK45. After 10 days, tumor sections were examined by confocal microscopy for the presence of GFP+ TCD8. Sections fixed with paraformaldehyde were costained for insulin and CD8 to localize TCD8 within the tumors (Fig. 3A). The results demonstrate that the GFP-expressing TCR-I T cells are localized within the insulin-producing stroma as described. In addition, the data indicate that FGK45 conditioning results in accelerated proliferation of TCR-I T cells following recognition of the endogenous T Ag, with ~10-fold expansion in 3 days.

**FIGURE 2.** Conditioning with FGK45 induces tumor infiltration and induction of TCR-I T cell function in RT4 mice. A, Six-month-old RT4 mice received 1×106 or 1×105 SJL/TCRI T cells (CD45.1+) plus 100 μg of FGK45 or control rat IgG the day before and the day after the transfer. A third group of mice received FGK45 alone without the adoptive transfer of TCR-I T cells. Ten days after adoptive transfer, mice were sacrificed, pancreatic tumors were enzymatically dissociated, and the resulting cell suspensions were stained with the indicated MHC tetramers and anti-CD8. The numbers shown in the dot plots indicate the percentage of total cells in each quadrant. Data shown are from a mouse that received 1×106 donor TCR-I T cells and are representative of five mice. Similar results were obtained from mice that received either dose of TCR-I T cells. B, RT4 mice treated similarly as in A were subjected to an in vivo killing assay. Ten days after the adoptive transfer a mixture of peptide-pulsed, CFSE-labeled target cells were injected into treated RT4 mice and untreated B6 mice. The population of CFSElow targets corresponds to cells incubated with control peptide NP366-374, whereas CFSELow targets correspond to cells incubated with control peptide NP366-374. C and D, Four- to five-month-old RT4 mice received donor CD45.1+ TCR-I T cells and FGK45 as in A. Ten days after adoptive transfer, suspensions prepared from spleens and tumors of representative mice were stained with anti-CD8, MHC tetramer, and anti-CD45.1 (C) or subjected to intracellular cytokine staining after a 6-h stimulation with the indicated peptide (D). Data from the spleens of two individual mice are shown. Cells derived from the tumors of three mice were pooled before analysis. The values in each dot plot indicate the percentage of CD8+ cells that stained with either MHC tetramer or anti-IFN-γ. The histograms in C show CD45.1 expression on the CD8+ Db/I tetramer+ (Tet+; top region, heavy line) and Db/I tetramer- (Tet−; bottom region, normal line) cells. This experiment was repeated with similar results.
Higher magnification revealed a close interaction between CD8⁺/H11001 GFP/fluorescent cells and insulin-producing tumor cells (Fig. 3A, right panels). To demonstrate that tumor cells expressing insulin simultaneously express T Ag, we costained the sections with anti-insulin and anti-T Ag Ab after performing the epitope retrieval approach (Fig. 3B). The images show that insulin (shown in red) was detected in the cytoplasm whereas the T Ag (shown in blue) was detected in the nucleus. This result indicates that the insulin-positive cells within the tumor stroma also express T Ag.

FIGURE 3. Visualization of tumor-infiltrating TCR-I T cells. Groups of three RT4 mice aged 5 to 6 mo were injected with 1 x 10⁶ TCR-I T cells expressing GFP (TCR-I.GFP) and treated with FGK45 the day before and after T cell injection. After 10 days, tumors were harvested and sections were stained and analyzed by confocal microscopy as described in Materials and Methods. A, TCR-I.GFP CD8⁺ T cells are detected within the tumor stroma and closely associate with insulin-expressing cells (shown in red). The areas outlined with white boxes are depicted at higher magnification in the right panels, which show DAPI staining of nuclei along with anti-CD8 staining (blue) of the TCR-I.GFP cells. B, Costaining of tumor cells for insulin (red) and T-Ag (blue). C, The specificity of the in situ tetramer staining is demonstrated on sections prepared from spleens of TCR-I transgenic mice (top row) and B6 mice (bottom row). The Db/I tetramer (green) bound specifically to the CD8⁺ cells (red) in the TCR-I mice, which is shown by the colocalization of both colors, whereas no tetramer staining or colocalization was detected in the control B6 mice. D, The presence of epitope I-specific TCR on GFP⁺ T cells is demonstrated by in situ tetramer staining with Db/I tetramer (red, top and middle row), whereas no staining was observed with control Db/Flu tetramer (bottom row). Scale bars, 40 μm (A–C) and 10 μm (D).
To further confirm that the GFP+ CD8+ cells detected within the tumors were epitope specific, we performed in situ tetramer staining. To first evaluate the specificity of the in situ tetramer technique, the spleens of TCR-I and B6 mice were stained with Db/I tetramer (Fig. 3C). The images show that the Db/I tetramer (shown in green) stained specifically the CD8+ cells (shown in red) in the TCR-I mice (top row) but not in the control B6 mice (bottom row). The same tumor samples as shown in Fig. 3A were then stained using an in situ tetramer detection technique. The images in Fig. 3D demonstrate that the Db/I tetramer, but not the control Db/Flu tetramer, colocalized with CD8 on the surface of the GFP+ cells. In conclusion, histological analysis of tumor sections demonstrates that TCR-I T cells activated by FGK45 conditioning infiltrate the tumors and closely associate with tumor cells to mediate the anti-tumor immune response.

Persistence of TCR-I T cells in RT4 mice after treatment with FGK45

We observed that conditioning with FGK45 induces vigorous expansion of adoptively transferred TCR-I T cells. To determine how long TCR-I T cells persist, FGK45-treated RT4 mice were given adoptive transfers with TCR-I T cells. Mice were assessed at 10 and 20 days after adoptive transfer for the presence of TCR-I T cells in the spleens and pancreatic tumors. The number of TCR-I T cells recovered was compared with B6 mice that were adoptively transferred with TCR-I T cells followed by immunization with B6/WT-19 T Ag transformed-fibroblasts expressing the wild-type T Ag. The data in Fig. 4A show that TCR-I T cells are detected at high levels in both the spleen (15.5% of TCD8) and the tumor (20.5% of TCD8) at day 10, comparable to the frequency induced in B6 mice following immunization (22% of TCD8). However, by 20 days after adoptive transfer the frequency of TCR-I T cells was reduced to 0.15% in the spleen and 2.6% in the tumor, whereas the number of TCR-I T cells detected in B6 mice remained high (16.2% of TCD8) (Fig. 4B). The rapid decline in the numbers of TCR-I T cells in both the spleen and the tumor was not overcome by the additional administration of FGK45 10 days after the initial adoptive transfer (Fig. 4C). Altogether, the results indicate that treatment with FGK45 leads to a transient high level accumulation of TCR-I T cells in RT4 mice.

Conditioning with FGK45 augments TCR-I T cell-mediated control of tumor progression

To determine whether the adoptive transfer of TCR-I T cells plus FGK45 had a significant effect on tumor growth and survival, groups of RT4 mice aged 5 to 5.5 mo were treated with FGK45 or rat IgG and given adoptive transfers with TCR-I T cells. A third group of RT4 mice received three treatments with TCR-I T cells plus FGK45 on days 0, 20, and 30 to determine the effect of multiple rounds of adoptive immunotherapy. By comparison with untreated RT4 mice (median age to time of death of 197 days; Fig. 5A), a single administration of TCR-I cells plus FGK45 increased the lifespan to a median age of 235 days (p = 0.0162). Survival was further increased in mice that received three rounds of immunotherapy (median age to time of death of 265 days; p = 0.0004). Although we observed an increase in lifespan after three treatments with TCR-I plus FGK45 compared with mice that received only a single treatment, the difference between the two groups was not statistically significant (p = 0.0827). No significant increase in survival was observed for mice injected three times with FGK45 alone (median age to time of death of 201 days; p = 0.7589) or for mice given TCR-I T cells plus control rat IgG (median age to time of death of 190 days; p = 0.8324).

Additionally, we monitored the concentration of blood glucose in mice that received a single treatment with TCR-I T cells and FGK45 or control rat IgG (Fig. 5B) as an indicator of the effect on the tumor. We found that the concentration of blood glucose increased between the initiation of treatment and day 10 in RT4 mice treated with TCR-I T cells and FGK45. This was followed by a gradual decrease over the next 50 days. This observation suggests that TCR-I-based immunotherapy initially eliminated some of the insulin-producing tumor cells and is in agreement with the increased survival of this group of RT4 mice (Fig. 5A). Subsequent tumor progression after day 10 is associated with the loss of TCR-I T cells from RT4 mice between days 10 and 20 after adoptive transfer (Fig. 4). In contrast, blood glucose levels in RT4 mice treated with TCR-I T cells and control rat IgG remained stable or declined between day −1 and day 10 and is associated with lack of expansion of TCR-I T cells after such treatment (Fig. 1A). Over half of the mice in this control group died before day 60 after adoptive transfer. Taken together, these results demonstrate that the enhanced accumulation of TCR-I T cells within established tumors of RT4 mice is associated with the control of tumor progression and the prolonged survival of tumor-bearing mice.
FIGURE 5. Treatment with TCR-I T cells plus FGK45 reduces tumor progression in RT4 mice. Groups of RT4 mice aged 5 to 5.5 mo received adoptive transfer of $1 \times 10^6$ naive TCR-I T cells and were treated with either FGK45 or control rat IgG. A third group of RT4 mice received three treatments with TCR-I T cells plus FGK45 at days 0, 20, and 30. A fourth group of RT4 mice remained unmanipulated. A, Lifespans were monitored and plotted as the percentage of surviving animals vs age. The number of mice per group ($n$) and the median age of survival are indicated. Significance was determined by log rank compared with untreated RT4 mice. The bracket indicates a comparison of mice that received three doses of TCR-I T cells vs mice that received only a single dose. Asterisks indicate the curves that are significantly different from that of the untreated control group. B, Blood glucose levels in RT4 mice from A that received either a single treatment with TCR-I T cells and FGK45 or control rat IgG were measured and plotted over time beginning at 11 days before adoptive immunotherapy (day −11). Each line represents an individual mouse.

Discussion

The $T_{CD8}$ response to cancer is often characterized by epitope multiplicity, providing several potential targets for immunotherapy (3). Effective immunotherapy may require targeting multiple epitopes to avoid Ag loss variants and to promote efficient control of tumor progression (40). One complication of this strategy is that $T_{CD8}$ responding to some epitopes may be more sensitive to the effects of peripheral tolerance than others (8, 9, 41). This may be due to, among other factors, the biological properties of the epitope, the nature of the tumor or self-Ag-expressing tissue, or the sensitivity of the responding T cells to tolerance-inducing mechanisms. In this study, we demonstrate that conditioning the host with the anti-CD40 agonist Ab FGK45 can postpone the loss of $T_{CD8}$ responsiveness toward an epitope that is highly tolerogenic, resulting in a delay in the progression of established tumors.

The SV40 T Ag epitope I was shown previously to form relatively long-lived complexes with H-2D$^b$ molecules on the cell surface compared with the other H-2$^d$-restricted SV40 T Ag epitopes (27, 42). Although the naturally processed form of this peptide has not been defined, previous studies showed that the synthetic 10-mer peptide SAINNYAQKL rather than one of the 9-mer peptides binds most efficiently to H-2D$^b$ (42). The relatively high stability of epitope I/D$^b$ complexes could potentially lead to their high level accumulation at the tumor cell surface but also might play a role in sustained Ag presentation on the surface of professional APCs following cross-presentation compared with the other T Ag epitopes (43). Under noninflammatory steady-state conditions, high-level epitope presentation on professional APCs may result in tolerance induction of naïve epitope I-specific $T_{CD8}$ (35).

The speed with which TCR-I T cells are eliminated from RT4 mice provides a difficult challenge for the development of immunotherapeutic strategies. In the present study, the conditioning of tumor-bearing RT4 mice with FGK45 results in both an increase in the number of cells that accumulate in the lymphoid organs early after TCR-I T cell transfer and a dramatic infiltration into pancreatic tumors. The lack of infiltration of tumor-specific T lymphocytes into the tumor stroma has been viewed as a major obstacle to immunotherapeutic approaches to cancer (37, 44, 45). When T lymphocyte infiltration does occur, it has been shown to correlate with a positive disease prognosis (38, 39, 46, 47). Histological analysis of tumors has revealed that lymphocytes often fail to penetrate into the tumor stroma. Instead, T lymphocytes are detected at the tumor margin and fail to result in tumor elimination (48, 49).

Mechanisms that may limit T cell infiltration include immunologic ignorance (50), the presence of an irregular vasculature (37), and lack of proper homing receptors or chemokine production within the tumor (11). Additional treatments such as exposure to radiation or intratumoral injection of proinflammatory substances (e.g., CpGs) may be necessary to “open” the tumor stroma for efficient T cell infiltration (37, 51, 52). Our data clearly demonstrate that TCR-I T cells heavily infiltrate insulinomas and closely associate with tumor cells after the administration of FGK45, consistent with decreased tumor progression. It is not clear from the results presented here whether FGK45 conditioning of the host directly results in access to the tumor stroma or is a consequence of other events initiated following the induction of a robust immune response toward epitope I. In vivo conditioning of mice with
FGK45 could potentially lead to enhanced T lymphocyte trafficking into the tissues due to the triggering of CD40 on vascular endothelial cells that results in enhanced expression of adhesion molecules and increased lymphocyte binding (53, 54).

Based on previous studies in related mouse models, the mechanism by which anti-CD40 conditioning leads to enhanced priming against the endogenous T Ag is likely through the activation of professional APCs that have taken up T Ag via cross-presentation (10, 18, 19, 55). Thus, the positive effect of anti-CD40 conditioning may be most useful for tumor Ags that are efficiently cross-presented in vivo. A similar enhancement of TCD8 accumulation toward self-Ag following anti-CD40 conditioning has been observed in several other models (14, 56). Hernandez et al. (56) found that the anti-CD40 conditioning of mice that express influenza hemagglutinin from the rat insulin promoter induces a robust accumulation of adoptively transferred hemagglutinin-specific TCR transgenic T cells in the pancreatic lymph node. However, these T cells were deficient for IFN-γ production unless mice were also injected with IL-12. The authors suggest that CD40 ligation only partially substitutes for T cell help (56). We made a similar observation in RT4 mice in which a subset of tumor-infiltrating TCR-I TCD8 were able to produce IFN-γ, suggesting that a proportion of the tumor-infiltrating TCR-I T cells are anergized by 10 days after adoptive transfer despite promoting a delay in tumor progression. Using a model in which the lymphocytic choriomeningitis virus glycoprotein 33 (GP33) epitope is expressed ubiquitously from the H-2Kb promoter, Pircher and colleagues (14, 57) found that adoptively transferred TCR transgenic TCD8 specific for GP33 were rapidly tolerized in vivo. However, the treatment of GP33 transgenic mice with anti-CD40 induced a massive expansion of adoptively transferred TCR transgenic T cells that resulted in the destruction of both lymphoid organs and the liver, a clear sign that these T cells had acquired effector functions. It should be noted that T cell activation in the GP33 model is not dependent on cross-presentation of the Ag, because all cells are capable of presenting the epitope directly. Together, these studies demonstrate that anti-CD40 conditioning can result in a delay in TCD8 tolerance due to an increase in the magnitude of T cell expansion following the recognition of self-Ag in vivo and the subsequent infiltration of the Ag-presenting tissues.

The TCD8-mediated reduction in tumor progression shown in the current study contrasts with the results from a previous study in which TCD8 targeting the H-2Kb-restricted epitope IV, recruited from the endogenous repertoire of RT4 mice by immunization, were able to persistently infiltrate established tumors but were unable to alter tumor progression (9). In that case, the epitope IV-specific, tumor-infiltrating TCD8 retained their effector function and were not deleted from RT4 mice. Thus, the escape of insulinomas from TCD8 specific for these two epitopes might be mediated by different mechanisms. In the case of the endogenous epitope IV-specific TCD8, escape may be controlled by changes within the tumor that protect the tumor cells from the resident effector T cells (58). In contrast, tumor escape from epitope I-specific TCD8 is based on elimination of the responding TCD8. These results indicate that TCD8-based strategies for cancer that successfully target one epitope may not apply to all tumor-associated epitopes, even within the same tumor Ag.

We also note that the effect of conditioning with FGK45 on TCD8 persistence can vary dramatically when targeting the same epitope in two different models of self-Ag expression. Previously, we found that TCR-I T cells are also suboptimally triggered against endogenous T Ag in line 501 mice (5). These mice express T Ag from the α-amylase promoter, resulting in the development of osteogenic osteosarcomas (59). After initial proliferation, adaptively transferred TCR-I T cells are eliminated from the lymphoid tissues within 3 wk (5). However, FGK45 conditioning of line 501 mice led to long-term persistence of TCR-I T cells for >1 year vs the transient effect that was observed in RT4 mice. One possible explanation for this difference is that the presence of rapidly progressing tumors in RT4 mice leads to accelerated deletion and a more immunosuppressive environment than in the more slowly developing tumor environment of line 501 mice. This explanation seems unlikely, however, because TCR-I transfer plus FGK45 conditioning of RT4 mice before tumor formation results in similar kinetics of T cell response and deletion from the peripheral lymphoid tissues (data not shown). Alternatively, this difference may be explained by the unique nature of tissues expressing T Ag. Recent evidence suggests that pancreatic islet cells may be involved in the maintenance of self-tolerance. In a mouse model of diabetes, expression of the inhibitory ligand programmed death ligand 1 (PD-L1) on the surface of pancreatic islet cells was reported to be critical for inhibiting autoreactive PD-1-expressing CD4+ T cells (60). Moreover, deletion of autoreactive TCD8 targeting an Ag expressed by the pancreatic β cells was shown to be PD-L1-dependent, although the requirement for PD-L1 expression on the pancreatic cells themselves was not demonstrated (61). Thus, one explanation for the rapid onset of tolerance in line RT4 mice despite the robust triggering following anti-CD40 conditioning is that T cell interactions with Ag presented by the pancreatic islet cells accelerates the deletion of activated T cells.

Rapid elimination of TCD8 following anti-CD40 administration has been observed previously in several systems (14, 56, 62). In particular, Kedl et al. (62) demonstrated that the administration of anti-CD40 into mice inoculated 1 wk earlier with OVA-expressing B16 melanoma cells enhanced TCD8 accumulation within the tumors, but these TCD8 were eliminated from the host after 1 week. This rapid elimination could be prevented if the mice received simultaneous immunization with a recombinant vaccinia virus expressing the tumor Ag. The authors suggested that additional inflammatory signals are required to promote a long-lived T cell response. In RT4 mice the rapid deletion of TCR-I T cells from the lymphoid tissues is unlikely to be due solely to the administration of anti-CD40, because we previously found that immunization of RT4 mice with wild-type T Ag-transformed cells the day following adoptive transfer resulted in a similar expansion and attrition from the lymphoid organs (9). Thus, in the case of RT4 mice, rapid loss of the responding TCR-I T cells appears to be an intrinsic property of the immunologic environment. A recent study by Ahonen et al. (63) showed that a combination of signaling through CD40 and TLRs induces a more potent TCD8 response than triggering either pathway independently and leads to the induction of stable T cell memory. Thus, the provision of additional inflammatory signals in RT4 mice before or following the adoptive transfer of TCR-I T cells may prolong TCD8 survival and increase the control of tumor progression.

Additional help from CD4+ T cells may be required to establish a more prolonged tumor-specific TCD8 response (56, 64–66). In a model similar to that of RT4 mice, Ganss and colleagues (36) showed that T Ag-specific CD4+ T cells can inhibit the onset of tolerance among T Ag-specific TCD8. The adoptive transfer of T Ag-specific, in vitro activated CD8+ and CD4+ TCR transgenic T cells plus the application of Cpg G adjuvant resulted in T cell infiltration of tumors and the long-term survival of experimental mice. These data and similar results obtained in InsHA (56) and RIP-OVA (67) mice define the need for additional help from CD4+ T cells to achieve the long-term survival of tumor-specific TCD8 in the continued presence of self-Ag. Indeed, the onset of peripheral tolerance toward epitope I is accelerated in T Ag transgenic mice.
in the absence of CD4\(^+\) T cells (52). Identifying which components of T cell help contribute to this enhanced survival of TCD8 will be critical for the further design of immunotherapeutic approaches.

In conclusion, the present results show that the development of peripheral tolerance can be delayed by the administration of an anti-CD40 agonist Ab, resulting in enhanced priming against the endogenous T Ag. The fact that the endogenous expression of a self-Ag can be used for the expansion of tumor-reactive T cells without any need for specific immunization following the administration of an anti-CD40 mAb might be an important consideration for therapy in humans. Efficient accumulation of functional tumor-specific TCD8 within established tumors was associated with a significant delay in tumor progression. Thus, even highly tolerogenic tumor Ags can be targeted for TCD8-mediated tumor immunotherapy.

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Disclosures

The authors have no financial conflict of interest.

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