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The Rate of the CD8-Dependent Initial Reduction in Tumor Volume Is Not Limited by Contact-Dependent Perforin, Fas Ligand, or TNF-Mediated Cytolysis

Joseph A. Hollenbaugh,*† Joyce Reome,* Mark Dobrzenski,* and Richard W. Dutton2*

Established EG7 tumors expressing OVA and growing at an intradermal site become rapidly reduced in size following adoptive therapy with in vitro-generated type I CD8 T cell (Tc1) effectors generated from naive CD8 T cells from transgenic TCR OVA-specific mice. Tc1 effectors kill EG7 target cells in vitro by a perforin-dependent mechanism. However, we show that there is no quantitative diminution of the initial phase of antitumor activity in vivo, whether the Tc1 effectors are derived from perforin-, Fas ligand-, or TNF-deficient transgenic TCR mice or whether the recipients are perforin deficient. Tumors are also equally well controlled whether the Tc1 effectors come from mice deficient in perforin plus Fas ligand or perforin plus TNF. Control of tumor growth is diminished when Tc1 effectors generated from IFN-γ-deficient mice are used. We conclude that control of tumor growth is not in any way affected by loss of contact-mediated lytic mechanisms, and conclude that the CD8 effectors must act by recruiting host effector mechanisms to control tumor growth. The Journal of Immunology, 2004, 173: 1738–1743.

Initially, CD8 T cells were distinguished from CD4 T cells by their ability to bring about Ag-specific contact-mediated lysis of target cells (1, 2). In short-term assays of cytolytic function, it was shown that perforin-dependent lysis was the most prominent mechanism, but that Fas ligand (Fas-L)3 and TNF interactions could also bring about target death, albeit much less effectively (3).

CD8 T cells have been shown to be effective in the elimination of tumors in a number of experimental models (reviewed in Ref. 4) and it was generally assumed that perforin-mediated lysis was involved in the antitumor effects. With the advent of perforin-deficient mice, it was shown that perforin-deficient mice were more susceptible to the development of tumors in a variety of situations (5). The inability of perforin-deficient mice to reject tumors (6, 7) was not necessarily due to a lack of CD8 T cell-mediated protection because perforin is expressed in a variety of other cells that may be involved in tumor rejection. More recently, a number of investigators have shown that adoptively transferred CD8 T cells from perforin-deficient mice were in some cases less effective than cells from wild-type (wt) mice (8), but in other cases could still bring about tumor rejection in a number of models (9, 10). In the study by Poehlein and colleagues (10) it was shown that control of tumor by perforin IFN-γ double-knockout cells could be blocked by injection of Ab to TNF, but it was not clear from which cells the TNF was derived. In others, it was shown that control of tumor growth was Fas-L dependent rather than perforin dependent (7). In many of the cases where it was shown that cells from perforin-deficient mice could still reject tumors, the measure used to gauge the efficacy of adoptive therapy was prolongation of survival or the generation of tumor-free mice, and there was no quantitative measure of the efficiency of rejection by deficient vs wt cells (11, 12). These measures give an overall picture of the effect of therapy but do not provide any kinetic information about the control of tumor growth.

Thus, although it is clear that CD8-dependent rejection of some tumors can occur in the absence of perforin, it is still unclear whether perforin-mediated lysis can facilitate tumor rejection.

Two key questions are addressed in the current study, with respect to the role of CTL activity of CD8 T cells in the control of tumor growth. The first is whether CD8-dependent tumor rejection is more efficient in the presence of perforin, and the second is whether other cytolytic mechanisms can compensate when perforin is missing.

We have used a tumor growing in a superficial site whose size can be monitored daily and we have established that there are two phases to the antitumor response. First, there is an initial phase of rapid reduction of tumor size (Phase 1) following adoptive transfer of effectors that commences 2–4 days after transfer and is complete by day 7–14. There is then a second phase (Phase 2) to day 22 and beyond, during which the tumor may or may not regress. The purpose of this current study is to examine the mechanisms involved in the initial phase.

We have used this model to examine the role of perforin-, Fas-L-, or TNF-mediated lysis of the tumor in the Phase 1 reduction in tumor volume brought about by the adoptive transfer of type I CD8 T cell (Tc1) effectors. We find that Tc1 CD8 effectors from deficient mice are quantitatively as effective as those from wt mice as we had previously shown for Phase 2 (13).

In addition, we have shown that CD8 effectors from perforin plus Fas-L doubly deficient mice and effector from perforin plus TNF-α/lymphotoxin-α (LT-α)-deficient triply deficient transgenic TCR (OT-1) mice have similar efficacy to effectors from wt mice in bringing about the initial reduction in tumor volume in Phase 1.

We conclude that the in vitro-generated CD8 effectors do not kill the tumor target by direct contact-mediated lysis but merely...
serve as the trigger to some host-mediated killer mechanism. The nature of the mechanism or mechanisms whereby the adoptively transferred cells and the host may bring about the initial attack on the tumor is discussed.

Materials and Methods

Mice

C57BL/6J, B6Smn.C3H-Fasl (generalized lymphoproliferative disease (GLD)), C57BL/6-Prf1tm1Sdz/J, and C.129S7(B6)-Ifngtm1Ts/J, were obtained from Jackson Laboratories or bred in the Trudeau Institute and used as recipient mice. TCR transgenic OT-I mice (13) specific for the OVA peptide, SIINFEKL, were originally obtained from Dr. S. Hedrick (University of California at San Diego, La Jolla, CA) and maintained as heterozygotes, were backcrossed for multiple generations to C57BL/6J, and were used as the source of donor cells. TNF-α/H9251/LT-β/H9251 mice were obtained from Dr. J. D. Sedgwick (DNAX, Palo Alto, CA) (14). In some experiments, donor cells were obtained either from wt OT-1 mice, or OT-1 mice crossed with C57BL/6 perforin/H11002 mice, GLD mice, or TNF-α/H9251/LT-β-deficient mice. Donors were also obtained from OT-1 perforin/H11002 mice crossed to OT-1.TNF-α/H9251/LT-β-doubly deficient mice to produce perforin/TNF-α/H9251/LT-β-triply deficient donor mice.

Cell lines

The parent H-2b cell line EL4 and the OVA-transfected EG7 were obtained from American Type Culture Collection (Manassas, VA).

Cell preparations

CD8 T cells were isolated from the spleen and lymph nodes of the OT-1 TCR transgenic mice and single-cell preparations were made. CD8 cells were positively selected using the MACS system (Miltenyi Biotec, Auburn, CA). The freshly isolated CD8 T cell populations were 95–98% CD8+Vβ8+ T cells. Splenic APCs (B cell blasts) were enriched from C57BL/6 mice by T cell depletion using anti-Thy1.2 (HO13.14), anti-CD4 (RL172.4), anti-CD8 (3.155), and anti-CD3 mAbs and complement.

Tc1 effector generation

CD8 cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with penicillin, streptomycin, glutamine, 2-ME, and 7% FCS (HyClone, Logan, UT). Three-day LPS-stimulated B cell blasts from C57BL/6 mice were used as APCs and were loaded with the OVA peptide (11 μM) at 37°C for 30 min, treated with mitomycin C (100 μg/ml; Sigma-Aldrich, St. Louis, MO) at 37°C for 40 min and washed three times before use. CD8 effector T cells were prepared from the OT-1 transgenic mice by 4-day culture under polarizing conditions as previously described (15). On day 4 of culture, effectors were 98–99% CD8+Vβ8+ cells.

CTL assays

Tc1 effector cell cytolytic activity was determined in 48-h assay using BODIPY Red (Molecular Probes, Eugene, OR) -labeled EL4 (control) and CFSE (Molecular Probes) -labeled peptide-pulsed EL4 (target) and fluorescent microspheres as described in the text.

Intradermal tumor establishment

Syngeneic C57BL/6 mice were injected intradermally with 3 x 10⁶ tumor cells EG7 tumor cells in 50-μl volumes of sterile PBS in the right flank skin. Tumor cells for injection were recovered from log-phase in vitro growth under 400 μg of G418 (Invitrogen). Tumors were clearly visible after 1 wk and grew progressively in untreated mice.

Tumor measurement and conditional survival

Tumors were measured on two perpendicular axes using a Vernier caliper. Tumor sizes were approximated by multiplying the measured lengths. Tumor size was determined every 2–3 days. Percentage of tumor reduction was determined by using the tumor size average of the test group divided by the tumor size average of the PBS-injected control for that recipient strain on day 6 posttreatment. Nonparametric ANOVA or Kruskal-Wallis were used to determine the significance of differences between treatment effectors.

![Image](http://www.jimmunol.org/Downloadedfrom/1739TheJournalofImmunology)

FIGURE 1. C57BL/6 mice were injected with 3 x 10⁶ tumor cells intradermally on day −7. The mice were then left untreated (a) or injected in the tail vein with the indicated numbers of wt Tc1 effectors (b–f) from OT-1 mice. Tumor size was measured from day 0 to 22. Plots represent tumor growth in individual mice.
Adoptive immunotherapy of tumors

Varying numbers of Tc1 effector cells were injected in 500-μl volumes of sterile PBS. Injections were delivered i.v. in the tail vein.

Results

Control of tumor growth by adoptively transferred Tc1 effectors occurs in two phases

In our earlier published studies with the EG7 thymoma (15), we had measured tumor growth at 7-day intervals, which did not reveal the early events following adoptive therapy. In the current studies, we have measured tumor size every 2 days and found that the tumor continues to grow for 2–3 more days after adoptive therapy and then shrinks rapidly in size and is almost eliminated by day 8–14. We have called this Phase 1. This is followed by a second phase (Phase 2) to day 22 during which the tumor may or may not regrow. The separation of the response into a Phase 1 and Phase 2 allows us to determine the difference in which factors are important in each phase.

Groups of eight individual wt B6 mice were injected intradermally with 3 × 10^6 tumor cells on day –7. PBS or increasing numbers (from 3 × 10^3 to 3 × 10^6) of Tc1 effectors were injected i.v. on day 0. The growth curves for the tumor in individual mice in each group are shown in the six panels of Fig. 1 (a–f). In the untreated controls (a), the tumor grew exponentially and the mice had to be sacrificed by day 6–10. As few as 3 × 10^3 Tc1 (b) brought about a marked pause in tumor growth that began at day 4. At 3 × 10^5 Tc1 (d), the inhibitory effect was apparent by day 2 and most of the tumors were no longer detectable by day 14. The rate of tumor growth and the effectiveness of in vitro-generated Tc1 effectors varied somewhat from experiment to experiment, but the overall pattern of events was seen in all experiments.

We used the control of tumor growth in Phase 1 to establish a quantitative relationship between the reduction in tumor growth and the number of adoptively transferred Tc1 effectors. This is shown in Table I. In the first four columns, we have calculated the percentage of reduction in tumor size for Phase 1, and the second four columns show the results of a statistical analysis of any differences. The third and fourth group of four columns provide a numerical analysis of the course of tumor growth in Phase 2. This was then used to compare the efficiency of populations of effectors from wt or genetically deficient mice.

The efficiency of Tc1 effectors from mice deficient in perforin

OT-1 was crossed to perforin-deficient mice to provide perforin "−/−" OT-1 mice. Graded numbers of Tc1 effectors from wt or perforin "−/−" OT-1 mice were adoptively transferred into wt or perforin "−/−" recipients bearing tumors arising from injection of EG7 7 days previously. The complete data set is presented for this first experiment and only summary data are presented for the remaining experiments.

Fig. 2a plots the average tumor size in each group of eight mice against time and shows that the tumor grew progressively in the control mice injected with PBS. Mice with the largest tumors were sacrificed after measurement of the tumor size at day 6 (as can be seen from the data in Table I, Conditional Survival), leading to an apparent drop in average tumor size at day 8. We used the data at day 6, the last day that all of the mice remained in the study, to calculate the percentage of reduction of tumor after adoptive therapy with each number of Tc1 effectors as shown in Table I (Percent Tumor Reduction).

Injection of increasing numbers of Tc1 from wt or perforin "−/−" mice into wt or perforin "−/−" recipients led to an increasing control of tumor growth (Fig. 2, b–f). Statistical comparisons using the Kruskal-Wallis Test (nonparametric ANOVA) were made at day 6, when all mice were still in the data pool, and there were no significant differences between the four groups, except in the one case indicated, in which 3 × 10^5 Tc1 effectors from wt mice into perforin "−/−" host were actually more effective than wt into wt (see Table I, Statistical Analysis). We conclude that there was no involvement of either donor or host perforin in the control of tumor growth in Phase 1.

Table I (Tumor Free) shows the number of tumor-free mice at day 22 and it can be seen that perforin-deficient donors or recipients were no less effective in generating tumor-free mice in Phase 2 after day 12. Mice were sacrificed when the tumor grew to be 19 mm in diameter or greater, and Table I also shows what we are calling the “conditional survival” (to indicate those alive after sacrificing the mice with large tumors) for the same four groups of mice. It shows that again there were no significant differences between groups.

The data presented in Table I (Percent Tumor Reduction), showing the percentage of reduction in average tumor size against the number of adoptively transferred Tc1 effectors, is graphed in Fig. 3a and demonstrates two important facts: 1) The percent reduction

<table>
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<th>Host Mouse Donor Cells</th>
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<th>Tumor Free (D22)d</th>
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a The data collected as shown in Fig. 1 show the tumor size for eight individual mice for each number of transferred Tc1 cells every 2 days, following transfer from day 0 to 22. The data is presented here in summary form.

b First, the average tumor size is calculated for each group of mice at day 6, and the percent reduction in tumor size compared with the untreated mice is shown for each dose of Tc1 cells transferred. The data refer to the effects seen in Phase 1.

c The statistical comparisons refer to the differences observed at day 6 when all mice are still in the data pool and indicate the p value for which reductions differ between deficient and wt mice. The data refer to the effects seen in Phase 1.

d The number of mice that are tumor free against the total number of mice in each group. The data refer to effects seen in Phase 2.

e The number of mice with conditional survival against the total number. The conditional survival is the number of mice that have not been sacrificed because their tumors had grown larger than 19 mm in diameter. The data refer to effects seen in Phase 2.
in tumor size at day 6 increases with the logarithm of the number of Tc1 effector cells transferred; and 2) the effect of Tc1 effectors from wt and perforin-deficient mice is quantitatively equivalent.

**Tumor cell lysis by in vitro-generated Tc1 effectors is perforin dependent in an extended 48-h in vitro assay**

We had previously shown that Tc1 effectors from perforin-deficient OT-1 mice kill EG7 targets in vitro <1% as efficiently as those from wt OT-1 mice (13, 15). However, we worried that we were comparing a 4-h in vitro Cr release assay with a potentially much longer contact period in vivo. Therefore, we developed a 48-h in vitro assay in which we could compare the efficiency of the Tc1 effectors.

Peptide-pulsed EL4 target cells were stained with CFSE, and control EL-4 cells without peptide were stained with BODIPY Red. Tc1 effectors were generated by 4-day culture of peptide-stimulated naïve CD8 T cells from OT-1 wt and perforin-deficient mice under cytokine-polarizing conditions, and their function and phenotype were confirmed (data not shown). Equal numbers of target cells and control cells were incubated together with graded numbers of CD8 effectors and the ratio of the numbers of the two dye-labeled cells determined by flow cytometry at the end of the timed incubation period. The percentage of lysis of the target cells was then calculated. The calculation is similar to that for the 4-h assay, but the situation is somewhat more complicated than in the conventional assay, as both the control and still-surviving target tumor cells divide during the course of the assay.

The reduction in the number of target cells can in part be due to cell lysis and in part due to inhibition of cell growth. To monitor the extent of growth we added fluorescent microbeads to cultures with control cells at the end of the incubation period immediately before assay, and compared the ratio of cells to beads with the ratio in cultures when microbeads were added and assayed at the start before culture. The percentage of killing is calculated as 1 minus the ratio of target to control over the ratio of target to control in the absence of Tc1. We have used this dye label assay to look at cytolyis using longer incubation times. As can be seen in Fig. 4, 100 times more effector cells from perforin-deficient mice are required to achieve the same amount of lysis or inhibition of tumor growth seen with wt effectors even in a 24-h or 48-h assay.
In the same experiments, we found that Fas-L-deficient hosts were as able to control tumor growth as wt hosts. TNF-α/LTα-deficient recipients, lacking organized lymph nodes, were less able to control tumor growth in Phase 2 (data not shown).

The efficiency of Tc1 effectors from IFN-γ-deficient mice

We had previously shown that Tc1 effectors from IFN-γ-deficient OT-1 mice were less effective than those from wt OT-1 mice but had used a model that did not distinguish between Phase 1 and Phase 2 events (15). Therefore, we repeated the analysis following the same protocol as described above so that we could make direct comparison with the current data on mice with lytic defects. As can be seen in Fig. 3d, the Tc1 effectors from IFN-γ-deficient OT-1 mice were markedly less efficient in bringing about the rapid initial Phase 1 reduction in tumor volume. The tumor was very poorly controlled when low numbers of Tc1 effectors were injected but the adoptively transferred cells were able to control tumor growth when large numbers were injected.

Discussion

In the current studies, we made a detailed analysis of the control of tumor growth following immunotherapy and found that the effect on tumor growth can be separated into two phases (Fig. 1). In Phase 1, the tumor continues to grow for 2–4 days following adoptive therapy, presumably while sufficient Tc1 effectors migrate to the tumor site. This is followed by a rapid reduction in tumor size so that the tumor is no longer detectable by visual inspection or palpation by day 7–14. In successful immunotherapy, the tumor remains undetectable for at least 22 days but can reappear if the therapy is inadequate. This we have called Phase 2.

We had previously shown that in vitro cytolytic activity of Tc1 effectors from OT-1 mice was >99% perforin dependent in the standard 4-h chromium release assay (16, 17) and was undiminished in Tc1 or Tc2 effectors from either Fas-L or TNF-α/LTα-deficient mice. We realized that we were comparing a 4-h in vitro reduction when large numbers were injected.

The efficiency of Tc1 effectors from mice deficient in multiple lytic mechanisms

We were concerned that effectors deprived of one lytic mechanism might control tumor growth by an alternate lytic pathway. First, we compared the effect of Tc1 from wt and from perforin and Fas-L doubly deficient mice (Fig. 3b), and second, the effect of Tc1 from wt and perforin plus TNF-α/LTα triply deficient mice (Fig. 3c) using the same protocols described above. It can be seen that OT-1 Tc1 effectors from donor mice deficient in any of the lytic mechanisms tested were quantitatively as effective as those from wt mice.

FIGURE 3. The percent reduction in tumor growth after adoptive therapy with graded numbers of Tc1 effectors was calculated from the average tumor growth curves. The percent reduction in tumor growth was plotted against the number of Tc1 effectors injected on day 0 into mice injected with 3 × 10^6 tumor cells on day –7. a. Tc1 effectors from wt mice (■) vs from perforin-deficient mice (□) injected into wt recipients; b. Tc1 effectors from wt mice (■) vs from perforin and Fas-L doubly deficient mice (□) injected into wt recipients; c. Tc1 effectors from wt mice (■) vs from perforin and TNF-α/LTα triply deficient mice (□) injected into wt recipients; and d. Tc1 effectors from wt mice (■) vs from IFN-γ-deficient mice (□) injected into wt recipients.

FIGURE 4. Peptide-pulsed EL-4 targets were labeled with CFSE and control EL-4 (without peptide) were labeled with BODIPY Red. Equal numbers of target and control cells were incubated together with Tc1 effectors from wt and perforin-deficient mice in various E:T ratios and cultured for 4, 24, or 48 h. The number of CFSE- and BODIPY-labeled cells were determined by flow cytometry, and the percentage of target lysis was calculated by the formula 1 minus EL4 peptide/EL4 control divided by EL4 target/EL4 control times 100.

EG7 expresses Fas (CD95) in vitro and we presume that it expresses Fas in vivo. We speculated that effectors might kill tumors by Fas-L-mediated lysis in the absence of perforin expression. However, we found that effectors from the Fas-L-deficient OT-1 GLD mice were also as effective as those from wt OT-1 mice (data not shown). This contrasts with the situation when adoptive immunotherapy is used to control B16 melanoma growing in the lung. Fas is up-regulated in B16 on exposure to IFN-γ in vitro and Tc1 effectors from Fas-L-deficient OT-1 mice are less effective at controlling tumor growth (16–18). Of more concern, it was possible that effectors could kill target cells by either perforin or Fas-L-mediated lysis. However, we found that Tc1 effectors from OT-1 mice doubly deficient in perforin and Fas-L were still quantitatively as effective as those from wt OT-1 mice (Fig. 3b).

Perforin and Fas-L are considered to be the major instigators of the lytic pathway, but TNF-α and LT-α can bind to TNFR1 and trigger apoptotic pathways and mediate contact-dependent lysis of target cells, and have been implicated in tumor control in other models (10). Therefore, we determined the efficiency of Tc1 effectors from OT-1 mice deficient in TNF-α/LT-α (data not shown) and effectors from mice deficient in perforin and TNF-α/LT-α in controlling EG7 growth. In neither case was there any reduction in the efficiency of the initial Phase 1 control of tumor size (Fig. 3c).

Therefore, we conclude that direct contact-mediated lysis has no
role in the mechanism(s) by which control of tumor growth is achieved. Tc1 effectors from IFN-γ-deficient mice were less effective than those from wt mice (Fig. 3d) in the rapid initial reduction in tumor volume. This is in accord with our previous findings in less discriminatory models (15, 19, 20) and those of others (10–12, 21–23), and provides the positive control for the negative finds with the lytic mechanisms.

In retrospect, it is perhaps not surprising that direct contact-mediated lysis does not seem to be involved in tumor reduction. When Tc1 effectors are incubated with target cells in the 48-h in vitro CTL assay, measurable killing is seen down to the E:T ratio of 0.2:1. It is difficult to determine the E:T ratio in vivo and one can only make a guess as to how low it might be. If we assume that the initial inoculum of 3 × 10⁶ EG7 cells grows during the 7 days before adoptive therapy, we can guess, conservatively, that after five doublings that number of tumor cells is of the order of 10⁸ by the time the Tc1 effectors arrive at the tumor site. The tumor is >10 mm in diameter by day 10, which gives a volume of around one cubic millimeter commensurate with a content of 10⁸ thymocytes. Rapid and almost complete reduction in tumor volume can be seen after i.v. injection of as few as 10⁶ cells. However, it is doubtful whether such low ratios could ever bring about the rapid lysis of the tumor that we observe and at best could only kill enough tumor cells to trigger some host response.

So how do the Tc1 CD8 effectors actually bring about the initial rapid reduction in tumor volume? Some investigators believe that CD8 T cells do kill by a cell-mediated lytic mechanism but that it is necessary to knock out all of them to see an effect (10). Others have suggested that CD8 T cells kill by some indirect mechanism involving host cells, or by cutting off the blood supply or the supporting stroma. We argue against the first explanation based on our results, reported in this study. Although we have not made and tested effectors from mice deficient in all known lytic pathways, mice deficient in two or three of the known pathways were quantitatively as effective as those from wt mice and we are also impressed with the theoretical argument, made above, that the E:T ratios can be expected to be too low to be effective. The second hypothesis invokes a role for host cells. This could take the form of some non-Ag-specific process, which could potentially eliminate bystander tumor cells lacking the tumor Ag. It is also possible that the CD8 delivers some Ag-specific signal to the target that does not bring about lysis per se, but renders the target vulnerable to attack by some host-mediated process, such as attack by activated macrophages, NK cells, neutrophils, or eosinophils. This would preserve the Ag-specific nature of the rejection process.

The CD8 effectors can be expected to secrete cytokines and chemokines and these will recruit host cells to the tumor site, and more inflammatory chemokine secretion may be induced by the cytokines, such as IFN-γ released by the donor T cells. In our earlier study (15), we saw increased B cell, NK cell, macrophage, and neutrophil recruitment to the draining lymph nodes following adoptive transfer with Tc1 effectors, and the amount of recruitment was diminished when the effectors came from IFN-γ-deficient mice. The role of these cells is under investigation.

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References