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*J Immunol* 2013; 191:3534-3544; Prepublished online 9 September 2013; doi: 10.4049/jimmunol.1300803

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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2013/09/09/jimmunol.1300803.DC1

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Rapid Deletion and Inactivation of CTLs upon Recognition of a Number of Target Cells over a Critical Threshold

Sandro Prato,*†‡ Yifan Zhan,* Justine D. Mintern,*§ and Jose A. Villadangos*‡§

Initiation of CTL responses against foreign pathogens also primes anti-self CTLs. Mechanisms of CTL inactivation inhibit anti-self CTLs to prevent tissue damage. These mechanisms are exploited by pathogens and tumors to evade the immune response, and present a major hurdle to adoptive CTL therapies. It is unclear whether CTL inactivation is Ag specific and, if so, which APCs are involved. Potential candidates include the target cells themselves, dendritic cells, myeloid-derived suppressor cells, and macrophages. In this study, we show that lymphoma-specific CTLs are rapidly deleted in an Ag-specific manner after adoptive transfer into lymphoma-bearing mice, and the surviving CTLs are functionally impaired. The only APCs responsible were the target cells directly presenting Ag, notwithstanding the presence of myeloid-derived suppressor cells, and CD8+ dendritic cells cross-presenting tumor Ag. The capacity to inactivate CTLs critically depended on the number of tumor/target cells; small numbers of targets were readily killed, but a large number caused quick deletion and functional inactivation of the CTLs. Application of mild, noninflammatory, and nonlymphoablative chemotheraphy to specifically reduce tumor burden before CTL injection prevented CTL deletion and inactivation and allowed eradication of tumor. Our results advocate the use of adoptive CTL therapy soon after mild chemotherapy. They also suggest a simple mechanism for Ag-specific impairment of anti-self CTLs in the face of an active anti-foreign CTL response. The Journal of Immunology, 2013, 191: 3534–3544.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1300803
elimination. Therefore, selective reduction of target cell numbers may be a relatively simple, but effective, strategy to maintain CTL activity against tumors and chronic infections.

**Materials and Methods**

**Mice**

Mice were maintained and bred under specific pathogen-free conditions in the Walter and Eliza Hall Institute and Bio21 Institute animal facilities, C57BL/6J (Ly5.2), C57BL/6-Pep3b (Ly5.1), F1 (Ly5.1 x B6.CH-2mml [bm1]), MHC I (+) (17), IFN-γ (+) x OT-I (18, 19), B6.BafF1 (+) (20), Ly5.1 x OT-I (19), and gB7T-I (21) mice were used between 6 and 12 wk of age; experimentation was performed in accordance with the Institutional Animal Care and Use Committee guidelines at the Walter and Eliza Hall Institute and the University of Melbourne.

**Ep-myc lymphomas**

Generation, i.v. inoculation, and growth of the Ep-myc-GFP and Ep-myc-OVA lymphomas have been described (27).

**In vitro activation of OT-I and gB7T-I**

Activated OT-I (wild type [WT] or IFN-γ+/−) or gB7T-I cells were generated as described (22). Cultures typically contained 94–98% CD8+V2+ cells.

**CTL analysis following transfer into Ep-myc lymphoma-bearing mice**

Ly5.1 x gB7T-I CTLs (6 x 10^5) and/or Ly5.1 x OT-I CTLs (5 x 10^5) or IFN-γ−/− OT-I CTLs were injected i.v. and spleens or lymph nodes were harvested at the indicated times for analysis. Organs were disrupted through a 70-μm cell strainer (BD Falcon). Cell suspensions were treated with red cell removal buffer, washed, and resuspended in an Ab mixture containing fluorochrome-bound anti-CD8 (YTS 169.4), B220 (RA3-6B2), with red cell removal buffer, washed, and resuspended in an Ab mixture containing fluorochrome-bound anti-CD8 (YTS 169.4), B220 (RA3-6B2), and CD4 (GK 1.5), and OT-I CTLs (Ly5.1+CD8+) were purified, washed twice, and incubated in the Ab mixture containing fluorochrome-bound anti-CD11c, CD11b, F4/80, and the activation marker anti-MHC II. Where indicated, LPS (0.5 μg/ml) was added for 12 h. After incubation, cells were analyzed by flow cytometry.

**Presentation of lymphoma-associated OVA in vivo**

Ag in mice bearing bm1.Ep−myc cells or myeloid cells were prepared by cytospin centrifugation onto glass slides and stained with May-Grunwald-Giemsa. Slides were viewed and photographed using a compound microscope (Zeiss) and a digital camera (Axiocam; Zeiss).

**Cytokine-production assay**

Sera collected from tumor-bearing mice were investigated for cytokine content using the 23-plex assay on the BioPlex 2200 system, according to the manufacturer’s protocol (Bio-Rad, Hercules, CA) (26).
5 × 10^4 CFSE-labeled OT-I cells. The number of proliferating OT-I cells was determined 60–65 h later. Each assay was performed in duplicate.

**Cyclophosphamide treatment**

Tumor-bearing mice were injected i.p. with vehicle (H_2O) or cyclophosphamide (CTX; Sigma). To determine the effect of combined chemoinmunotherapy, tumor-bearing mice were injected i.v. with 5 × 10^5 OT-I CTLs 1 or 3 d following CTX treatment.

**Data analysis**

Quantitation of CTLs, tumor cells, or other cell types was performed by addition of 5 × 10^5 blank calibration particles (6.0–6.4 μm; BD Biosciences) per sample prior to flow cytometry. The p values were calculated using a two-tailed unpaired Student t test or a two-tailed Mann–Whitney U test when data failed normality tests.

**Results**

**Deletion and inactivation of adoptively transferred antilymphoma CTLs in mice with high tumor burden**

We described two new variants of the mouse Eμ-myc lymphoma, a well-established model of human non-Hodgkin lymphoma. They express GFP alone (Eμ-myc-GFP control tumor) or with OVA as a model neoantigen (Eμ-myc-OVA tumor) (27). The Eμ-myc-OVA tumor recapitulates several features described for human lymphomas: it is susceptible to anti-OVA CTL killing but is ignored by the immune system of tumor-bearing mice (i.e., it does not elicit anti-OVA CD8+ T cell priming spontaneously), and although it is possible to prime endogenous anti-OVA CD8+ T cells in Eμ-myc-OVA tumor-bearing mice with an anti-OVA vaccine, the T cells do not become effective CTLs (27). In this scenario, adoptive cell therapy with high-avidity anti-OVA CTLs (OT-I) might be a suitable approach to overcome immunosuppression of the endogenous repertoire.

Mice in which Eμ-myc-GFP or Eμ-myc-OVA tumors (FSC^high B220^intGFP+) had been growing for 3–5 d were treated with anti-OVA OT-I CTLs generated in vitro (22) (Fig. 1A). Eμ-myc-OVA lymphoma was almost eradicated within 48 h if OT-I CTLs were transferred 3 d after tumor inoculation (Fig. 1A, 1B). This was Ag specific because control Eμ-myc-GFP tumors were not affected. Furthermore, Eμ-myc-OVA tumor–bearing mice receiving CTLs eventually succumbed to tumor growth, but the expanded tumor did not express GFP and was no longer recognized by anti-OVA CTLs (Fig. 1C and data not shown). Therefore, OT-I CTLs injected at day 3 exerted enough pressure on the tumor to select immunoedited cells, a clear indication of effective Ag-specific antitumor activity. In contrast, if the OT-I CTLs were injected 4 d after tumor inoculation, tumor cell killing was less effective; injection on day 5 had no significant effect (Fig. 1A, 1B). To address whether this was due to loss of CTL activity upon encounter of large tumors, we compared CTL recovery, phenotype, and function two days after injection of the CTL at the three time points.

Mice bearing Eμ-myc-GFP or Eμ-myc-OVA for 3 d were injected with OT-I CTLs. Two days later, the number of CTLs in spleen was equivalent (Fig. 2A). These two groups of mice contained more CTLs than did mice with no tumor, probably because the larger size of the spleens in the tumor-bearing mice provided more “space” for CTL accumulation. The number of CTLs recovered from Eμ-myc-OVA–bearing mice that received OT-I CTLs 4 d after tumor inoculation was more variable (Fig. 2B). Few CTLs were recovered from mice treated 5 d after tumor inoculation (Fig. 2C). The remaining CTLs expressed low TCR and high PD-1, signature markers of exhausted T cells (2) (Fig. 2D, 2E). The phenotype of the CTLs injected on day 5 (large tumor) contrasted with the phenotype of CTLs injected on day 3 (small tumor). Indeed, virtually all CTLs displayed PD-1 upregulation in both situations, indicative of Ag encounter, but those injected at day 5 expressed higher levels of PD-1 and had downregulated TCR expression (Fig. 2D, 2E). The killing activity of the OT-I was assessed in vivo by injecting peptide-pulsed splenocytes 2 d after injecting CTLs (Fig. 2F). Splenocytes were killed in Eμ-myc-GFP tumor–bearing mice receiving OT-I CTLs 3 or 5 d after tumor inoculation or in mice bearing a low burden of Eμ-myc-OVA tumor (day 3) but not in mice with a high Eμ-myc-OVA tumor burden (day 5, Fig. 2F). This was not due to competition between the target splenocytes and the tumor cells. Indeed, the CTLs remaining in mice treated at day 5 were intrinsically defective, because when we purified them from mice treated at day 5 and assessed their killing activity ex vivo, they displayed little lytic activity (Fig. 2G), consistent with their incapacity to secrete cytokines upon restimulation (Fig. 2H). This experiment also showed that the tumor had not become resistant to CTLs at day 5, because it could be killed ex vivo (Fig. 2H, and see below). In summary, in mice bearing large lymphomas most of the injected CTLs were eliminated, and those remaining had lost effector function so that they were incapable of killing tumor cells and other cells, in vivo or ex vivo.

**CTL inactivation requires direct encounter of cognate Ag**

Next, we established to what extent Ag-specific CTL inactivation required direct contact with an APC. An alternative possibility was that Ag recognition by a few CTLs triggered sup-
pressive cytokine release that indirectly impaired the majority of transferred CTLs. Distinguishing between these two scenarios is important because if most CTLs are inactivated indirectly by cytokines, neutralization of these cytokines might restore activity, but if inactivation is mediated by direct CTL–APC contact, identification of the relevant APC becomes critical.

Mice bearing Eμ-myc-GFP or Eμ-myc-OVA lymphoma for 5 d were coinjected i.v. with equal numbers of gBT-I CTLs and CFSE-labeled OT-I CTLs (Fig. 3A). The transgenic gBT-I CTLs recognize a H-2Kb–restricted HSV-1 glycoprotein B epitope (gB498–505) (21), absent in the mice. Three days later, mice bearing control Eμ-myc-GFP lymphoma contained similar numbers of gBT-I and OT-I CTLs, with normal TCR and PD-1 levels (Fig. 3B). In contrast, in mice containing Eμ-myc-OVA lymphoma, gBT-I CTLs displayed normal TCR and PD-1 expression, whereas OT-I CTLs exhibited an exhausted phenotype (Fig. 3C). Furthermore, the number of OT-I CTLs in spleens of mice bearing Eμ-myc-OVA lymphoma was lower than in Eμ-myc-GFP–bearing counterparts, whereas the number of gBT-I CTLs was three times higher (Fig. 3B, 3C). This suggests that tumor Ag recognition by OT-I CTLs created an environment conducive to nonspecific CTL (gBT-I) expansion, but those CTLs that directly recognized Ag (OT-I) were deleted and inactivated. The killing activity of OT-I and gBT-I CTLs was determined by measuring lysis of third-party cells in spleens of recipient mice (Fig. 3A). Splenocytes coated with either gB498–505 or OVA257–264 peptide were killed in spleens of mice bearing control Eμ-myc-GFP lymphoma (Fig. 3B). In contrast, in Eμ-myc-OVA lymphoma–bearing mice, OT-I CTL killing activity was reduced ~50%, whereas gBT-I CTL killing was not impaired (Fig. 3C). Our results demonstrate that for the CTLs to be inactivated, they are required to recognize their cognate Ag on an APC. Which APC?
Lymphoid organs contain multiple types of DCs that play distinct functions in Ag presentation, immunity, and tolerance (28, 29), so we first addressed whether the number and phenotype of DCs were altered in lymphoma-bearing mice. We did not observe significant alterations in the proportion and number of any DC population in spleen or lymph nodes of lymphoma-bearing mice (Fig. 4A, 4B). Likewise, expression of the DC activation markers MHC II, CD86, and CD69 was equivalent in DCs from normal and tumor-bearing mice, except for a slight increase in CD86 expression on splenic conventional DCs (cDCs) and CD69 on plasmacytoid DCs (pDCs) (Fig. 4C, 4D).

To test whether the presence of the lymphoma made the DCs refractory to stimulation, we purified them from normal or tumor-bearing mice and incubated them in vitro. The results of two independent experiments, with each symbol representing an individual mouse (n = 8), were analyzed using a two-tailed Mann-Whitney U test; **p < 0.005, ***p < 0.0001, two-tailed unpaired Student t test; n.s., not significant (p > 0.05).

**CTL inactivation is mediated by direct Ag recognition on tumor cells**

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**Target cells that cause CTL inactivation are not resistant to killing**

When CTLs engage target cells they release IFN-γ, which reportedly induces other cells to acquire an inactivating phenotype characterized by the upregulation of inhibitory receptors, such as PD-L1 (36). We observed a small upregulation of this marker in Eμ-myc-OVA tumor cells, as well as in B cells, cDCs, pDCs, and MDSCs, upon injection of OT-I CTLs in mice bearing the Eμ-myc-OVA tumor (Fig. 6A). The effect was mediated by IFN-γ and required Ag recognition because it was not observed in mice bearing Eμ-myc-GFP tumors or if the CTLs lacked IFN-γ (Fig. 6A). Notably, IFN-γ-deficient CTLs were deleted and acquired an inactivated phenotype in Eμ-myc-OVA tumor–bearing mice (Fig. 6B, 6C). This implies that CTL impairment was not caused by acquisition of an IFN-γ–induced inactivating phenotype by the target cells. We also addressed whether tumor cells became resistant to CTL killing at the time that they induced inactivation. To do this, we injected splenocytes from mice bearing tumors for 5 d and labeled them with a fluorescent violet dye. The cells (of which approximately one quarter were tumor cells) were inoculated into naive mice. One day later, one group of mice was injected with CTLs or OT-I CTLs and another was not. After 2 d, the tumor and nontumor cells could be distinguished because the former had divided and were less bright in the Violet channel (Fig. 6D). Control and CTL–injected mice contained similar numbers of nontumor (Violethigh) transferred cells, but >75% of the tumor cells had been eliminated in the mice injected with CTLs (Fig. 6B).
Therefore, the ability of day-5 tumors to cause CTL inactivation was not due to an intrinsic capacity to avoid CTL killing. Selective reduction of target cell number with mild chemotherapy prevents CTL inactivation

Next, we tested the hypothesis that CTL fate might be dictated by target cell density: low in mice bearing the tumor for 3 d and high in mice bearing the tumor for 5 d. If so, reducing tumor size in the latter group might prevent inactivation of subsequently injected CTLs. We titrated the effect of the cytotoxic drug CTX to identify a single low-dose injection (40 mg/kg) sufficient to kill most tumor cells within 24 h (Fig. 7A, day 6) without affecting DCs or MDSCs (data not shown). The tumor rebounded and was detectable 2 d later if mice were left untreated but not if they were treated

![Graphs showing the number and phenotype of cDCs and pDCs in lymphoma-bearing mice.](http://www.jimmunol.org/)

**Figure 4.** The number and phenotype of cDCs and pDCs remain largely unchanged in the presence of lymphoma. Graphs display absolute number of pDCs and cDCs purified from spleen (A) or lymph nodes (B) of naive and Em-myc-OVA–bearing (GFP-OVA) mice after 5 d. Bar graphs display mean ± SD of pooled data from two independent experiments (n = 2). In each experiment, four pooled spleens were used for analysis. DC surface expression of MHC II, CD86, and CD69 was determined by flow cytometry for freshly isolated pDCs (CD11cintCD45RA+), CD8+ DCs (CD11c intCD45RA–CD8+), CD8– DCs (CD11cintCD45RA–CD8–), and migratory (migr) DCs (CD11cintCD45RA–CD8–CD205aint/high) purified from spleens (C) or lymph nodes (D, axillary, brachial, inguinal) of naive mice (filled graph) or mice bearing the lymphoma (solid black line). The dotted line represents background staining. All graphs are representative of two to five independent experiments, with four mice used in each analysis. (E) cDCs were purified from naive mice or mice bearing GFP-OVA tumor for 7 d and maintained overnight at 4˚C (fresh) or cultured at 37˚C in the presence of LPS. Expression of MHC II and CD86 was determined by flow cytometry. Data in (C–E) are representative of two independent experiments.

**Figure 5.** Direct Ag presentation by lymphoma cells, but not cross-presenting CD8+ DCs or MDSCs, induces deletion and inactivation of adoptively transferred lymphoma Ag-specific CTLs. (A) CD8+ DCs, CD8– DCs and MDSCs, were purified from spleens of mice bearing Em-myc-OVA for 7 d and incubated with naive CFSE-labeled OT-I cells in vitro. The number of proliferating OT-I cells was determined after 60–65 h of culture. Experiments were performed in duplicate, and data represent the mean ± SD. The graph is representative of two or three independent experiments. (B) C57BL/6 or Batf3−/− mice were injected with OT-I CTLs 5 d after Em-myc-OVA (GFP-OVA) injection. The number of tumor cells (left panel), OT-I CTLs (middle panel), and OT-I CTLs that produced IFN-γ upon restimulation in vitro (right panel) was determined 2 d later. Graphs represent data pooled from two independent experiments, with each symbol representing an individual mouse (n = 5–6). (C) B6×bm1 (F1) mice inoculated with Em-myc-OVA (GFP-OVA), Em-myc-GFP, or bm1.Em-myc-OVA (bm1-OVA) tumor were injected with OT-I CTLs 5 d later. After 3 d, the number of OT-I CTLs (left panel) and tumor cells (middle panel) in the spleens of lymphoma-bearing mice was determined. In addition, OT-I CTL killing in the spleen was measured 2 d after CTL injection (right panel). Data are pooled from two independent experiments, with each symbol representing an individual mouse (n = 5–11). **p < 0.005, ***p < 0.0001, two-tailed unpaired Student t test, with the exception of the left panel in (C), for which the two-tailed Mann–Whitney U test was used. n.s., not significant (p > 0.05).
with OT-I CTLs 1 d after CTX (Fig. 7A, day 8). This correlated with a lack of OT-I CTL deletion and inactivation in CTX-pretreated mice (Fig. 7B), supporting our hypothesis that both effects were caused by encounter with a high density of target cells.

We ruled out that CTX was acting to increase CD8+ DC cross-presentation of tumor Ag released by dying cells, given that analysis of Batf3<sup>-/-</sup> mice showed CD8+ DCs were not involved (Supplemental Fig. 3). Another possibility was that CTX-induced inflammation, as the result of the release of “danger” signals by dying tumor cells, prevented CTL inactivation (37). We did not observe upregulation of DC maturation markers (Fig. 7C, Supplemental Fig. 4) or increased serum inflammatory cytokines (Supplemental Fig. 4) in CTX-treated animals. Furthermore, lower doses of CTX (30 mg/ml) could kill 75% of tumor cells (Fig. 7D), which should promote the release of significant danger signals, but this treatment was insufficient to prevent CTL inactivation and tumor re-expansion (Fig. 7E), suggesting that, with this dose of CTX, the number of tumor cells had not been reduced below the inactivating threshold.

We then examined the fate of CTLs if they were injected 3 d, and not 1 d, after CTX treatment to allow tumor re-expansion before CTL inoculation (Fig. 7A). In this scenario, CTLs were again deleted and inactivated, with no effect on tumor reduction (Fig. 7F). This result supports the notion that the lag between CTX treatment and CTL injection enabled the tumor burden to again reach an inactivating number threshold.

Efficient Ag-specific elimination of lymphoma in mice treated with CTX and CTLs

The effect of combined low-dose CTX therapy and adoptive transfer of tumor-specific CTLs on the survival of E<sub>μ</sub>-myc-OVA
tumor–bearing mice was assessed. Despite the strong tumor re-
duction caused by low-dose CTX (Fig. 7A), mice given only this
treatment survived just 2 d longer than untreated animals (Fig.
8A). Injection of CTLs without previous CTX treatment conferred
no benefits, as expected (Fig. 8A). However, if mice were injected
with OT-I CTLs 1 d after CTX, their lifespan nearly doubled (Fig.
FIGURE 8. Efficient lymphoma elimination in mice treated with CTX and CTL. (A) Tumor-bearing mice were treated with vehicle (H2O) or 40 mg/kg CTX 5 d after tumor inoculation. One day later, OT-I CTLs were adoptively transferred. Kaplan–Meier survival curves are shown. The black line with filled circles includes the following groups: GFP or GFP-OVA tumor–bearing mice treated with H2O (n = 4 mice/group) and GFP or GFP-OVA tumor–bearing mice treated with H2O + OT-I CTLs (n = 4 mice/group). The dashed line with open circles dots includes the following groups: GFP or GFP-OVA tumor–bearing mice treated with CTX (n = 8 mice/group) and GFP tumor-bearing mice treated with CTX + OT-I CTLs (n = 8 mice/group). The dotted line with open triangles includes GFP-OVA tumor–bearing mice treated with CTX + CTL (n = 7 mice/group). The p value (log-rank analysis) for GFP-OVA tumor (+CTX) versus GFP-OVA tumor (+CTX + OT-I CTLs) is shown. (B) FACS plots of GFP expression (a surrogate of OVA expression) by Eμ-myc-GFP cells (FSChighB220+) in tumor-bearing mice that received CTX alone (left panel) or CTX + OT-I CTLs (right panel). GFP expression by endogenous B cells is indicated as background staining (filled graph). Results are representative of two independent experiments.

8A). This was Ag-specific because it was not observed in mice bearing Eμ-myc-GFP tumors. Indeed, the tumors that eventually killed the mice treated with the combined therapy expressed low GFP (Fig. 8B), indicating that OVAlow tumors had been selected by immunoeediting under the pressure of OT-I CTLs (38).

Discussion

This study was based on analysis of CTL activity against Eμ-myc tumors, a standard model of human non-Hodgkin lymphoma. Eμ-myc-OVA lymphoma is susceptible to CTL killing but is ignored by the immune system of naive recipients (27). Injection of high-affinity anti-OVA CTLs generated in vitro should be a suitable immunotherapy against this tumor; however, as often observed in trials of adoptive T cell therapy (39, 40), high-affinity anti-lymphoma CTLs were deleted. In addition, the surviving CTLs exhibited an inactivated phenotype, suggesting more than one mechanism of T cell impairment. We do not know whether inactivation of surviving CTLs was due to exhaustion, anergy, or a novel mechanism. Exhaustion and anergy often result from long-term (several days to weeks) interaction between T cells and APCs (41, 42), but we observed deletion and functional CTL impairment within 2 d. Although several mechanisms of antitumor T cell tolerance have been documented (43), little progress has been made toward characterizing APCs that mediate CTL inactivation (2). The purpose of the current study was to establish the Ag specificity and dissect the contribution of different APCs to CTL impairment. It is unclear to what extent such mechanisms are Ag specific as opposed to resulting from overt immunosuppression.

An important conclusion of our study is that deletion and inactivation only affected CTLs that recognized cognate Ag. By-stander CTLs (gBT-I) coinjected with tumor-specific CTLs did not suffer the same fate. Our results do not exclude a role for inactivating surface receptors or soluble factors in CTL impairment, but we show that these molecules cannot exert their function without TCR engagement. We hypothesize that CTL inactivation is triggered by excessive formation of immunological synapses, either serially or simultaneously. Although studies of the dynamics of CTL–APC interactions have defined key attributes of CTL-mediated tumor cell killing, transferred CTLs remained functional in all of these models (44–47). To our knowledge, this is the first description of CTL inactivation following the engagement of large numbers of immunological synapses during adoptive T cell therapy.

The major goal of this study was to identify the APC responsible for CTL inactivation. Tumor cells themselves were obvious candidates, but so were DCs and MDSCs, both of which can reportedly induce cross-tolerance (48–50). In contrast to previous reports (49, 51, 52), we did not observe tumor Ag cross-presentation by MDSCs. Furthermore, these cells did not inhibit T cell priming by other cells (CD8+ DCs), at least in vitro. The only APCs responsible for CTL inactivation in our system were the tumor cells themselves. This was demonstrated using the bm1.Eμ-myc-OVA lymphoma, which enabled definitive demonstration that DCs (50), MDSCs, or any other host cell capable of cross-presentation (e.g., macrophages) (35) were not the APCs responsible for CTL inactivation. Furthermore, we could not attribute CTL inactivation to the acquisition of a “suppressive” phenotype by the tumor cells (53).

Our observations suggest that target cell density is the critical parameter that dictates CTL fate. We propose that encounter of a number of lymphoma cells above a critical threshold causes inactivation of anti-tumor CTLs. In agreement with our study, Budhu et al. (54) support the concept that an appropriate CTL E:T ratio is critical to achieve successful tumor reduction, although in that report inactivation of CTLs was not examined. In the present study, we show that mild CTX therapy of mice with large tumors prevents the inactivation of CTLs injected 1 d later. Importantly, although CTX therapy alone drastically reduced Eμ-myc lymphoma size, it afforded the mice a relatively small gain in survival (2 d). Of note, the CTX dose that we used did not cause lymphoablation, so its benefit could not be attributed to providing more “space” for injected CTLs, nor was it overtly inflammatory. Analysis of Batf3-deficient mice (20) also excluded the possibility that CTX-mediated tumor killing enhanced cross-priming by CD8+ DCs, which then prevented CTL inactivation. The most logical explanation for the effect of CTX is that it reduced tumor burden below the critical density responsible for CTL inactivation. As a result, CTL injection soon after chemotherapy provoked a dramatic outcome, almost doubling the survival of tumor-bearing mice. The tumor likely would have been completely eradicated if the Ag targeted by the CTL had been derived from a protein critical to tumor survival and not the model Ag OVA, because the tumor that eventually killed the mice treated with CTX plus CTL was immunoeedited and expressed low OVA levels. Our results support the concept of applying adoptive T cell therapy soon after mild chemotherapy to treat minimal residual dis-
ease, without waiting for the tumor to become detectable again. Such an approach may also avoid the use of immunosuppressive cytokines or neutralizing Abs together with adoptive cell therapy treatments, which can promote unwanted side effects, such as autoimmunity (55). Elimination of the APCs that mediate CTL inactivation may be more feasible than using blocking Abs in cases where several receptors (e.g., PD-1, lymphocyte-activation gene 3 (T) cell Ig and mucin domain-containing molecule-3) act simultaneously to inactivate CTLs (6–8, 56).

It is pertinent to ask whether the conclusions of our study are applicable to other tumor and nontumor settings. A limitation of our lymphoma model is its rapid growth (27), which may not allow the development of other tumor-resistance mechanisms. Regardless, it provides a unique system with which to address, in vivo, the APC contribution to CTL deletion and inactivation in mice with low versus large tumor burden. Similar outcomes to those described in this article were observed in a model of adoptive CTL transfer in which the target cells were pancreatic β cells (57). In this case, memory CTLs are inactivated following encounter of Ag presented by a sufficiently large number of different types of APCs (57). Therefore, this suggests that our observations are not unique to the lymphoma model that we used.

We studied a major hurdle encountered during adoptive T cell therapy of cancer: the loss and inactivation of infused antigen-specific CTLs. Significant research efforts have attempted to define strategies that improve the expansion and survival of infused CTLs (39). In this study, we identified the in vivo APCs that mediate the loss and/or inactivation of transferred CTLs in mice bearing lymphoma. Furthermore, we propose that CTL inactivation by encounter with a high density of target cells may be a mechanism for the prevention of immunopathology and autoimmunity. Anti-self CTLs can also be generated during immune responses against pathogens (e.g., virus) and must be inactivated by a mechanism that operates only on antiself, but not antivirus, CTLs. A mechanism that is based on the frequency of target cell encounters would enable that distinction because, at least during the initial phase of infection, the number of virus-infected cells is small relative to the number of cells that express self-Ag. Antiself CTLs would be inactivated, whereas antivirus CTLs would maintain their capacity to kill, a scenario that we observed in Eμ-myc-OVA tumor-bearing mice injected simultaneously with OT-I and gB7-T CTLLs. This is a hypothesis that awaits testing in other experimental systems.

Acknowledgments

We thank all members of the Flow Cytometry and Animal Services Facilities at the Walter and Eliza Hall Institute, and the University of Melbourne (Department of Immunology and Microbiology and Bio21 Institute), for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure Legends

Figure S1: The lymphoid organs of lymphoma-bearing mice accumulate CD11b^Gr-1^ myeloid cells that resemble phenotypically mature monocytes.

(A) Ly5.1 mice were injected with E\(\mu\)-myc-OVA tumor cells (Ly5.2) and spleens were analysed by flow cytometry at various days following tumor inoculation. The top contour plots were obtained by removing Ly5.2^+ tumor cells, CD19^+ and CD3^+ cells from analysis. The bottom contour plots show SSC and Gr-1 expression of gated cells (CD11c^{int}CD11b^+, dashed gate) and identify immature monocytes (iMono, SSC^{low}Gr-1^+), neutrophils (Neutro, SSC^{int}Gr-1^{high}), eosinophils (Eosino, SSC^{high}Gr-1^{int}) and mature monocytes (mMono, SSC^{low}Gr-1^{int/low}). The appearance of SSC^{low}Gr-1^{int} cells at day seven and ten is indicated (arrow).

(B) Graph shows the accumulation of myeloid cells (CD11c^{int}CD11b^+) in the spleen of tumor-bearing mice with time. The results are the mean (n=2) ± SD of a representative experiment.

(C) Ly5.1 mice were injected with E\(\mu\)-myc-OVA tumor cells and ten days later spleens were analysed by flow cytometry. The contour plot was obtained by removing Ly5.2^+ tumor cells, CD19^+ and CD3^+ cells from analysis. Histograms show expression of CD43, Gr-1, F4/80, Ly6C, Mac3, CD115(M-CSFR), MHC II and CD86 on gated cells (dashed circle). All FACS plots are representative of 2-5 independent experiments using 2-4 mice in each analysis.

(D) Tumor cells (FSC^{high}B220^+GFP^+) or myeloid cells (CD11c^{int}CD11b^+CD43^{high}) isolated from spleen of mice bearing E\(\mu\)-myc-OVA cells for ten days were purified by flow cytometry, cytocentrifuged and stained with May-Grünwald Giemsa.

(E) Myeloid cells (CD11c^{int}CD11b^+CD43^{high}) were purified by flow cytometry and cultured for eight days in medium alone (top row), GM-CSF (middle row) or M-CSF (bottom row). Expression of CD11c, CD11b, MHC II or F4/80 is shown. The dotted line represents background staining. FACS plots are representative of two independent experiments.

(F) Myeloid cells (CD11c^{int}CD11b^+CD43^{high}) were purified by flow cytometry and cultured for five days in medium alone, GM-CSF or M-CSF after which cells were stimulated with LPS for 12 hours. Histograms are representative of two independent experiments.
**Figure S2: Antigen presentation by DC, MDSC and bm1.Eμ-myc-OVA lymphoma cells**

(A) CD8⁺ DC (circle), CD8⁻ DC (square) and MDSC (triangle) were purified from spleens of mice bearing Eμ-myc-OVA for seven days and incubated with titrating concentration of peptide OVA257-264. The number of proliferating OT-I cells was determined after 60-65 hours of culture. Experiments were performed in duplicate and results represent the mean ± SD. The graph is representative of two independent experiments.

(B) CD8⁺ DC were purified from spleens of mice bearing Eμ-myc-OVA tumor for seven days. Titrated number of myeloid cells, CD8⁻ DC or MHC I⁻ splenocytes was added to 25,000 CD8⁺ DC and CFSE-labelled OT-I cells. The number of proliferating OT-I cells was determined after 60-65 hours of culture. Experiments were performed in duplicate and results represent the mean ± SD. The graphs are representative of 2-3 independent experiments.

(C) bm1.Eμ-myc-OVA (bm1-OVA) cells were purified from spleens of tumor-bearing mice, irradiated and directly incubated with CFSE-labelled OT-I cells. The number of proliferating cells was determined after 60-65 hours of culture. Experiments were performed in duplicate and results represent the mean ± SD. The graph is representative of two independent experiments.

(D) CD8⁺ DC (circle) or CD8⁻ DC (square) purified from spleens of naïve mice were incubated with the indicated numbers of irradiated bm1-OVA tumor cells and CFSE-labelled OT-I cells. The number of proliferating cells was determined after 60-65 hours of culture. Experiments were performed in duplicate and results represent the mean ± SD. The graph is representative of two independent experiments.

(E) CD8⁺ DC (open and filled circles) or CD8⁻ DC (open and filled squares) were purified from spleens of mice bearing bm1-OVA (filled symbols) or GFP-OVA (open symbols) tumor for seven days and incubated directly with CFSE-labelled OT-I cells. The number of proliferating cells was determined after 60-65 hours of culture. Experiments were performed in duplicate and results represent the mean ± SD. The graph is representative of two independent experiments.

**Figure S3: CTL are deleted and inactivated in tumor-bearing mice that lack CD8⁺ DC.**

(A) C57Bl/6 or Batf3⁻⁻ mice were injected with OT-I CTL five days after Eμ-myc-OVA (GFP-OVA) injection and spleens were analysed two days later by flow cytometry (left experimental outline). Alternatively, tumor-bearing mice were treated with 40 mg/kg CTX on day five followed
by OT-I CTL one day later. Spleens were analysed two days later by flow cytometry (right experimental outline).

(B) Graphs show the absolute number of tumor cells (left), OT-I CTL (middle) and the percentage of CD8+IFNγ+ cells (right) in C57Bl/6 or Batf3−/− mice that received CTL only (panel A, left experimental outline) or 40 mg/kg CTX and CTL (panel A, right experimental outline). Graphs represent data pooled from two independent experiments with each circle representing one mouse (n=5-6).

(C) Histograms show (TCR) Vα2 expression and PD-1 by CTL. Data is representative of two independent experiments.

Figure S4: The activation status of DC is not altered in tumour-bearing mice treated with CTX.

(A) Eμ-myc-OVA-bearing mice were treated with vehicle (H2O) or 40 mg/kg CTX five days following tumor inoculation. One day later, DC were purified from spleen of tumor-bearing mice and analysed by flow cytometry. Contour plots on the top row were obtained by gating on CD19−CD3−CD49b−CD11cint/high cells and show cDC and pDC identified as indicated. The plots on the bottom row were obtained by gating on CD11chighCD45RA− cells to identify CD8+ and CD8− DC populations. Results are representative of 2-5 independent experiments.

(B) Histograms show surface expression of CD86, CD40, MHC II and CD69 in freshly isolated pDC (CD11cintCD45RA+) and CD8+ DC (CD11chighCD45RA−CD8+) purified from H2O-treated (back line) or 40 mg/kg CTX-treated (dotted line) mice. The shaded grey represents background staining. Histograms are representative of 2-5 independent experiments.

(C) Eμ-myc-OVA-bearing mice were treated with vehicle (H2O) or 40 mg/kg CTX five days following tumor inoculation. One day later, serum was analysed for cytokines content by Bio-plex 2200 system. The results are representative of two independent experiments with each circle representing one mouse. The line represents the mean (n=5-11) ± SD. Statistical analysis is two-tailed unpaired Student t test. **p <0.005.
Fig. S4