

# Inefficient boosting of antitumor CD8<sup>+</sup> T cells by dendritic-cell vaccines is rescued by restricting T-cell cytotoxic function

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**Abbreviations:** Ag, antigen; CFSE, 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester; CTL, cytotoxic T lymphocyte; CTO, CellTracker™ Orange; CMTMR, [5-(and-6)-((4-chloromethyl)benzoyl) amino]tetramethyl-rhodamine; DC, dendritic cell; DR5, death receptor 5; FASL, FAS ligand; IFN $\gamma$ , interferon  $\gamma$ ; IMDM, Iscove's modified Dulbecco medium; LN, lymph node; NK, natural killer; PKO, perforin knockout; TCR, T-cell receptor; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; Treg, regulatory T cell; WT, wild-type

Dendritic cells (DCs) are powerful activators of primary and secondary immune responses and have promising activity as anticancer vaccines. However, various populations of immune cells, including natural killer cells, regulatory T cells and especially cytotoxic T lymphocytes (CTLs), can inhibit DC function through cytotoxic clearance. Spontaneous tumor-specific CTL responses are frequently observed in patients before immunotherapy, and it is unclear how such pre-existing responses may affect DC vaccines. We used an adoptive transfer model to show that DC vaccination fails to induce the expansion of pre-existing CTLs or increase their production of interferon  $\gamma$  (IFN $\gamma$ ). The expansion and effector differentiation of naïve host CD8<sup>+</sup> T cells was also suppressed in the presence of CTLs of the same specificity. Suppression was caused by the cytotoxic activity of the adoptively transferred CTLs, as perforin-deficient CTLs could respond to DC vaccination by expanding and increasing IFN $\gamma$  production. Proliferation and effector differentiation of host CD8<sup>+</sup> T cells as well as resistance to tumor challenge were also significantly increased. Expression of perforin by antitumor CTLs was critical in regulating the survival of vaccine DCs, while FAS/FASL and TRAIL/DR5 had a significant, but comparatively smaller, effect. We conclude that perforin-expressing CTLs can suppress the activity of DC vaccines and prevent the expansion of naïve and memory CD8<sup>+</sup> T cells as well as antitumor immune responses. We suggest that, paradoxically, temporarily blocking the cytotoxic functions of CTLs at the time of DC vaccination should result in improved vaccine efficiency and enhanced antitumor immunity.

## Introduction

Dendritic cells (DCs) are powerful antigen-presenting cells whose role in the initiation of primary CD4<sup>+</sup> and CD8<sup>+</sup> T-cell immune responses, as well as in the reactivation of memory CD8<sup>+</sup> T-cell responses, is well recognized.<sup>1</sup> Because of their high immunogenicity, DCs loaded with different types of antigenic tumor material, including peptides, tumor lysates or apoptotic tumor cells, have been used as vaccines in several studies of tumor immunotherapy (reviewed in ref. 2). The results of these

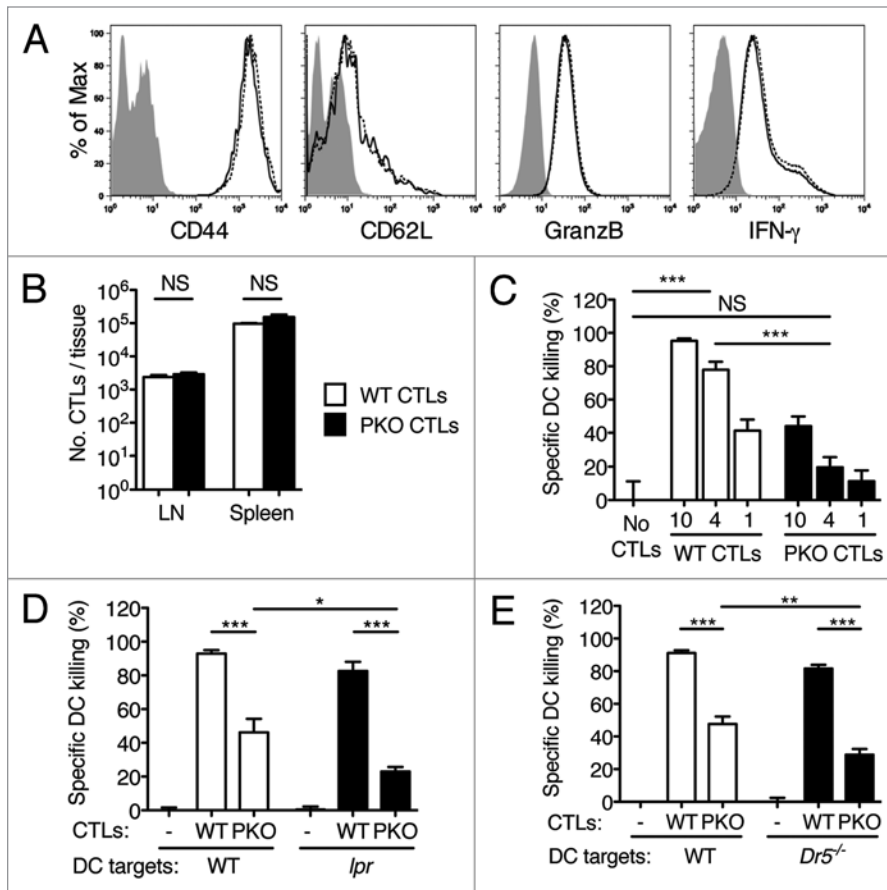
studies have confirmed that DC-based vaccines can successfully elicit antitumor immune responses and, in some cases, generate objective clinical responses.

Tumor progression is the result of an ongoing reciprocal interaction between malignant cells and the immune system.<sup>3</sup> As a consequence, patients frequently show spontaneous immune response to antigens expressed by tumor cells.<sup>4</sup> Tumor-specific CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) can be observed in the peripheral blood, secondary lymphoid organs and at the tumor site. These T cells may show an activated phenotype<sup>5</sup> but their

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**Figure 1.** CTL-mediated killing of DCs in vivo is mainly perforin-dependent and is modestly affected by inactivation of the FAS/FASL or TRAIL/DR5 pathways. (A–E) WT and PKO CTL were generated in vitro by culturing TCR-transgenic T cells with DCs and Ag. Activated T cells were expanded in IL-2 for 1–2 d, and transferred i.v. into naïve syngeneic recipients. One day later recipient mice were injected s.c. with DCs loaded or not with Ag. DC survival was determined in the draining LN 48 h after DC injection. (A) CTL phenotype before transfer. WT and PKO L318 CTLs were generated as described, re-stimulated with specific peptide (IFN $\gamma$  only) and examined for the expression of the indicated activation markers by flow cytometry. Filled histograms: unstained CTLs; solid line, WT CTLs; dashed line, PKO CTLs. (B) Recovery of WT and PKO L318 CTLs from the spleen and LNs of naïve recipients one day after transfer. The bar graph shows means + SEM for 3 mice/group. (C) In vivo killing of Ag-loaded WT DCs by WT or PKO L318 CTLs. Recipient mice were injected with 10, 4 or 1 million CTLs as indicated; DC killing was examined in draining LN 48h after DC challenge. The bar graph shows means + SEM for 3 mice/group. (D and E) In vivo killing of Ag-loaded WT, Fas<sup>pr</sup> or Dr5<sup>-/-</sup> DCs by WT and PKO OT-I CTLs. Recipient mice were injected with  $5 \times 10^6$  CTLs and  $0.5 \times 10^6$  DCs; DC killing was examined in draining LN 48 h after DC challenge. Bar graphs show means + SEM for groups of 3–6 mice, and are representative of 2–8 experiments that gave similar results. NS, not significant; \* 0.01 < p < 0.05; \*\* 0.001 < p < 0.01; \*\*\* p < 0.001 by one-way ANOVA with Tukey's post-test.

characterization has often revealed various degrees of functional impairment<sup>6</sup> including signaling defects,<sup>7</sup> expression of markers associated with inactivation,<sup>8,9</sup> and altered cytokine responses.<sup>10,11</sup> In contrast, the cytotoxic activity of tumor-specific CD8<sup>+</sup> T cells is mostly preserved.<sup>12</sup> Immunotherapy aims at re-programming the immune response from a status of functional unresponsiveness to the activation of effector functions.<sup>13</sup>

A few studies have examined the impact of existing antitumor immune responses on the success of subsequent immunotherapies. In the case of DC-based vaccines, it is known that T cells have the capacity to clear antigen-presenting DCs,<sup>14,15</sup> but the

impact of such killing on antitumor T-cell responses has not been fully examined.

In this study, we examine the effect of CTL-mediated cytotoxicity on the induction of antitumor T cell responses by DC vaccines. We used mutant CTLs and/or DCs to characterize the contribution of different cytotoxic mechanisms to DC killing. We show that wild-type (WT) CTL, but not CTL that are defective in cytotoxic function, strongly inhibit the induction of primary and memory T-cell responses by DC vaccines as well as the boosting of antitumor immune responses. Our results suggest that existing antitumor immune responses can have a substantial effect on the impact of DC-based immunotherapies and predict that temporary inhibition of cytotoxic function may result in improved therapeutic effect of DC vaccination.

## Results

**Multiple cytotoxic mechanisms contribute to the clearance of vaccine DCs by CTLs in vivo.** We wished to examine the effect of CTL- and non CTL-mediated cytotoxicity on the ability of DC vaccines to re-stimulate antitumor immune responses. We used an adoptive transfer model in which T-cell receptor TCR-transgenic WT or perforin knockout (PKO) CTLs were activated in vitro and injected i.v. into C57BL/6 hosts. In these mice, specific CTLs differentially expressed perforin, while all other cell populations including natural killer (NK) cells and regulatory T cells (Tregs) were perforin sufficient. WT and PKO CTLs were recovered from in vitro cultures in similar numbers (not shown), and expressed similar levels of activation markers and of the cytotoxic granule protein granzyme B (Fig. 1A). Antigen (Ag)-dependent interferon  $\gamma$  (IFN $\gamma$ ) production (Fig. 1A), FASL expression and granule exocytosis (data not shown) by the two populations were also similar. After adoptive transfer, WT and PKO CTLs were recovered from both lymphoid (Fig. 1B) and non-lymphoid (not shown) tissues in similar numbers. Thus, WT and PKO CTLs did not differ to notable extents in terms of activation status or in vivo distribution.

We examined the survival of vaccine DCs in C57BL/6 mice injected with WT or PKO CTLs. WT CTLs effectively killed Ag-loaded WT DCs injected s.c., and prevented their accumulation in draining lymph nodes (LNs), while PKO CTLs were about 10-fold less effective in doing so (Fig. 1C). To examine cytotoxic mechanisms other than those mediated by perforin, mice were

injected with WT or PKO CTLs and then challenged with WT DCs or DCs lacking death receptors. This setup enabled us to test the combined effect of multiple defects in cytotoxic function. As shown in **Figure 1D**, Ag-loaded WT DCs were almost completely eliminated by WT CTLs. Perforin deficiency reduced the effectiveness of DC killing by half, whereas FAS inactivation had a more modest effect. When both perforin and FAS were simultaneously inactivated, DC killing was significantly reduced as compared with the inactivation of perforin alone, but was not completely abolished. Similar results were obtained using DCs from *Dr5<sup>-/-</sup>* mice (**Fig. 1E**). Thus, inactivating DR5 had a small but significant effect on the susceptibility of DCs to killing by PKO CTLs, but the simultaneous inactivation of both perforin and the DR5/TRAIL pathway was not sufficient to abolish killing. Thus, perforin is the main effector mechanism underlying the killing of DCs by CTLs, while FASL and TRAIL have a measurable, but less prominent, role.

**PKO CTLs respond more vigorously than WT CTLs to s.c. vaccination with DCs loaded with Ag.** We compared the response of WT and PKO CTLs to DC vaccination. Vaccination with DCs and no Ag induced some expansion of CTLs in the LN draining the vaccination site. However, the size of these cells remained relatively small (**Fig. 2A**). Immunization with DCs loaded with Ag led to a modest increase in the cell size and the division of WT CTLs (**Fig. 2A and B**). In contrast, PKO CTLs increased in size and proliferated vigorously (**Fig. 2A and B**). Similarly, the numbers of WT CTLs in LN were only slightly increased in mice injected with DC loaded with Ag as compared with animals receiving DCs only, while the numbers of PKO CTLs were significantly higher (**Fig. 2B**). The numbers of WT CTLs found in the spleen on day 7 after vaccination (**Fig. 2C**), and their frequency in blood (not shown) were also consistently lower than those of PKO CTLs in similarly vaccinated mice. Thus, the response of PKO CTLs to DC vaccines is stronger than the response of WT CTLs.

The experiments described above used DCs loaded with peptide Ag. We wished to determine whether DCs loaded with whole protein Ag would show a superior ability to induce the proliferation of WT CTLs *in vivo*. We used OT-I CTLs and DCs loaded with ovalbumin (OVA) at 2 mg/mL, after preliminary experiments showed that this dose of OVA is sufficient for efficient cross-presentation and results in Ag presentation in LNs. As shown in **Figure 2D**, immunization with DCs loaded with peptide or protein Ag induced comparable extents of division of WT CTLs as well as comparable numbers of CD8<sup>+</sup> T cells in the draining LN. Therefore, loading DCs with protein vs. peptide Ag does not change the response of WT CTLs to DC vaccines.

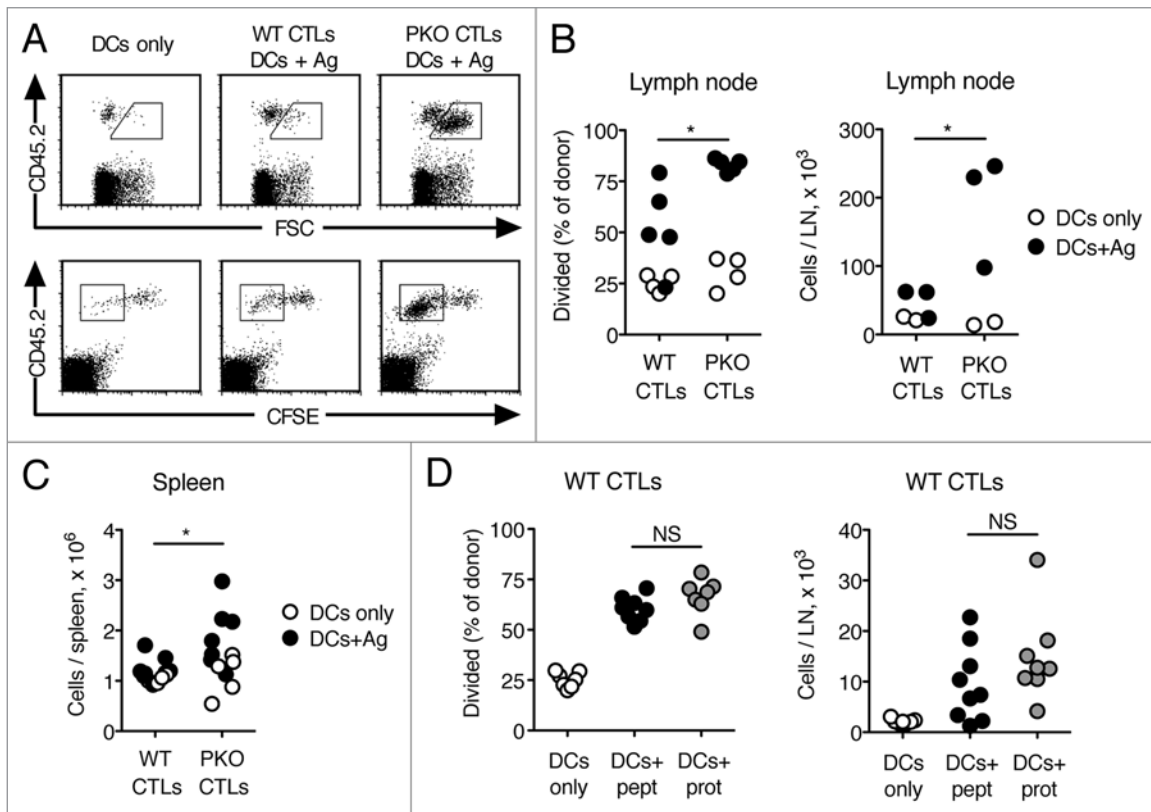
**The increased response of PKO CTLs to DC vaccination is cell extrinsic.** The stronger proliferation of PKO CTLs compared with that of WT CTLs might be due to the intrinsic toxicity of perforin,<sup>16</sup> or to extrinsic factors. To address this possibility, we designed an experiment in which WT and PKO CTLs were exposed to similar numbers of Ag-loaded DCs in the LN, as cell-intrinsic effects of perforin should still affect the response in this scenario. Naïve recipient mice were injected with Ag-loaded DCs first, and then with CTLs at a later time point. In these conditions, CTLs can stop the accumulation of DCs in the LN, but

have little effect on the DCs that are already in the LN or its close proximity (**Fig. 3A**).

Mice were injected with Ag-loaded DCs, and then given CFSE-labeled WT or PKO CTLs 24 h later. CTL proliferation was evaluated in the draining LN 66 h after the transfer of CTLs. As shown in **Figure 3B**, both WT and PKO CTL populations increased in cell size and divided in response to DCs loaded with Ag, with the responses of these CTL populations being similar to each other. The percentages of WT and PKO CTLs were also comparably increased (**Fig. 3C**), suggesting a similar expansion of the two populations. We conclude that WT and PKO CTLs have a similar ability to respond to Ag when this is appropriately presented in the LN.

**WT, but not PKO, CTLs inhibit the response of naïve CD8<sup>+</sup> and CD4<sup>+</sup> T cells to DC vaccination.** We next asked whether WT and PKO CTLs would differentially affect the proliferation of naïve T cells of the same specificity. Naïve C57BL/6 mice were injected *i.v.* with CFSE-labeled CD45.1<sup>+</sup> naïve CD8<sup>+</sup> T cells together with CD45.1<sup>-</sup> WT CTLs, PKO CTLs, or no CTLs. Mice were vaccinated with DCs loaded with Ag or DCs only, and CD8<sup>+</sup> T-cell proliferation was evaluated in the draining LN 3 d later. As shown in **Figure 4A**, proliferation was observed only in mice vaccinated with DCs loaded with Ag. In the absence of CTLs, naïve CD8<sup>+</sup> T cells increased in cell size and underwent several cycles of division. When WT CTLs were present, CD8<sup>+</sup> T cells underwent fewer divisions, and their cell size was only modestly increased. In contrast, PKO CTLs had almost no effect on the response of CD8<sup>+</sup> T cells, which proliferated and increased in cell size almost as vigorously as in the absence of CTLs. The results of this experiment are summarized in **Figure 4B**, which shows the percentage of CD45.1<sup>+</sup> CD8<sup>+</sup> cells that divided 5 times or more. Similarly, **Figure 4C** shows that, in mice vaccinated with DCs loaded with MHC Class I and Class II-restricted peptides, the number of CD8<sup>+</sup> and CD4<sup>+</sup> T cells, respectively, that underwent division in the draining LN was significantly lower in the presence of WT CTLs. Inactivation of perforin-dependent killing in CTLs partly reversed this decrease, while inactivation of FAS-dependent cytotoxicity had a small, non-significant effect. When perforin-dependent and FAS-dependent killing were simultaneously inactivated by using PKO CTLs and *lpr* DCs, the effect of CTLs on the division of naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells was mostly reversed.

**Increased cytokine production by CD8<sup>+</sup> T cells after DC vaccination is inhibited by WT but not PKO CTLs.** We wished to determine whether differential CD8<sup>+</sup> T-cell proliferation in the presence of WT or PKO CTLs is also associated with differences in cytokine production. Mice were injected with CTLs and vaccinated with DCs as described above, and intracellular cytokine production was examined in the spleen 7 d later. As shown in **Figure 5A and B**, WT and PKO CTLs from mice vaccinated with DCs only were able to produce IFN $\gamma$  upon *in vitro* re-stimulation, as would be expected for pre-activated memory cells, while cytokine production by host CD8<sup>+</sup> T cells was undetectable. Vaccination with DCs loaded with Ag did not detectably increase IFN $\gamma$  production, either by WT CTLs, or by the host CD8<sup>+</sup> T-cell population, suggesting that DC vaccination had little impact on



**Figure 2.** PKO CTLs respond more vigorously than WT CTLs to vaccination with Ag-loaded DCs. (A–D) WT CTLs and PKO CTLs were generated as described in the legend to Figure 1, labeled with CFSE and transferred i.v. into CD45-congenic recipients. One day later recipient mice were vaccinated s.c. with Ag-loaded DCs or DCs only, and CTL responses were determined as detailed below. (A) Representative flow plots of WT and PKO L318 CTLs recovered from draining LN 3 d after immunization with DCs only or Ag-loaded DCs. (B) Expansion of WT and PKO L318 CTLs in draining LNs was determined 3 d after vaccination with Ag-loaded DCs or DCs only. Data are from the same experiment shown in (A). (C) Numbers of WT and PKO L318 CTLs were determined in the spleen 7 d after s.c. vaccination with Ag-loaded DCs or DCs only. (D) Expansion of WT OT-I CTLs in draining LNs was determined 3 d after vaccination with DCs loaded with peptide Ag (DCs+pept), DCs loaded with protein Ag (DCs+prot), or DCs only. Each dot represents one mouse. Data are from one of 2–6 experiments that gave similar results (A and B), or combined from two independent experiments (C and D). Statistical analysis refers to the comparison between mice that received DCs loaded with Ag. NS, not significant; \* 0.01 < p < 0.05; \*\* 0.001 < p < 0.01 by Student's t-test (B and C) or one-way ANOVA with Tukey's post-test (D).

immune responses in these mice. Production of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) by WT and PKO CTLs was already very high (Fig. 5C), and did not further increase upon DC vaccination. In contrast, the production of IFN $\gamma$  by PKO CTLs was substantially increased after vaccination with Ag-loaded DCs, and was significantly higher than that by WT CTLs (Fig. 5B). Priming of host CD8<sup>+</sup> T cells to IFN $\gamma$  and TNF $\alpha$  production (Fig. 5B and C) was also observed, suggesting that in these mice vaccination with DCs loaded with Ag induces the activation of naïve cells. We conclude that WT CTLs inhibit the ability of DC vaccines to prime naïve CD8<sup>+</sup> T cells to IFN $\gamma$  and TNF $\alpha$  production.

**DC vaccines fail to boost antitumor immunity in mice adoptively transferred with WT CTLs.** We wished to determine whether increased responses to DC vaccination in mice receiving PKO vs. WT CTLs might also correlate with improved tumor resistance. We used a Lewis lung carcinoma (LL-LCMV) model, as our previous work indicates that IFN $\gamma$  is critical for rejection of this tumor,<sup>17</sup> while perforin is not required (data not shown). Mice received WT or PKO CTLs at a dose that confers only partial tumor protection, and they were vaccinated on the next day

with DCs without Ag or Ag-loaded DCs to boost the response. As shown in Figure 6A, mice injected with WT or PKO CTLs and vaccinated with DCs without Ag developed tumors at similar rates, which were slower than the rates observed in naïve mice. Vaccination with DCs loaded with Ag modestly delayed tumor growth in mice injected with WT CTLs, but significantly improved survival in mice injected with PKO CTLs. Thus, the vaccine activity of Ag-loaded DCs is inhibited by CTLs via their cytotoxic function.

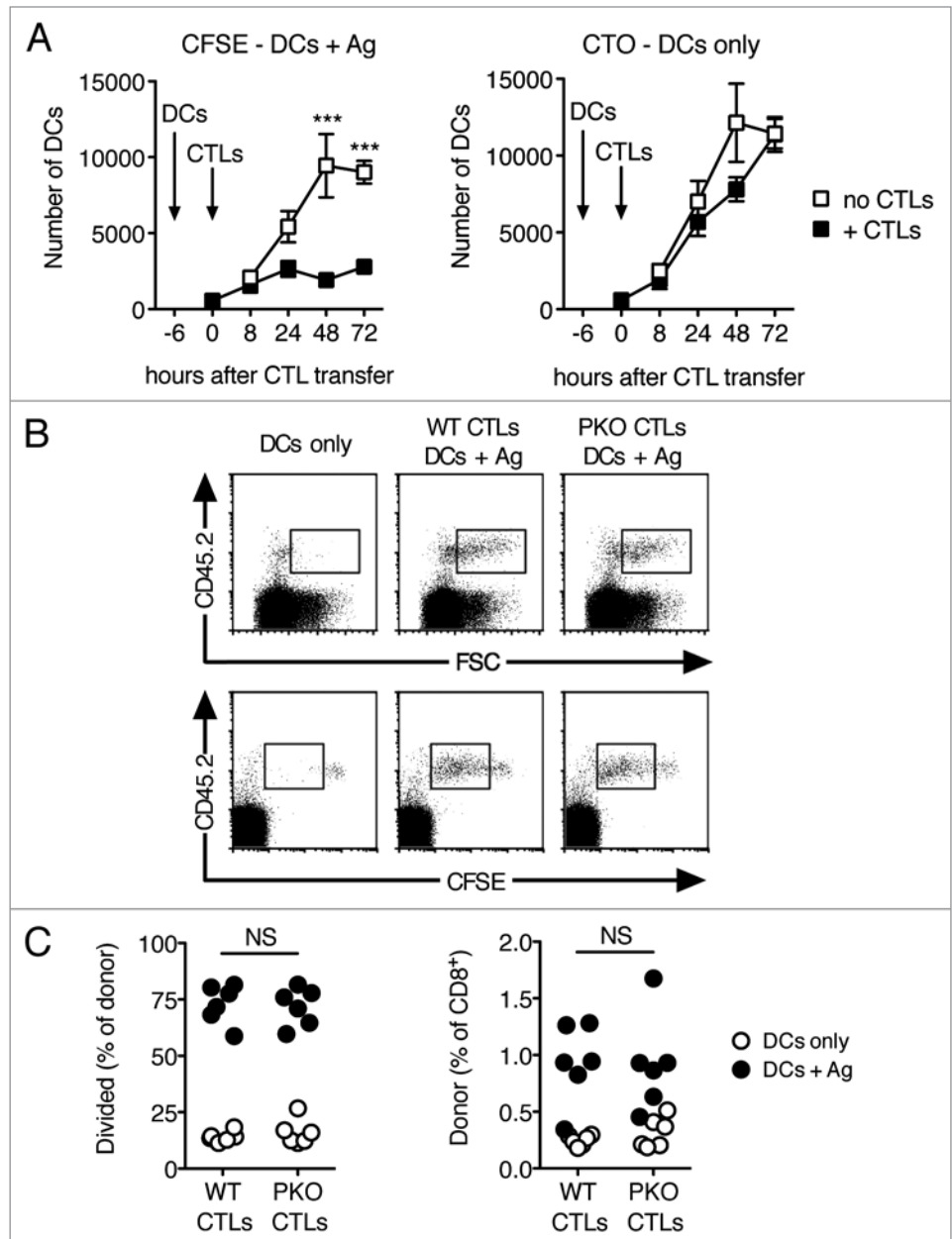
We then asked whether vaccination with DCs loaded with protein, instead of peptide, Ag might be more effective at improving the response of WT CTLs to an OVA-expressing B16 melanoma. PKO CTLs were not used in this experiment, since their ability to control the growth of B16-OVA tumors is impaired by perforin deficiency (data not shown). As shown in Figure 6B, vaccination with DCs loaded with peptide Ag or with protein Ag did not significantly boost the ability of WT CTLs to delay the growth of B16-OVA tumors. In addition, DCs loaded with protein Ag were not superior to DC loaded with peptide Ag in boosting the response of WT CTLs.

## Discussion

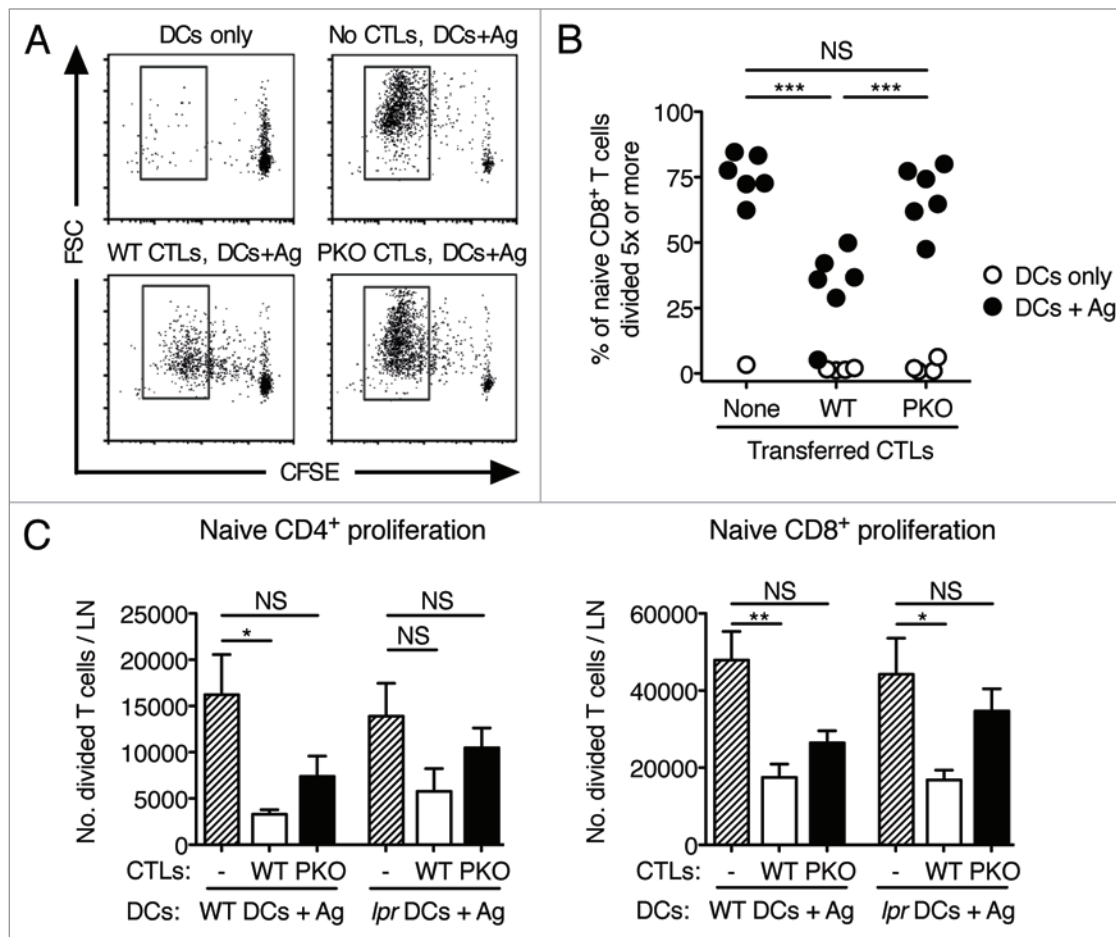
In this paper, we demonstrate that pre-existing cytotoxic antitumor immunity has a substantial inhibitory effect on the efficacy of DC vaccines. Our studies were based on an adoptive transfer model that enabled us to carefully control the activation status and numbers of T cells transferred *in vivo*, as well as to select experimental conditions whereby transferred CTLs exactly reproduced the findings in immunized mice,<sup>18</sup> hence generating results that are relevant to physiological responses. This experimental setting enabled us to closely examine the mechanism by which CTLs inhibit immune responses, by eliminating the contribution of other immune cells such as NK or Tregs, and by allowing for the discrimination between the responses of the CTLs themselves and those of naïve host cells. Using this model, we demonstrate that CTLs can suppress primary and secondary immune responses as induced by DC vaccination, leading to impaired boosting of antitumor immunity. Importantly, we also find that reducing the cytotoxic function of CTLs through the genetic inactivation of perforin results in a significantly improved ability to generate strong primary and memory immune responses to DC vaccines, as well as in improved tumor rejection. Thus, perforin expressed by CTLs mediates a powerful immunoregulatory role that limits the efficacy of tumor immunotherapy.

We considered the possibility that DCs loaded with peptide Ag, as used in several of our experiments, might be especially sensitive to CTL-mediated killing due to the high expression of MHC Class I-peptide complexes at their surface.<sup>19</sup> To assess this possibility, we compared DCs loaded with peptide Ag to DCs loaded with protein Ag, which requires processing and generates lower numbers of surface MHC Class I-peptide complexes.<sup>19</sup> Our previous experiments using DCs loaded with protein Ag confirmed that these DCs are indeed less susceptible to CTL-mediated killing than peptide-loaded DCs and are able to access the LN in detectable numbers.<sup>20</sup> Nonetheless, the

ability of protein-loaded DCs to re-stimulate CTL proliferation, or boost antitumor immune responses, was not improved, suggesting that Ag reaching the LN was not sufficiently immunogenic.



**Figure 3.** WT and PKO CTLs respond equally well to Ag presented in the LN. **(A)** Naïve mice were injected s.c. with a 1:1 mix of CFSE-labeled Ag-loaded DCs, and CTO-labeled DCs with no Ag. Six hours later, half of the mice were injected i.v. with OT-I CTLs activated *in vitro* as described in **Figure 1**. At the indicated time points, mice were sacrificed and the numbers of CFSE<sup>+</sup> and CTO<sup>+</sup> DCs in draining LNs were evaluated by flow cytometry. Each symbol shows mean  $\pm$  SEM for a group of 3–6 mice; data are from one of two experiments that gave similar results. **(B and C)** WT and PKO L318 CTLs were generated as described, labeled with CFSE and transferred i.v. into CD45-congenic recipients that had been injected s.c. one day earlier with Ag-loaded DCs or DCs only. CTL responses were examined in draining LNs 66 h after CTL transfer. **(B)** Representative flow plots of live CD8<sup>+</sup> cells recovered from the draining LNs of mice adoptively transferred with WT or PKO CTLs and immunized with DCs only or Ag-loaded DCs. Gates highlight large activated cells (top row) or divided cells (lower row). **(C)** Expansion of WT and PKO CTLs in draining LNs of mice vaccinated with DCs only or Ag-loaded DCs. Each dot represents one mouse, gating was as in **(B)**. Combined data from two independent experiments are shown. NS, not significant.



**Figure 4.** The proliferation of naïve CD4<sup>+</sup> and CD8<sup>+</sup> cells after vaccination with Ag-loaded DCs is inhibited by WT, but not PKO, CTLs. (A–C) WT and PKO CTLs were generated as described in the legend to **Figure 1** and transferred i.v. into syngeneic recipients together with CD45-congenic, CFSE-labeled naïve T cells. One day later, mice were vaccinated with Ag-loaded DCs or DCs only. Proliferation of the naïve T-cell population was assessed in draining LNs 3 d after vaccination. (A) Representative flow plots of naïve L318 CD8<sup>+</sup> T cells recovered from the draining LNs of mice adoptively transferred with L318 CTLs and immunized with DCs. (B) Percent naïve L318 CD8<sup>+</sup> T cells divided 5 times or more, recovered from the draining LNs of the indicated experimental groups. Data are from one of two experiments that gave similar results and are from the same experiment shown in (A). Each symbol corresponds to one mouse. (C) Numbers of divided, naïve OT-I CD8<sup>+</sup> T cells and OT-II CD4<sup>+</sup> T cells recovered from the draining LNs of mice that were adoptively transferred with  $5 \times 10^6$  WT or PKO OT-I CTLs, and immunized with  $0.5 \times 10^6$  WT or Fas $lpr$  DCs. Bar graphs show means + SEM for groups of 4–10 mice. NS, not significant; \*  $0.01 < p < 0.05$ ; \*\*  $0.001 < p < 0.01$ ; \*\*\*  $p < 0.001$  by one-way ANOVA with Tukey's post-test.

The relevance of our findings is 2-fold. First, our data are relevant in the context of DC-based immunotherapy. As shown in **Figure 6**, the cytotoxic ability of tumor-specific CTLs essentially prevented DC vaccine from boosting tumor-protective immunity, presumably by preventing the priming of host CD8<sup>+</sup> T cells as well as the acquisition of multifunctional effector activity by memory T cells that is normally observed after repeated antigenic stimulations.<sup>21</sup> DCs loaded with protein Ag were not significantly more effective than DCs loaded with peptide, suggesting that the failure of DC vaccination to boost antitumor immunity is not due to the method of antigen loading. Indeed, results from control experiments indicated that DCs loaded with OVA protein or an OVA-derived peptide were equally effective at inducing primary antitumor immune responses in naïve mice (data not shown). We did not investigate whether other types of immunotherapies, such as the administration of Ag together with adjuvant, are affected by CTL cytotoxic function. Given

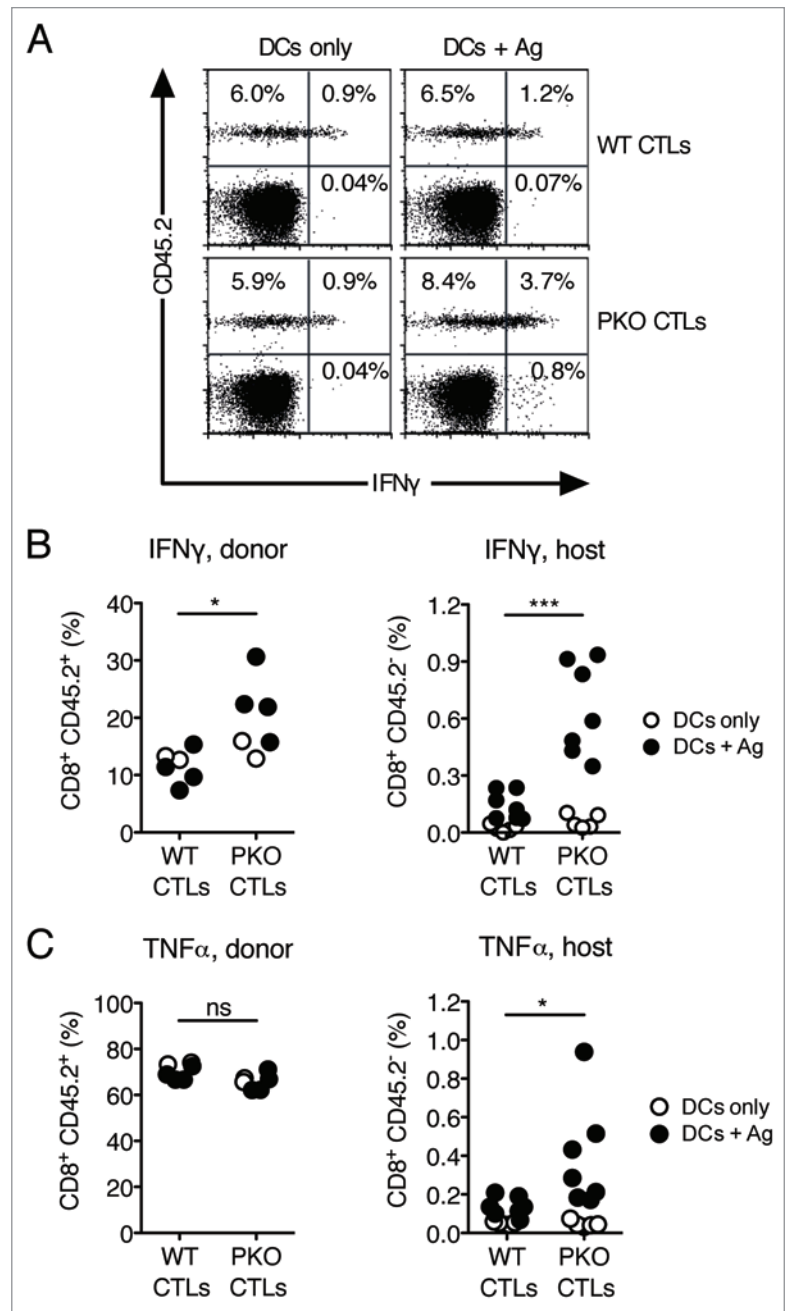
the central role of DCs in antigen presentation in vivo, we would expect most immunotherapies to be affected by CTL activity, at least to some extent. As tumor-specific CTLs are often observed in cancer patients even before treatment, our findings are likely to be relevant to a considerable proportion of patients before and/or during immunotherapy.

Second, our data provide a mechanism that potentially underlies increased immune responses as observed in perforin-deficient mice and humans.<sup>22,23</sup> For example, PKO mice generate increased immune responses upon repeated immunization or viral infection,<sup>18,22,24</sup> and may succumb to severe inflammation when vaccinated.<sup>25</sup> The interpretation of some of these studies is complicated by the use of infection models in which perforin-expressing cells also participate in the clearance of infectious agents.<sup>22,26</sup> The resulting increased Ag load makes it difficult to establish the precise cause of increased immune responses.<sup>26–28</sup> In addition, perforin has been reported to be necessary for the function

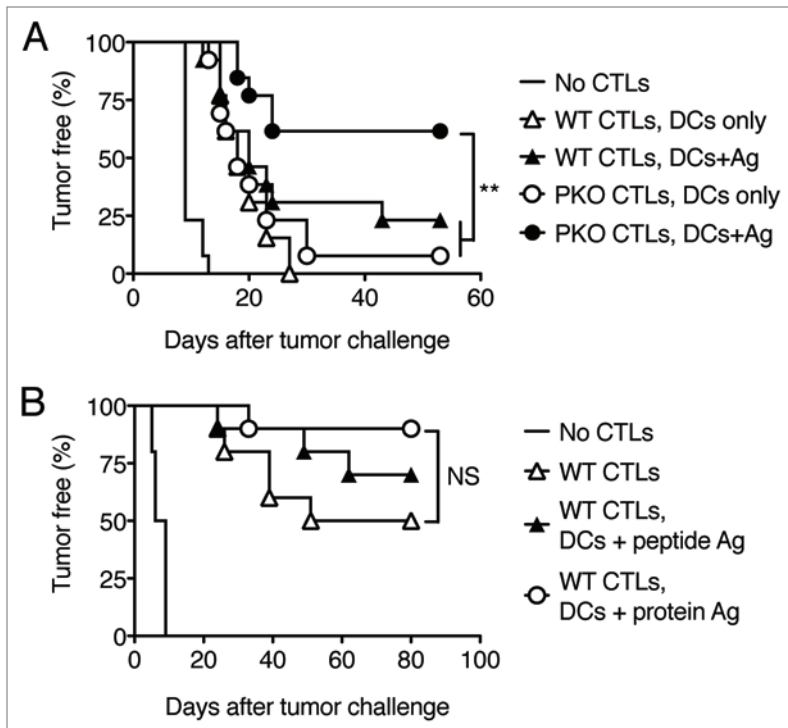
of Tregs,<sup>29,30</sup> suggesting that defective Treg function may contribute to enhanced immune responses. Our study used a non-replicating Ag to exclude effects of varying Ag load, and an adoptive transfer model in which only CD8<sup>+</sup> T cells differed in perforin expression. This simplified model nonetheless recapitulated several characteristics of perforin deficiency<sup>22,26</sup> such as increased expansion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as an increased capacity of T cells to produce cytokines. Thus, a selective deficiency of perforin in the CTL population combined with Ag presentation by DCs is sufficient to reproduce the main features of dysregulated immune responses observed in PKO hosts. Other authors have suggested that CD8<sup>+</sup> T cells regulate Ag presentation *in vitro*<sup>31</sup> and *in vivo*<sup>32,33</sup> and that perforin<sup>27</sup> and/or FAS<sup>34</sup> are critical mediators of this process. Some of these studies used naïve WT and PKO T cells, while our experiments used activated CTLs that were already cytotoxic before transfer, and expressed FASL (not shown) and presumably also perforin and TRAIL.<sup>35</sup> In these conditions, perforin was clearly the major mediator to regulate DC killing and T-cell responses, while FAS and DR5 played a smaller, but significant, role.

One additional mechanism that might explain increased T-cell responses in PKO mice is T-cell suicide or fratricide, whereby WT CTLs succumb to the toxic effect of endogenously expressed cytotoxic mediators,<sup>16,36</sup> or kill each other upon recognition of Ags acquired from antigen-presenting cells through trogocytosis.<sup>37</sup> To examine this possibility we performed a simple experiment in which mice were injected with DCs first, to enable DC migration to LNs, and then adoptively transferred with WT or PKO CTLs. If perforin was involved in T-cell suicide or fratricide, in such setting PKO T cells should still survive better than WT cells, and accumulate in LNs in larger numbers. However, this was not the case. The similar response of WT and PKO CTLs strongly suggests that the effect of perforin is not cell-intrinsic, but mediated via a second population of cells, most likely DCs.

In conclusion, our data describe a perforin-dependent mechanism of regulation of the immune response, and provide evidence that this mechanism significantly affects the efficacy of DC-based vaccines. We propose that this mechanism acts as an additional “immune checkpoint”<sup>38</sup> that counteracts DC-based immunotherapy and may be co-opted by tumors to escape the immune response.<sup>39</sup> We also present evidence that, in the context of DC immunotherapy, this perforin-dependent checkpoint operates during a brief period soon after DC administration. Therefore, our results suggest that, paradoxically, a temporary blockade of T cell-mediated cytotoxic function at the time of DC vaccination, as would be rendered possible by the



**Figure 5.** The increase in frequency of IFN $\gamma$ -producing CD8<sup>+</sup> T cells after vaccination with Ag-loaded DCs is inhibited by WT, but not PKO, CTLs. (A–C) CD45.1 mice were injected with CD45.2<sup>+</sup> WT or PKO L318 CTLs and vaccinated with Ag-loaded DCs or DCs only as described in the legend to Figure 2. Cytokine production by transferred CTLs and by the host naïve T-cell population was assessed in the spleen 7 d after DC immunization. (A) Representative flow plots of total CD8<sup>+</sup> T cells from spleens in the indicated treatment groups. Spleen cells were re-stimulated *in vitro* in the presence of specific Ag and stained for intracellular IFN $\gamma$ . (B) Percentages of adoptively transferred CTLs (left) and host resident CD8<sup>+</sup> T cells (right) that were IFN $\gamma$ <sup>+</sup> by *in vitro* re-stimulation and intracellular staining. (C) Percentages of adoptively transferred CTLs (left) and host resident CD8<sup>+</sup> T cells (right) that were TNF $\alpha$ <sup>+</sup> by *in vitro* re-stimulation and intracellular staining. Each dot plot shows combined data from two independent experiments. Each symbol corresponds to one mouse. Statistics refer to the comparison between mice adoptively transferred with WT or PKO CTLs, and immunized with Ag-loaded DCs. NS, not significant; \* 0.01 < p < 0.05; \*\*\* p < 0.001 by a two-tailed unpaired Student’s t-test.



**Figure 6.** Vaccination with Ag-loaded DCs boosts tumor protection in mice adoptively transferred with PKO, but not WT, CTLs. **(A and B)** WT CTL and PKO CTLs were generated as described in the legend to **Figure 1** and transferred i.v. into naive recipients. One day later recipient mice were vaccinated s.c. with DCs loaded with peptide Ag, DCs loaded with protein Ag, or DCs only, and challenged with tumor cells 5 d later. Tumor appearance was monitored every 2–3 d and mice were scored as tumor positive when tumor size was in excess of 4 mm<sup>2</sup>. **(A)** Mice were injected with  $5 \times 10^6$  WT or PKO L318 CTLs and challenged with LL-LCMV tumors. The graph shows combined data from two independent experiments with a total of 13 mice/group. **(B)** Mice were injected with  $1 \times 10^6$  WT OT-I CTLs and challenged with B16-OVA tumor cells. The graph shows combined data from two independent experiments with a total of 10 mice/group. NS, not significant; \*\*  $0.001 < p < 0.01$  by a log-rank test.

development of perforin inhibitors,<sup>40</sup> may maximize the efficacy of DC-based immunotherapy.

## Materials and Methods

**Mice.** All mice were maintained at the Malaghan Institute Biomedical Research Unit, Victoria University of Wellington. Experimental procedures were approved by the Victoria University Animal Ethics Committee and conducted in accordance with Institutional guidelines. C57BL/6 mice were originally from Jackson Laboratory; CD45-congenic B6.SJL-*Ptprc*<sup>a</sup> were from Animal Resources Centre; C57BL/6-*Fas*<sup>lpr</sup> mice were from the Walter and Eliza Hall Institute of Medical Research. The TCR transgenic strain Line318<sup>41</sup> was crossed to PKO mice<sup>42</sup> (Jackson Laboratories) for two generations to obtain PKO-Line318 mice. Line318, OT-I<sup>43</sup> and OT-II<sup>44</sup> mice were crossed to B6.SJL-*Ptprc*<sup>a</sup> mice for one generation to obtain CD45-heterozygous Line318, OT-I and OT-II mice, respectively. *Dr5*<sup>-/-</sup> mice<sup>45</sup> were bred at the Penn State Hershey Cancer Institute and used according to Institutional Animal Care and Use Committee guidelines.

**In vitro culture media and reagents.** All cultures were in complete Iscove-modified Dulbecco's medium (IMDM), consisting of IMDM supplemented with 5% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin and 55 µM 2-mercaptoethanol (2-ME) (all from Invitrogen Corp.). The gp33 (LCMV<sub>33–41</sub> KAV YNF ATM), OVA<sub>257–264</sub> (SII NFE KL) and OVA<sub>323–339</sub> (ISQ AVH AAH AEI NEA GR) peptides were purchased from Mimotopes Pty Ltd. OVA protein fraction V was from Sigma Aldrich Co.

**DC injection and immunization.** DCs were prepared from the bone marrow of C57BL/6 mice by culture in 10 ng/mL mouse recombinant granulocyte macrophage colony-stimulating factor (rGM-CSF) and 20 ng/mL mouse recombinant interleukin-4 (rIL-4), as previously described.<sup>46</sup> DCs were activated by adding 100 ng/mL of lipopolysaccharide (LPS, Sigma Aldrich Co.) on day 6, and loosely adherent cells were harvested by gentle pipetting on day 7.

For immunizations, DCs were harvested and re-suspended at  $1 \times 10^6$  cells/mL. Peptides were added to the cells at a final concentration of 1–10 µM and cells were incubated at 37°C for 2–4 h. For OVA protein loading, OVA was added to DCs in culture at a final concentration of 2 mg/mL for 48 h, LPS was added during the final 18–24 h. Cells were washed to remove excess Ag, and  $1–5 \times 10^5$  cells were injected s.c. in 100 µL.

**Generation of specific CTLs in vitro.** Total LN cells from L318, PKO L318, or OT-I mice were cultured with LPS-treated DCs and Ag as described,<sup>47,48</sup> and expanded in human rIL-2 for 1–2 d before transfer. This protocol routinely yielded cell populations that were 90–95% TCR Vα<sup>+</sup>Vβ<sup>+</sup> CD62L<sup>low</sup> by flow cytometry and highly cytotoxic.

**In vivo DC cytotoxicity assays.** LPS-treated DCs were labeled with fluorescent dyes so that their survival in vivo could be tracked.<sup>14</sup> One population of DCs was loaded with 1 µM gp33 peptide or 10 µM OVA<sub>257–264</sub> and labeled with the green fluorescent dye CFSE (Molecular Probes) while the second population of DC was left unloaded and labeled with the orange dye CTO (Molecular Probes), as described. Each mouse was injected intradermally into the distal forelimb or ear pinna with  $1 \times 10^6$  CFSE-labeled DCs and  $1 \times 10^6$  CTO-labeled DCs. At various times after DC injection, draining LNs were harvested, incubated for 1 h at 37°C in an enzyme cocktail containing 2.4 mg/mL collagenase II (Invitrogen) and 0.1 mg/mL DNase I (Sigma Aldrich), and passed several times through a 21G needle and then a gauze before flow cytometry analysis. The percent killed DC in individual LNs was calculated according to the formula:  $100 - [(no. Ag-loaded DCs / no. non Ag-loaded DCs in immune LN) \times 100 / (no. Ag-loaded DCs / no. non Ag-loaded DCs in control LN)]$ .

**Adoptive transfer experiments.** L318, OT-I and OT-II T cells were prepared from naïve LN and spleen cell suspensions, positively



selected using anti-CD8 and anti-CD4 magnetic beads (Miltenyi Biotech GmbH), respectively, and labeled with 1  $\mu$ M CFSE for 10 min at 37°C. Labeling was stopped by adding FCS and IMDM, and cells were washed extensively before injection. CTL populations generated in vitro as described above were labeled with CFSE using the same conditions. Cells were injected i.v. at the following doses: 5  $\times$  10<sup>6</sup> L318 or PKO L318 CTLs (or as otherwise indicated), 1–5  $\times$  10<sup>6</sup> OT-I CTLs, and 1  $\times$  10<sup>6</sup> naïve L318, OT-I or OT-II cells. In each experiment, the CFSE-labeled cells were also CD45 congenic with respect to the host and other adoptively transferred cells. Recipient mice were immunized with DCs with or without Ag, and/or challenged with tumor cells as indicated.

**Flow cytometry.** Cell suspensions were incubated with the appropriate concentration of antibodies in PBS containing 2% FCS and 0.01% sodium azide. The anti-Fc $\gamma$ R2 mAb 2.4G2 was used at 10  $\mu$ g/mL. All antibodies were from BD PharMingen, with the exception of anti-CD45.2, anti-granzyme B, anti-TNF $\alpha$  and appropriate isotype control antibodies, which were from eBioscience. Anti-CD8 (clone 2.43) was purified in-house from hybridoma supernatants. Samples were analyzed using a FACSCalibur (Becton-Dickinson) and FlowJo software (Tree Star). Propidium Iodide (BD PharMingen) was used to exclude dead cells.

**Intracellular cytokine staining.** Cell suspensions were cultured in complete IMDM containing 1  $\mu$ M gp33 and Golgistop<sup>®</sup> (BD Bioscience) for 4 h. Cells were harvested, surface stained, fixed and then permeabilized. Intracellular cytokines were

detected using the BD PharMingen intracellular staining kit and anti-IFN $\gamma$ , anti-TNF $\alpha$ , or appropriate isotype control antibodies as per manufacturer's specifications.

**Tumor challenge experiments.** The gp33-expressing Lewis lung carcinoma (LL-LCMV)<sup>49</sup> and OVA-expressing B16 melanoma (B16-OVA)<sup>50</sup> were maintained in complete IMDM containing 0.5 mg/mL Geneticin<sup>®</sup> (Invitrogen). Mice were challenged with 1  $\times$  10<sup>5</sup> LL-LCMV or 1  $\times$  10<sup>6</sup> B16-OVA cells injected s.c. in the left flank, and examined every 2–3 d to monitor tumor growth as described.<sup>49</sup> Mice were scored as tumor-free if the tumor mass did not exceed 4 mm<sup>2</sup>.

**Statistical analysis.** Statistical analyses were performed using the Prism GraphPad software. Statistical tests and significance thresholds employed are indicated in each figure legend.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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