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Recovery from Cyclophosphamide-Induced Lymphopenia Results in Expansion of Immature Dendritic Cells Which Can Mediate Enhanced Prime-Boost Vaccination Antitumor Responses In Vivo When Stimulated with the TLR3 Agonist Poly(I:C)[1]

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Abstract

Recent preclinical studies suggest that vaccination following adoptive transfer of $CD8^+$ T cells into a lymphopenic host can augment the therapeutic antitumor responses of the transferred cells. However, the mechanism by which the lymphopenic microenvironment benefits Ag-specific $CD8^+$ T cell responses remains elusive. We show herein that induction of lymphodepletion by a single 4 mg cyclophosphamide (CTX) treatment induces a marked expansion of immature dendritic cells (DCs) in the peripheral blood on days 8–16 post-CTX (termed restoration phase). In vitro, these DCs were functional, because they showed normal phagocytosis and effective Ag presentation capability upon activation. In vivo, administration of the TLR3 agonist poly(I:C) at the peak of DC expansion (day 12 postlymphopenia) induced inflammatory cytokine production and increases in the number of activated DCs in lymph nodes. Importantly, boosting with $gp100_{25–33}$ melanoma peptide combined with poly(I:C) 12 days after an initial priming with the same regimen significantly increased the expansion and the antitumor efficacy of adoptively transferred pmel-1 $CD8^+$ T cells. These responses were abrogated after depletion of activated DCs during Ag boosting. In conclusion, our data show that CTX treatment induces, during the restoration phase, expansion of immature DCs, which are functional and can be exploited in vivo to foster more effective antitumor adoptive immunotherapy strategies.

Recent studies have shown that preconditioning a host with lymphodepletion regimens such as total body irradiation (TBI)[3] or chemotherapy (e.g., cyclophosphamide (CTX)) can

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effectively augment the antitumor efficacy of adoptively transferred T cells in response to vaccination (1–6). Suggested mechanisms underlying this effect include enhanced engraftment and survival of the transferred T cells by creation of an immunological “niche” (7) with induction of survival cytokines (8,9); elimination of regulatory $CD4^+CD25^+$ T cells, NKT cells, and myeloid-derived suppressor cells (3,10–12); and depletion of endogenous cells that compete with the transferred T cells for cytokines “cytokine sink” (7,8,13). Recent studies suggest, however, that these mechanisms might not be the principal means by which lymphodepletion regimens augment adoptive immunotherapy (4,14–17). Moreover, these mechanisms have been investigated during the lymphopenic phase, and few studies addressed the role of cellular components that might be altered at the restoration phase (in which the host recovers from the induced lymphopenia; days 5–18). Therefore, it is relevant to test whether additional mechanisms are in effect during the lymphopenic or the restoration phases after lymphodepletion.

Given the importance of understanding the precise mechanisms underlying the beneficial effects of lymphodepletion on the clinical application of immunotherapy-based treatment regimens, we have addressed the role of different host mediators during CTX-induced lymphodepletion. Using the OT-1 transgenic (Tg) TCR model, we have recently reported that the adoptive transfer of $CD8^+$ T cells at the lymphopenic phase after CTX treatment resulted in dramatic increases in postvaccination T cell responses, including enhanced expansion and function, and delayed contraction of adoptively transferred $CD8^+$ T cells. These effects were associated with rapid activation of dendric cells (DCs) in the liver and spleen during this early phase of lymphopenia (days 1–4) (14). Earlier studies have also reported rapid activation of DCs and follicular DCs in the spleen and lymph nodes (LNs) of mice rendered lymphopenic by TBI (3) or CTX (18,19). This rapid activation of DCs during the lymphopenic phase has been suggested to be due to the rapid systemic release of LPS in response to mucosal damage after lymphopdepletion (3). Further, we reported that the beneficial effects of CTX to OT-1 T cell responses were dependent on the presence of CD11b-expressing cells (14), indicating to the possible significance of DCs during the lymphopenic phase. Wrzesinski et al. have reported that using a myeloablative (rather than nonmyeloablative) approach, coupled with subsequent hematopoietic stem cell transplantation, can render adoptively transferred pmel-1 T cells independent of a requirement for prior in vitro activation. This observation was felt to be most likely due to enhanced MHC class I-restricted Ag presentation by elements of the transplanted bone marrow (BM) (4). To directly understand the role of DCs, we focused our present studies on evaluating the numbers, phenotype, and function of DCs at the restoration phase postlymphodepletion induced by CTX treatment.

With our most recent studies, we have made the interesting observation that CTX treatment can induce a substantial expansion of biologically functional immature DCs during the restoration phase, peaking on day 12. Activation of this large pool of DCs by the TLR3 ligand (TLR3L) poly(I:C) resulted in their activation and the appearance of activated $CCR7^+$ DCs in LNs. Furthermore, boosting with peptide/poly(I:C) at the peak of this DC expansion (day 12 postdepletion) after an initial Ag priming led to an enhanced $CD8^+$ T cell antitumor response in the tolerogenic pmel-1 TCR Tg mouse model. Our data would suggest a novel rationale for the design of cancer immunotherapy in the clinical setting based on targeting DCs at certain phases after application of lymphodepletion regimen.

[3]Abbreviations used in this paper: TBI, total body irradiation; BM, bone marrow; cDC, conventional DC; CTX, cyclophosphamide; DC, dendritic cell; DTx, diphtheria toxin; DTR, diphtheria toxin receptor; LN, lymph node; OVAp, OVA albumin peptide; PBL, peripheral blood leukocyte; pDC, plasmacytoid DC; Tg, transgenic; TLRL, TLR ligand; TLR3L, TLR 3 ligand.

Materials and Methods

Mice

B6.SJL (Ly5.1), C57BL/6 (Ly5.2; B6), OT-1 and pmel-1 TCR Tg (B6 background), and CD11c-diphtheria toxin receptor (DTR) Tg mice (B6 background) were purchased from The Jackson Laboratory. OT-1 mice were bred with wild type B6.SJL mice to generate $Ly5.1^+/Ly5.1^+$ mice heterozygous for the OT-1 TCR (Vα2/Vβ5) transgene, which was confirmed by flow cytometry with mAb specific for Vα2 and Vβ5. $CD8^+$ T cells in OT-1 mice recognize the MHC-I OVA albumin peptide SIINFKEL (OVAp). Pmel-1 (Ly5.2) mice were bred with Ly5.1 wild type mice to generate Ly5.1 mice heterozygous for the pmel-1 TCR Vα1/Vβ13 transgene. The transgene expression was confirmed by PCR analysis. $CD8^+$ T cells expressing the Vα1/Vβ13 TCR specifically recognize the $H\text{-}2D^b$-restricted human $gp100_{25–33}$ epitope (KVPRNQDWL: $gp100_{25–33}$). This peptide represents an altered form of the murine $gp100_{25–33}$ (EGSRNQDWL) with improved binding to the MHC class-I. All animals were housed under specific pathogen-free conditions in accordance with institutional and federal guidelines at the Medical University of South Carolina.

Cell lines, Abs, and reagents

B16 melanoma ($H2K^b$) cells were maintained in complete RPMI 1640 medium. Anti-CD16/CD32, and FITC-, PE-, APC-, and cychrome-conjugated mAbs were purchased from Pharmingen and eBioscience. Human $gp100_{25–33}$ EGSRNQDWLL melanoma and SIINFEKL OVA peptides (American Peptide Company) were dissolved in 10% DMSO (Sigma) and diluted in PBS. GM-CSF and IL-4 (R&D Systems) were stored as a lyophilized powder at −20°C, and reconstituted immediately before use in 0.1% BSA in PBS. CTX, diphtheria toxin (DTx; Sigma), and DQ-OVA (Invitrogen) were reconstituted in PBS and frozen until used. The TLR3L poly(I:C) was purchased from InvivoGen.

Flow cytometry

Mice were bled from the retro-orbital plexus, and blood samples were then processed for VetScan HM5 Hematology (Abaxis) to assess the total count of peripheral blood leukocytes (PBL). Spleen and LN single-cell suspensions were prepared and counted as previously described (20). For flow cytometry, fresh cells ($0.5–1 \times 10^6$) were treated with anti-CD16/CD32 for 5 min on ice, stained with the indicated conjugated mAbs, and incubated for 30 min on ice. The cells were washed twice and resuspended in 0.3 ml of 0.5% BSA, 0.02% sodium azide solution. Cells were then washed, and analyzed by flow cytometry using the Cell Quest software package (BD Biosciences). The absolute numbers of different cell populations in each compartment were calculated as: % cells from flow cytometry × total number of cells/100.

Phagocytic function of DCs

Mice were bled and the PBL were prepared, incubated with FITC-labeled dextran (Sigma) for 1 h at 37°C or on ice as negative control. Then cells were washed extensively and stained with anti-CD11c and anti-CD11b mAbs, and the numbers of $FITC^+$ DCs were analyzed by flow cytometry as described before (21).

Ag-presenting function of DCs

DCs ($CD11c^+CD11b^+$) were sorted by flow cytometry from the peripheral blood samples pooled from 20 mice after staining the samples with anti-CD11c and anti-CD11b mAbs for 30 min at 4°C. Sorted cells were washed and resuspended in RPMI 1640 medium and cultured in 6-well plate for 2 days in RPMI 1640 medium containing GM-CSF and IL-4 (10 ng/ml each). DCs were irradiated, pulsed with 1 μg/ml OVAp for 2 h at 37°C, washed three

times, and then cocultured (1×10^4) with purified naive OT-1 cells (1×10^5) for 3 days. Cultures were pulsed with thymidine for the last 18 h, and then cells were harvested to measure thymidine uptake by γ-counter (22).

CTX preconditioning, adoptive T cell transfer, and vaccination

Recipient wild type naive Ly5.2 mice were treated with PBS or 4 mg/mouse CTX (14,23) and adoptively transferred 1 day later by lateral tail vein injection with one million of naive pmel-1 Ly5.1 cells. The mice were then vaccinated with s.c. injection of 100 μg/mouse $gp100_{25–33}$ melanoma peptide with or without 200 μg/mouse poly(I:C) on day 2 and/or day 12 after PBS or CTX treatment. The mice were bled and killed at multiple time points to analyze the expansion and contraction of pmel-1 cells. When mentioned, CD11c-DTR Tg mice were treated with single i.p. injection of 90 ng/kg DTx, as previously described (24), to deplete DCs.

Tumor challenge

Naive B6 mice were challenged with s.c. injection of 2.5×10^5 fresh B16 tumor cells. Ten days after tumor inoculation, mice were treated with PBS or CTX and then adoptively transferred with naive pmel-1 cells followed by vaccination at the indicated time points. Tumor growth was measured by caliper every 3 days and the tumor surface area (mm^2) was calculated by length × width. Survival of tumor bearing mice was also recorded.

Statistics

Statistical analyses were performed using the Student's *t* test. Log-rank nonparametric analysis using GraphPad Prism (GraphPad Software) was used to graph and analyze the survival data. All *p* values were two sided, with $p < 0.05$ considered significant.

Results

CTX treatment induces DC expansion with immature phenotype during the restoration phase

Consistent with previous studies (12,16,25), we found that single i.p. injection of CTX induced rapid lymphopenia (days 1–5) in the peripheral blood (Fig. 1*A*, *left panel*), spleen, and BM (Fig. 1*A*, *right panel*) followed by a restoration phase, in which the cellular components started to recover from lymphopenia. The restoration from lymphopenia in the spleen and BM was observed on day 6 post-CTX treatment. The lymphodepletion in PBL, however, was much more prolonged and hardly reached the basal level by day 18. In all experiments, control mice were treated with PBS and the levels of cells present are represented as day 0 in the figures. Despite the fact that a significant lymphopenia was still persistent in the peripheral blood, substantial increases in the relative numbers of DCs ($CD11c^+CD11b^+$) were observed in this compartment from days 9–14 as compared with PBS-treated mice ($p = 0.0005$) (Fig. 1*B*), peaking on day 11–12 post-CTX treatment and representing ~25% of total PBL. When PBL harvested 12 days after CTX treatment was incubated overnight in serum-free medium, it showed numerous adherent DCs as compared with PBL harvested from PBS-treated mice (Fig. 1*C*). The increase in the relative numbers of DCs in PBL on day 12 post-CTX treatment was correlated with a significant increase in the absolute numbers of these cells (greater than 2-fold, $p = 0.007$) (Fig. 1*D*, *left panel*), which was more pronounced when it was calculated per one million PBL (Fig. 1*D*, *right panel*). Of note, expansion of DCs was also found in the spleen of CTX-treated mice; the DC expansion was dose- and Flt3L-dependent (M. L. Salem, A. A. AL-Khami, S. A. EL-Naggar, C. M. Díaz-Montero, Y. Chen, O. Naga, and D. J. Cole, manuscript in preparation). To understand whether DC expansion in the peripheral blood was due to the proliferation of

DC progenitors in BM or in the blood, we analyzed the proliferation of DCs in these compartments by BrdU incorporation assay at multiple time points post-CTX treatment. As shown in the Supplementary Fig. 1, *A* (PBL) and *B* (BM),[4] the relative numbers of $BrdU^+CD11c^+CD11b^+$ DCs were higher only in BM, but not in the blood, as compared with control mice when analyzed 2 days post-CTX treatment. By day 6, however, the relative numbers of $BrdU^+$ DCs in the blood of CTX-treated mice were high and those in BM were low. On the whole, the increase in the numbers of the proliferating DCs in BM at an early time point following CTX treatment, together with the appearance of $BrdU^+$ DCs in the blood on day 6 (i.e., at least 3 days before the initiation of DC expansion), would suggest that DC expansion in the peripheral blood is, at least in part, due to the migration of proliferating DC progenitor from BM to the circulation.

DCs in the peripheral blood can be categorized into conventional DCs (cDCs; $CD11c^{high}CD11b^{high}Gr.1^{low}B220^{low}$) and plasmacytoid DCs (pDCs; $CD11c^{high}CD11b^{low}Gr.1^{high}B220^{high}$) (26–28). To determine which DC subset was expanded in response to CTX treatment, PBL from PBS- or CTX-treated mice were analyzed for the presence of cDC and pDC specific markers (Supplementary Fig. 2).[4] Blood from PBS-treated mice showed ~4% cDCs vs ~1–2% pDCs. By contrast, blood from CTX-treated mice showed ~25–30% cDCs and ~2–4% pDCs (Fig. 1*E*). We then analyzed the activation phenotype of DCs at multiple time points after CTX treatment. Consistent with previous studies (14,29), we found that CTX treatment induced an up-regulation of CD80 expression in DCs on day 3 posttreatment. From day 3 onward, however, expression of CD80 (Fig. 2*A*) and CD40, CD86, and MHC class-II (data not shown) showed low levels in DCs (Fig. 2*A*). In conclusion, CTX-induced expansion of DCs in circulation during the recovery phase favored the generation of cDCs bearing an immature phenotype.

Post-CTX expanded DCs are biologically functional

To address whether post-CTX expanded DCs were functional, we assessed their phagocytic and Ag presentation capabilities, which are the hallmarks of functional immature and mature DCs, respectively. Using the FITC-labeled dextran in vitro phagocytosis assay, we found that the phagocytic capability of circulating DCs on day 12 after CTX treatment did not significantly differ from those of circulating DCs in PBS-treated control mice (Fig. 2*B*, *lower panel*). Cells incubated with FITC-dextran-FITC at 4°C, as negative control, did not show phagocytosis (Fig. 2*B, upper panel*). We further tested whether DCs could uptake and process Ag in vivo, using DQ-OVA as a model Ag. DQ-OVA is a fluorogenic reagent which is undetectable in its unprocessed form because of autoquenching, but becomes fluorescent upon entry into acidic endosomal cellular compartment (30). PBS- and CTX-treated mice were s.c. challenged with DQ-OVA on day 12 after treatments. DCs from PBS- and CTX-treated mice acquired similar amounts of DQ-OVA (Fig. 2*C*). Then, we assessed the Ag presenting capability of DCs. Thus, DCs ($CD11c^+CD11b^+$) were sorted from PBL pooled from 20 mice after 12 days of PBS or CTX treatment. Similar numbers of sorted DCs from PBS- and CTX-treated mice were matured by incubation with GM-CSF/IL-4 for 2 days, pulsed with OVAp, and cocultured with naive OT-1 cells (1:1 ratio) for 3 days. Fig. 2*D* shows that DCs sorted (>98% pure) 12 days post-CTX treatment were able to more efficiently present OVAp to OT-1 cells than DCs purified from PBS-treated mice, as evidenced by the proliferation levels of OT-1 cells. OVAp-pulsed DCs, generated in vitro from BM of naive B6 mice in the presence of GMCSF and IL-4, were used as a positive control APCs (data not shown).

[4]The online version of this article contains supplemental material.

Triggering TLR3 signaling at the peak of post-CTX DC expansion induced an inflammatory milieu, resulting in appearance of activated DCs in LNs

Because post-CTX expanded DCs at the restoration phase expressed an immature phenotype (Fig. 2), we attempted to activate these cells with TLR ligands (TLRL), which are potent activators of innate immune cells, in particular DCs (31–38). We have recently reported that the TLR3L poly(I:C) is a potent adjuvant for $CD8^+$ T cell responses (20,38,39), and that DCs ($CD11c^+CD11b^+$) sorted from PBL harvested 12 days after PBS and CTX treatments showed similar expression levels of TLR3 (data not shown). Therefore, we used poly(I:C) to activate CTXexpanded cDCs. It was found that administration of poly(I:C) on day 12 after CTX treatment induced a significant decrease in the number of cDCs in PBL, coinciding with a significant increase in the number of these cells in LNs compared with CTX-treated controls (Fig. 3*A*). Of note, poly(I:C) did not alter the numbers of DCs in blood, spleen, or LNs of naive mice (data not shown). The cDCs that appeared in the LNs expressed an activation phenotype, as evidenced by the expression of high levels of CD40 and CD80 (Fig. 3*B*). Interestingly, after poly(I:C) treatment, cDCs in PBL of CTX-treated mice expressed low levels of CCR5 and CCR7, but those in LNs and spleen expressed low levels of CCR5 and higher levels of CCR7 (Fig. 3*C*), which is essential for DCs to migrate to LNs (40).

To address how post-CTX expanded DCs were activated after poly(I:C) treatment, we measured the serum levels of inflammatory cytokines after injection of poly(I:C) on day 12 post-PBS- or CTX-treatment. Consistent with our recent report (38), higher levels of TNF-α, MCP-1, IL-6, and IFN-γ were induced in sera collected 4 h after poly(I:C) administration into PBS-treated mice (Fig. 4). The levels of these cytokines, however, were higher in the sera of CTX-treated mice than in control mice. These data would suggest that poly(I:C) induces activation of CTX-expanded DCs through creation of an inflammatory milieu even in the absence of active vaccination. To determine whether DCs or NK cells are the source of these cytokines, we injected poly(I:C) into DTR transgenic mice (in which DCs were depleted by i.p. injection of 90 ng/Kg of DTx) or into mice depleted of NK cells by i.p. injection of 50 μl/mouse anti-asialo GM1 Ab 24 h before poly(I:C) injection. As shown in Fig. 4, depletion of DCs abrogated the poly(I:C)-mediated induction of TNF-α, MCP-1, IL-6, and IFN-γ after poly(I:C) treatment. Depletion of NK cells, however, only abrogated the poly(I:C)-mediated induction of IFN-γ. These results indicate that DCs are a significant contributor to the inflammatory cytokines induced after administration of poly(I:C) at the peak of post-CTX DC expansion.

Vaccination with $gp100_{25–33}$/poly(I:C) at the peak of post-CTX DC expansion augmented $CD8^+$ T cell responses to self tumor Ag

To evaluate the significance of post-CTX DC expansion, we sought to determine whether it could affect T cell responses to self tumor Ag in vivo. To this end, we used the preclinical TCR Tg pmel-1 model. Thus, naive Ly5.2 mice were challenged with B16 tumor and treated 10 days later (the time point when the tumor was established) with PBS or CTX and adoptively transferred with 1×10^6 naive Ly5.1 pmel-1 cells followed by vaccination with $gp100_{25–33}$ ± poly(I:C) at day 12 post-CTX administration. Ag priming with $gp100_{25–33}$ alone did not induce pmel-1 cell expansion as measured by the low levels of the relative and absolute numbers of pmel-1 cells in PBL 5 days post vaccination. Ag priming with $gp100_{25–33}$/poly(I:C) in CTX-, but not PBS-, treated mice marginally enhanced the expansion of pmel-1 cells (Fig. 5*A*). The same results were seen when adoptive transfer of pmel-1 cells and $gp100_{25–33}$/poly(I:C) vaccination was performed on day 2 post-CTX treatment (data not shown). Given this low expansion of pmel-1 cells in response to a single vaccination, we sought to perform vaccination with $gp100_{25–33}$/poly (I:C) at both the lymphopenic and restoration phases (prime-boost regimen). Thus, mice were challenged with B16 tumor and treated with PBS or CTX as above and then primed and boosted with

$gp100_{25–33}$ ± poly(I:C) on days 2 and 12, respectively, posttreatments. Boosting with $gp100_{25–33}$/poly(I:C) in CTX-, but not PBS-, treated mice resulted in significant increases in the relative (shown above each column) and absolute numbers of pmel-1 cells analyzed in PBL (Fig. 5*B*, *upper panel*), LNs (Fig. 5*B*, *lower panel*), and spleen (data not shown) on day 3 after Ag boosting. These results indicate that activation of post-CTX expanded DCs could enhance the activity of vaccine primed pmel-1 cells better than naive cells, suggesting that a prime-boost vaccination regimen would be an effective approach to exploit the significance of CTX-induced DC expansion.

Augmentation of $CD8^+$ T cell expansion after $gp100_{25–33}$/poly(I:C) prime-boost regimen coincides with a temporal appearance of activated DCs in LNs

To determine whether the poly(I:C)-induced expansion of pmel-1 cells in CTX-treated mice was associated with trafficking of post-CTX expanded DCs into LNs, we analyzed the distribution of DCs 3 days after boosting with the same regimen described in Fig. 5*B*. As expected at this time point (day 15 post-CTX treatment), the relative and absolute numbers of DCs in PBL of CTX-treated mice were still higher than control mice (Fig. 5*C*). Interestingly, this increase in DC numbers in PBL of CTX treated mice were further accentuated upon vaccination with $gp100_{25–33}$ but not after vaccination with $gp100_{25–33}$/poly(I:C), which was associated with a marked increase in the numbers of DCs in LNs. These results suggest the migration of DCs from blood to lymphoid compartments such as LNs upon their activation with poly(I:C). A selective increase in the percentage and absolute numbers of $CD8\alpha^+$ cDCs over $CD8\alpha^-$ cDCs was observed in LNs (Fig. 5*D*) and spleen (data not shown) of CTX-treated mice boosted with $gp100_{25–33}$/poly(I:C). To test whether this selective effect of poly(I:C) on the number of these subsets of cDCs in LNs correlates with a selective effect on their activation phenotype, we repeated the experiment above and analyzed the activation phenotype of $CD8\alpha^-$ cDCs and $CD8\alpha^+$ cDCs in LNs. We found that vaccination with $gp100_{25–33}$/poly(I:C) induced significant up-regulation in the expression of CD40 in $CD8\alpha^-$ cDCs and $CD8\alpha^+$ cDCs in LNs and spleen, with more noticeable effect in CTX- vs PBS-treated mice (Fig. 6, *A* and *B*). The expression levels of CD86 were also increased in $CD8\alpha^-$ and $CD8\alpha^+$ cDCs in LNs (Supplementary Fig. 3).[4] Similar results were observed in the spleen (Fig. 6*B*). We then asked which of the cDC subsets in LNs and spleen is capable of Ag uptake. To this end, PBS- and CTX-treated mice were s.c. injected with DQ-OVA ± poly(I:C) on day 12 after treatments and killed 2 days later to assess the capability of $CD8\alpha^-$ cDCs and $CD8\alpha^+$ cDCs in LNs and spleen to uptake DQ-OVA. Consistent with previous studies (41), we found that the number of DQ-OVA^+ $CD8\alpha^+$ cDCs in LNs was higher than DQ-OVA^+ $CD8\alpha^-$ cDCs; opposite profile of DQ-OVA uptake by these cells was observed in spleen (Fig. 6*C*). Poly(I:C) treatment did not alter the Ag uptake profile. Taken together, the augmented systemic pmel-1 cell expansion in response to boosting with $gp100_{25–33}$/poly(I:C) at the peak of post-CTX DC expansion could be attributed to the temporal increase in the number of activated DCs in secondary lymphoid tissues, in particular LNs.

Depletion of DCs abrogates the augmented pmel-1 T cell responses to prime-boost vaccination

To directly test our hypothesis of the critical role of post-CTX-activated DCs in mediation of $CD8^+$ T cell expansion, DCs were depleted before Ag boosting using CD11c-DTR Tg mouse model, which is based on a system that allows the conditional ablation of $CD11c^{high}$ cDCs, but not pDCs. DTR expression in these mice is driven by a DNA fragment flanking the *Itgax* gene, which encodes the α_x subunit of the CD11c integrin (30). Thus, Ly5.2 wild type and CD11c-DTR Tg mice were treated with PBS or CTX, adoptively transferred one day later with Ly5.1 pmel-1 cells, and then primed and boosted with $gp100_{25–33}$ ± poly(I:C) on days 2 and 12, respectively, posttreatments. The CD11c-DTR Tg mice were treated i.p.

with DTx one day before Ag boosting to deplete DCs. The mice were killed 2 days later (day 14 post-CTX treatment) to analyze the numbers of DCs and the expansion of pmel-1 cells. As shown in Fig. 7*A*, treatment with DTx effectively depleted $CD11c^+CD8^+$ (*upper right* of the quadrant) and $CD11c^+CD8^-$ (*upper left* of the quadrant) in blood, spleen, and LNs of both PBS- and CTX-treated CD11c-DTR Tg mice. The relative and absolute numbers of DCs in the spleen and LNs before and after DTx injection into control and CTX-treated mice are shown in Fig. 7, *B* and *C*. Interestingly, depletion of DCs in CTX-treated mice markedly abrogated the expansion of pmel-1 cells, as indicated by the marked decreases in the relative and absolute numbers of pmel-1 cells in spleen, LNs (Fig. 7*D*), and PBL (data not shown). Of note, treatment of wild type mice with DTx did not alter the magnitude of pmel-1 cell expansion (data not shown). These data would indicate that post-CTX expanded DCs significantly contributed to the beneficial effects of CTX preconditioning regimen to the responses of effector cells to Ag recall.

Boosting with peptide/poly(I:C) at the peak of post-CTX DC expansion is essential to establish a therapeutic antitumor immunity to self tumor Ag

We finally evaluated the therapeutic antitumor efficacy of our treatment regimen consisting of CTX preconditioning, adoptive transfer of naive pmel-1 cells, and prime-boost vaccination with $gp100_{25–33}$/poly(I:C) at the lymphopenic and restoration phases. To this end, we tested the effects of one vs two vaccinations against established B16 tumor. Thus, B6 mice were challenged with B16 tumor. Tumors were allowed to grow for 10 days, and the mice were then treated with PBS or CTX. One day later, the mice were adoptively transferred with naive pmel-1 cells alone as control, or vaccinated with $gp100_{25–33}$ peptide along with poly(I:C) on day 2 only (lymphopenic phase), day 12 only (the restoration phase; the peak of DC expansion), or days 2 and 12 post-CTX treatment. As shown in Fig. 8*A*, CTX treatment alone significantly ($p = 0.001$) delayed the tumor growth compared with the PBS-treated tumor bearing mice, and then the tumor rapidly grew afterward. Single vaccination with $gp100_{25–33}$/poly(I:C) at the lymphopenic phase led to a significant delay ($p = 0.038$) in the tumor growth as compared with CTX-treated non-vaccinated group, and then the tumor rapidly grew afterward. Interestingly, prime-boost vaccination with $gp100_{25–33}$/poly(I:C) did not affect the tumor growth in PBS-treated mice, but completely prevented the tumor growth in CTX-treated mice. The degree of tumor growth under each treatment regimen correlate with the rate of survival of each group (Fig. 8*B*). Interestingly, the antitumor efficacy after prime-boost was further manifested by the development of vitiligo as compared with single vaccination (Fig. 8*C*).

Although the enhanced antitumor efficacy of pmel-1 cells in the presence of a large pool of activated DCs could be explained by the enhanced expansion (quantity) of pmel-1 cells, the quality of pmel-1 cells might also be enhanced. To evaluate the quality of pmel-1 cells, naive mice were inoculated with B16 cells, treated 7 days later with CTX, received one million pmel-1 cells, and then were vaccinated with $gp100_{25–33}$/poly(I:C) on day 2 or on days 2 and 12 post-CTX treatment. Mice were killed 5 days later and spleens and LNs were harvested and pooled. Equal numbers (1×10^5) of pmel-1 cells from each group were adoptively transferred into recipient mice (new recipients) challenged 7 days before with s.c. injection of 2×10^5 B16 cells. These new recipients were treated with CTX 1 day before the adoptive transfer of pmel-1 cells. PBS- and CTX-treated controls (with no adoptive T cell therapy) were challenged with B16 cells as controls. Tumor size was recorded at the indicated time points. As shown in Fig. 8*D*, adoptive transfer of donor cells harvested from the mice vaccinated with $gp100_{25–33}$/poly(I:C) on days 2 and 12 induced higher antitumor responses than those obtained after adoptive transfer of donor cells harvested from mice vaccinated with $gp100_{25–33}$/poly(I:C) only on day 2, indicating that the quality of pmel-1 cells generated in the presence of a large pool of activated DCs is a critical mechanism

mediating the antitumor responses of the prime-boost vaccination with peptide/poly(I:C). Of note, donor cells harvested from PBS-treated mice vaccinated once or twice with $gp100_{25–33}$/poly(I:C) could not induce antitumor response in the new recipients (data not shown). In conclusion, these results demonstrate that prime-boost vaccination with peptide/poly(I:C) at both the lymphopenic and restoration phases post-CTX therapy can markedly augment both the quantity and quality of the antitumor efficacy of adoptively transferred $CD8^+$ T cell therapy, perhaps by taking the full advantage of both the lymphopenic microenvironment and the CTX-induced expansion of DCs.

Discussion

In the present study we observed substantial increases in both the relative and absolute numbers of DCs with an immature phenotype during the restoration phase after CTX-induced lymphodepletion. This observation is consistent with recent studies that reported increases in the number of DCs during the restoration phase in the peripheral blood of cancer patients receiving combinatorial treatment with CTX and the growth factors G-CSF and GM-CSF (42–44). Although it was not clear in these studies whether the increase in the frequency of DCs was due solely to the effects induced by CTX or by the growth factors, our results demonstrate that CTX is capable of inducing DC expansion in the peripheral blood in a murine model. The potential clinical significance of our observation is evidenced by the substantial increases in the antitumor efficacy of adoptively transferred $CD8^+$ T cells when these cells were boosted with peptide vaccination and poly(I:C) at the peak of post-CTX DC expansion. These Ag recall responses were markedly abrogated when DCs were depleted during Ag boosting; indicating to the essential contribution of post-CTX expanded DCs.

Despite their immature phenotype, CTX-expanded DCs showed phagocytic (Fig. 2*B*) and Ag-presenting (Fig. 2*C*) capabilities, indicating that they are biologically functional. As expected, this post-CTX surge in DCs bearing immature phenotype did not enhance the responses after vaccination with $gp100_{25–33}$ alone (Fig. 5*A*). Provision of poly(I:C) at this surge of DCs, however, created an inflammatory microenvironment (TNF-α, MCP-1, IFN-γ, and IL-6) (Fig. 3*D*), resulting in the appearance of activated $CCR7^+$, $CD40^+$, and $CD80^+$ DCs in LNs (Fig. 3, *B* and *C*), which could be contributing to the augmented Ag-specific responses of $CD8^+$ T cells to $gp100_{25–33}$ peptide when delivered along with poly(I:C) at the peak of DC expansion (Fig. 5*A*). The appearance of DCs in LNs after poly(I:C) was associated with a significant decrease in the numbers of DCs in the peripheral blood. Given that CCR7 is essential for migration of activated DCs to LNs (40), its up-regulation would suggest that the appearance of DCs in LNs after poly(I:C) treatment is, at least in part, due to their homing from PBL into LNs after maturation. Because resident DCs in LNs express CD8α while migratory DCs do not (41), and because DCs expanded in the blood were $CD8\alpha^-$ and those increased in LNs were $CD8\alpha^+$, we do not exclude the possibility of local expansion and activation of the resident DCs in LNs of CTX-treated hosts in response to poly(I:C). Alternatively, migratory blood-born DCs may acquire the phenotype of lymphoid (CD8α) DCs upon their arrival to LNs as previously reported (45,46), and thus become indistinguishable from resident DCs. Regardless of the mechanisms mediating the increase in the number of DCs in LNs, depletion of these cells before boosting of both PBS- and CTX-treated hosts with peptide/poly(I:C) significantly abrogated the expansion of effector pmel-1 cells (Fig. 6), indicating to the importance of DCs to the Ag recall responses of $CD8^+$ T cells to active vaccination in general, and to CTX preconditioning in particular. Similar requirement of DCs to Ag recall responses has been reported in the viral setting (41). Indeed, recent studies by Wang et al. have pointed to the importance of hematopoietic-derived APCs during the lymphopenic phase to the Ag priming of $CD8^+$ T (pmel-1) cells

(47). Together, DCs appear to be required during Ag priming and boosting for generation of memory $CD8^+$ T cells.

The expansion of DCs during the restoration phase post-CTX induced lymphodepletion was preceded by the activation of these cells during the lymphopenic phase (Fig. 2*A*). Indeed, previous reports, including ours, have demonstrated a rapid activation of DCs at the lymphopenic phase after chemotherapy and TBI, associating with significant increases in the Ag-specific responses of adoptively transferred T cells (3,14,18,19,48,49). Paulos et al. demonstrated that this rapid DC activation is attributed to the significant damage to the integrity of mucosal barriers and translocation of bacterial products (LPS; a TLR4 ligand) (3). These events would lead to induction of inflammatory cytokines (8,9,38) and activation of DCs. Therefore, the transient activation of DCs during the lymphopenic phase (up to day 4) after CTX treatment may be because of the rapid clearance of LPS from circulation. There is further evidence that exogenous LPS can substitute for the endogenous TBI-induced LPS for augmentation of the antitumor responses of $CD8^+$ T cells to active vaccination when they were adoptively transferred to immune cell (NK cells and CD4 T cells)-ablated recipient mice (3). Similarly, we have reported that addition of poly(I:C) to OVAp vaccination during the lymphopenic phase after CTX preconditioning markedly augmented $CD8^+$ T cell expansion, which was associated with significant activation of DCs in spleen and liver (14). In the present study, we were able to extend this observation by showing the capability of poly(I:C) to induce activation of DCs expanded during the restoration phase, resulting in robust increases in the $CD8^+$ T cell responses to active vaccination (Fig. 5, *A* and *B*). Taken together, we would suggest that the host microenvironment created after induction of lymphodepletion can be successfully exploited both at the lymphopenic and restoration phases by TLRL (e.g., TLR3L and TLR4L) to benefit adoptive T cell therapy combined with active vaccination.

The activation of DCs during the lymphopenic phase and their expansion during the restoration phase may suggest a harmonized hierarchy of multiple mechanisms involved in the beneficial effect of CTX on adoptive T cell therapy. This hierarchy warrants the reconsideration of the timing between adoptive T cell transfer into a CTX-lymphodepleted host and any subsequent vaccinations. We tested this hypothesis by applying prime-boost vaccination with $gp100_{25–33}$/poly(I:C) at the lymphopenic and restoration phases, so that pmel-1 cells could benefit from the activated DCs at the lymphopenic phase as well as from the activated large pool of DCs at the restoration phase. This regimen induced a substantial expansion of pmel-1 cells and prevention of B16 tumor growth (Fig. 8), which absolutely required CTX treatment because the same prime-boost regimen in PBS-treated mice could not prevent B16 tumor growth. Our results point to the role of post-CTX expanded DCs in this antitumor effects of pmel-1 cells because boosting of the CTX-treated mice with $gp100_{25–33}$/poly(I:C) at the peak of DC expansion resulted in recruitment of activated DCs in LNs and spleen (Fig. 5*B*), increases in the numbers (quantity) of the Ag-specific pmel-1 cells in LNs and spleen (Fig. 5*D*), and tumor regression and better survival (Fig. 8). These results suggest that tumor regression was mediated by pmel-1 cells expansion in the presence of a large pool of activated DCs. Because DCs were required for pmel-1 cell expansion (Fig. 7*D*), our results suggest that DCs are important for the antitumor effects of pmel-1 cells by augmenting the quantity of the latter. DCs might also play a role in the tumor regression by augmenting the quality of the Ag-specific pmel-1 cells because adoptive transfer of donor cells harvested from mice vaccinated with $gp100_{25–33}$/poly(I:C) on days 2 and 12 induced higher antitumor responses than those harvested from mice vaccinated with $gp100_{25–33}$/poly(I:C) only on day 2. Thus, we suggest that the presence of a large pool of activated DCs augment both the quantity and quality of the tumor-specific responses of pmel-1 cells.

Recent studies have also reported the capability of naive and effector $gp100_{25–33}$-specific pmel-1 cells to induce regression of established B16 tumor. The antitumor effects in these studies, however, required aggressive treatment protocols consisting of TBI-induced lymphodepletion or myelodepletion followed by hematopoietic stem cell transplant, adoptive transfer of in vitro cytokine-conditioned Ag-stimulated pmel-1 cell, vaccination with 2×10^7 plaque forming units of a recombinant fowlpox virus encoding $gp100_{25–33}$ or with repeated ex vivo vaccination with peptide-pulsed DCs, and exogenous administration of high doses of IL-2 (4,47,50). In addition to these established therapeutically effective antitumor regimens, our data presents another therapeutically effective antitumor regimen based on targeting a large pool of DCs by TLRLs during peptide vaccination, obviating the need for more complicated and potentially toxic treatment regimens such as IL-2 therapy.

We believe that our in vivo DC-based prime-boost vaccination with peptide/TLR3L at defined phases post-lymphodepletion is an effective treatment approach because it induces in vivo maturation of DCs by TLRLs, which, in contrast to ex vivo DC-based vaccination, also stimulate other critical factors in the host microenvironment. In line with this notion, several recent studies reported substantial improvement in T cell responses to active vaccination and generation of efficacious antitumor responses upon in vivo activation of Flt3L-mobilized DCs with TLRLs, in particular the TLR9L CpG (51–55). In conclusion, our data showed that CTX therapy induces a biphasic effect on DCs. During the lymphopenic phase, DCs are activated, while during the restoration phase DCs are expanded but express immature phenotype, which can be exploited in vivo to favor generation of robust antitumor immunity against self-tumor Ag. Our data provide a useful foundation for a rationale design of anti-cancer immunotherapy regimens by combining lymphodepletion, adoptive T cell therapy, and TLRL-based tumor vaccines.

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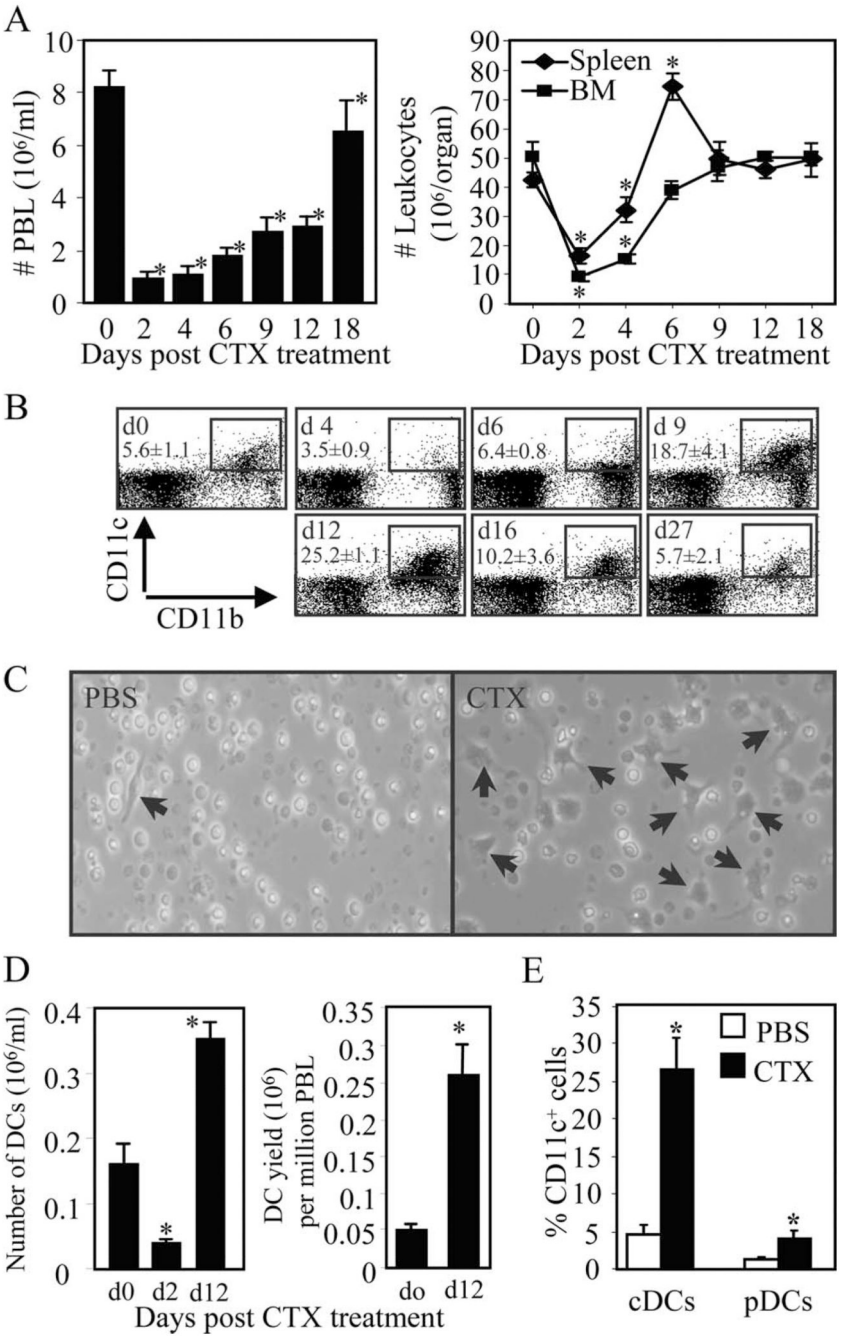

FIGURE 1.

CTX treatment induced increases in the numbers of circulating cDCs during the restoration phase. *A*, Female naive B6 mice (*n* = 4/group) were i.p. treated with PBS (represented as day 0) or CTX (4 mg/mouse) and killed at the indicated time points. The total number of mononuclear cells was determined in blood (*left panel*), and spleen and BM (*right panel*). Naive B6 mice (*n* = 4/group) were treated as in *A* and bled at the indicated time points. *B*, A representative flow data showing percentage of DCs ($CD11c^+CD11b^+$) in PBL at multiple time points post-CTX treatment after staining with anti-CD11c and anti-CD11b mAbs. *C*, PBL samples were prepared from blood harvested on day 12 after treatment with PBS or CTX and then incubated for 4 h at 37°C to allow DCs to adhere. DCs were defined by their

appearance as adherent cells, indicated by arrows. *D*, *left panel*, The absolute numbers of DCs ± SD determined as percentage of DCs in each PBL samples (from PBS- or CTX-treated mice) × total number of PBL/100. *D*, *right panel*, The absolute numbers of DCs per one million PBL of the blood of PBS-(represented as day 0) and CTX-treated mice. *E*, Average ± SD of the relative numbers of cDCs ($CD11c^{high}CD11b^{high}Gr.1^{low}$ $B220^{low}$) and pDCs ($CD11c^{high}CD11b^{low}Gr.1^{high}B220^{high}$) in the PBL of PBS and CTX-treated mice after 12 days of treatments. *, $p < 0.05$. In all experiments control mice were treated with PBS and the levels of cells present are represented as day 0 in the figures. These experiments were performed at least five times.

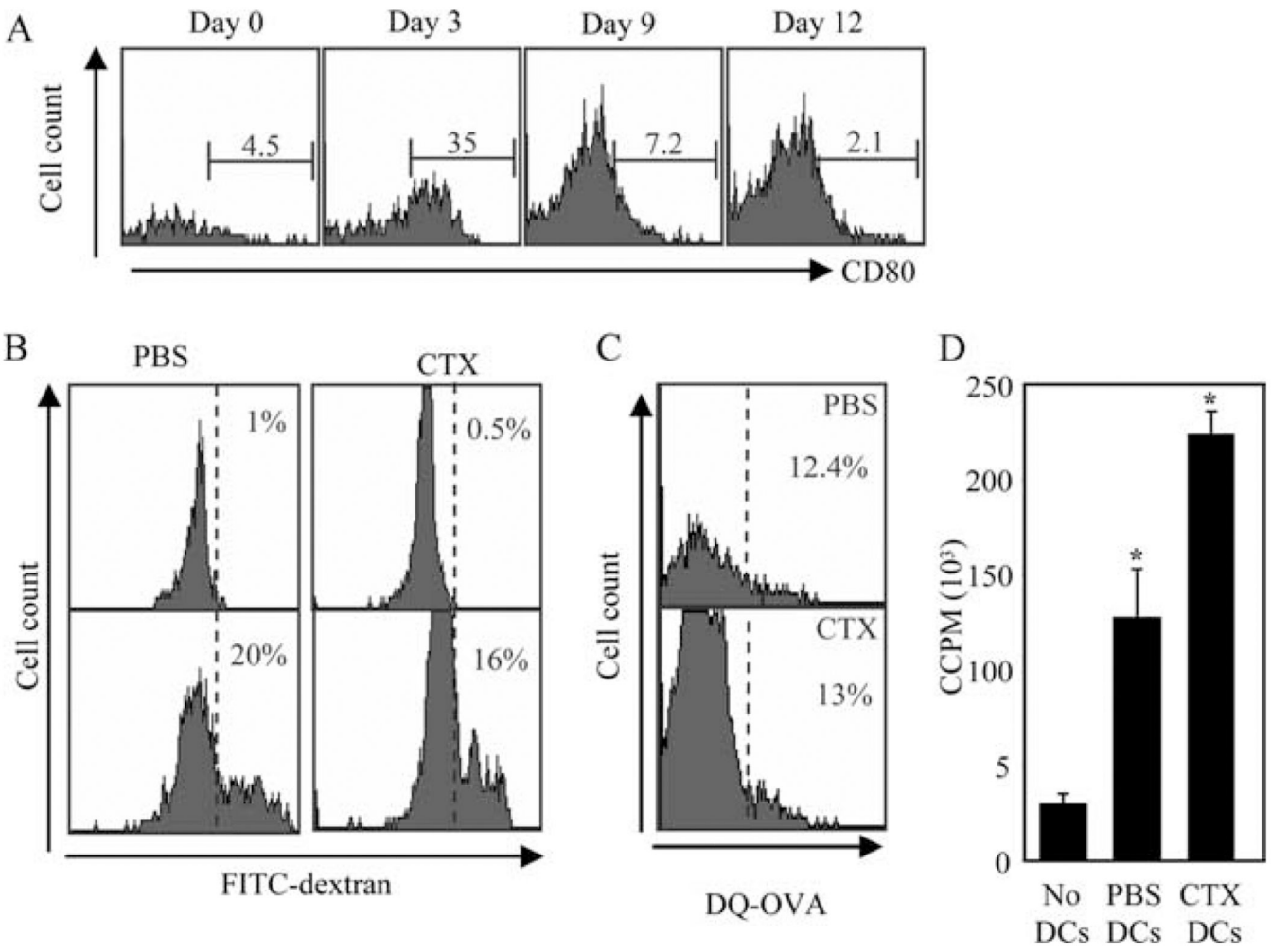

FIGURE 2.

CTX treatment induced an expansion of functional cDCs with immature phenotype. *A*, Naive B6 mice (n = 4/group) were treated i.p. with PBS or CTX, bled at the indicated time points, and the PBL were stained with anti-CD11c, anti-CD11b, and anti-CD80 mAbs to measure the CD80 expression on DCs. *B*, PBS- and CTX-treated B6 mice (n = 6/group) were bled 12 days posttreatments and PBL samples were incubated with FITC-labeled dextran at 4°C as negative controls (*upper panel*) or at 37°C (*lower panel*). Percent $FITC^+CD11c^+CD11b^+$ DCs were analyzed by flow cytometry. *C*, Naive B6 mice (n = 4/group) were i.p. treated with PBS or CTX and challenged 12 days later with s.c. injection of 50 μg/mouse DQ-OVA. Histograms show DQ-OVA uptake by $CD11c^+CD11b^+$ in blood 1 day after DQ-OVA injection. *D*, In vitro Ag presenting function of DCs sorted from PBL of PBS- or CTX-treated mice. Cells were stimulated with GM-CSF + IL-4 (10 ng/ml each) for 2 days, pulsed with 1 μg/ml OVAp, irradiated, and cocultured with purified naive OT-1 cells. Proliferation of OT-1 cells was measured by thymidine assay after 3 days. *, $p < 0.05$. These experiments were performed at least two times.

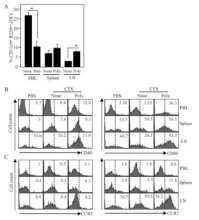

FIGURE 3.

Triggering TLR3 signaling at the peak of post-CTX DC expansion resulted in appearance of activated DCs in LNs. *A*, Naive B6 mice (n = 4/group) were treated with PBS or CTX and i.p. injected 12 days later with PBS or 200 μg/mouse poly(I:C). Mice were killed 24 h after poly(I:C) injection and percentage of $CD11c^{high}B220^{low}$ DCs were determined in PBL, spleen, and LNs. CD40 (*B*, *left panel*), CD80 (*B*, *right panel*), CCR5 (*C*, *left panel*), and CCR7 (*C*, *right panel*) expression in DCs in PBL, spleen, and LNs was analyzed by flow cytometry. These experiments were performed three times.

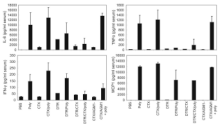

FIGURE 4.

Triggering TLR3 signaling at the peak of post-CTX DC expansion created an inflammatory milieu dependent on DCs. Wild-type (n = 4/group) or DTR Tg (n = 3/group) B6 mice were treated with PBS or CTX and i.p. injected 12 days later with PBS or 200 μg/mouse poly(I:C). One day before poly(I:C) injection (i.e., day 11 post-PBS or -CTX treatment), half of the wild-type mice were treated with anti-asialo-GM1 Ab to deplete NK cells, and all of the DTR Tg mice were treated with DTx to deplete DCs. Mice were bled 4 h after poly(I:C) injection and the sera were collected to measure the levels of TNF-α, IL-6, MCP-1, and IFN-γ by flow cytometric beads array. These experiments were performed three times.

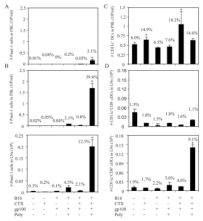

FIGURE 5.

Boosting with peptide/poly(I:C) at the peak of post-CTX DC expansion is essential to augment $CD8^+$ T cell response to self-tumor Ag. *A*, Naive Ly5.2 mice were inoculated with 2.5×10^5 B16 cells in their right flank and treated 10 days later (the time point when the tumor was established) with PBS or CTX and adoptively transferred with 1×10^6 unfractionated naive spleen cells from Ly5.1 donor pmel-1 Tg mice. Mice were left without further manipulation or vaccinated with $gp100_{25–33}$ ± poly(I:C) 12 days post-PBS or -CTX treatment. Mice were then bled on day 5 post-vaccination to assess the percentage and absolute numbers of pmel-1 cells in PBL. *B–D*, Mice were treated as in *A* except that they were vaccinated with $gp100_{25–33}$ ± poly(I:C) on days 2 (priming) and 12 (boosting) post-PBS or -CTX treatment. Mice were killed on day 3 after Ag boosting (day 15 post-CTX treatment) to assess the percentage and absolute numbers of pmel-1 cells in PBL (*B*, *upper panel*) and LNs (*B*, *lower panel*) and percentage and absolute numbers of $CD11c^+$ DCs in PBL (*C*), $CD11c^+CD8^-$ cDCs (*D*, *upper panel*), and $CD11c^+CD8^+$ cDCs (*D*, *lower panel*) in LNs. The absolute numbers of pmel-1 cells and DCs were determined as: percentage of $Ly5.1^+V\beta13^+$ and $CD11c^+$ cells × total number of cells/100. *, $p < 0.05$. These experiments were performed at least three times.

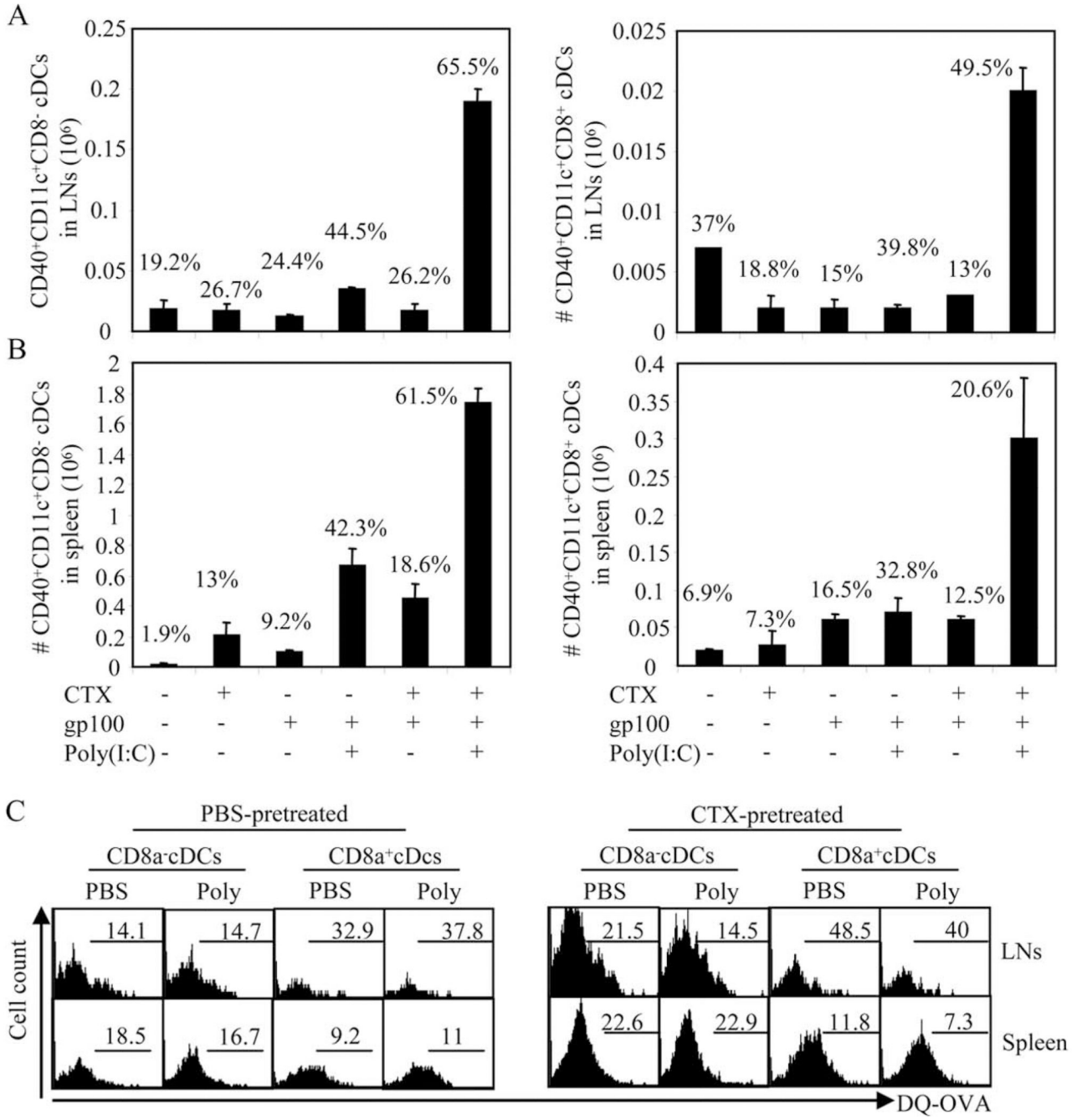

FIGURE 6.

Boosting with peptide/poly(I:C) at the peak of post-CTX DC expansion increases the number of activated DCs in LNs. Mice were treated with PBS or CTX and adoptively transferred with 1×10^6 un-fractionated naive spleen cells from Ly5.1 donor pmel-1 Tg mice. Mice were left without further manipulation, or vaccinated with $gp100_{25–33}$ ± poly(I:C) 2 and 12 days post-PBS or -CTX treatment. Mice were then killed on day 3 post vaccination to assess the percentage and absolute numbers of CD40-expressing $CD11c^+CD8^-$ cDCs (*A, left panel*) and $CD11c^+CD8^+$ cDCs (*A, right panel*) in LNs and $CD11c^{high}CD8^-$ cDCs (*B, left panel*) and $CD11c^+CD8^+$ cDCs (*B, right panel*) in spleen. The absolute numbers of activated DCs were calculated as the percentage of DCs × total

number of DCs/100. *C*, Naive B6 mice (n = 4/group) were i.p. treated with PBS or CTX and challenged 12 days later with s.c. injection of 50 μg/mouse DQ-OVA. Histograms show DQ-OVA uptake by $CD8\alpha^-$ cDCs and $CD8\alpha^+$ cDCs in LNs and spleen 1 day after DQ-OVA injection. These experiments were performed three times.

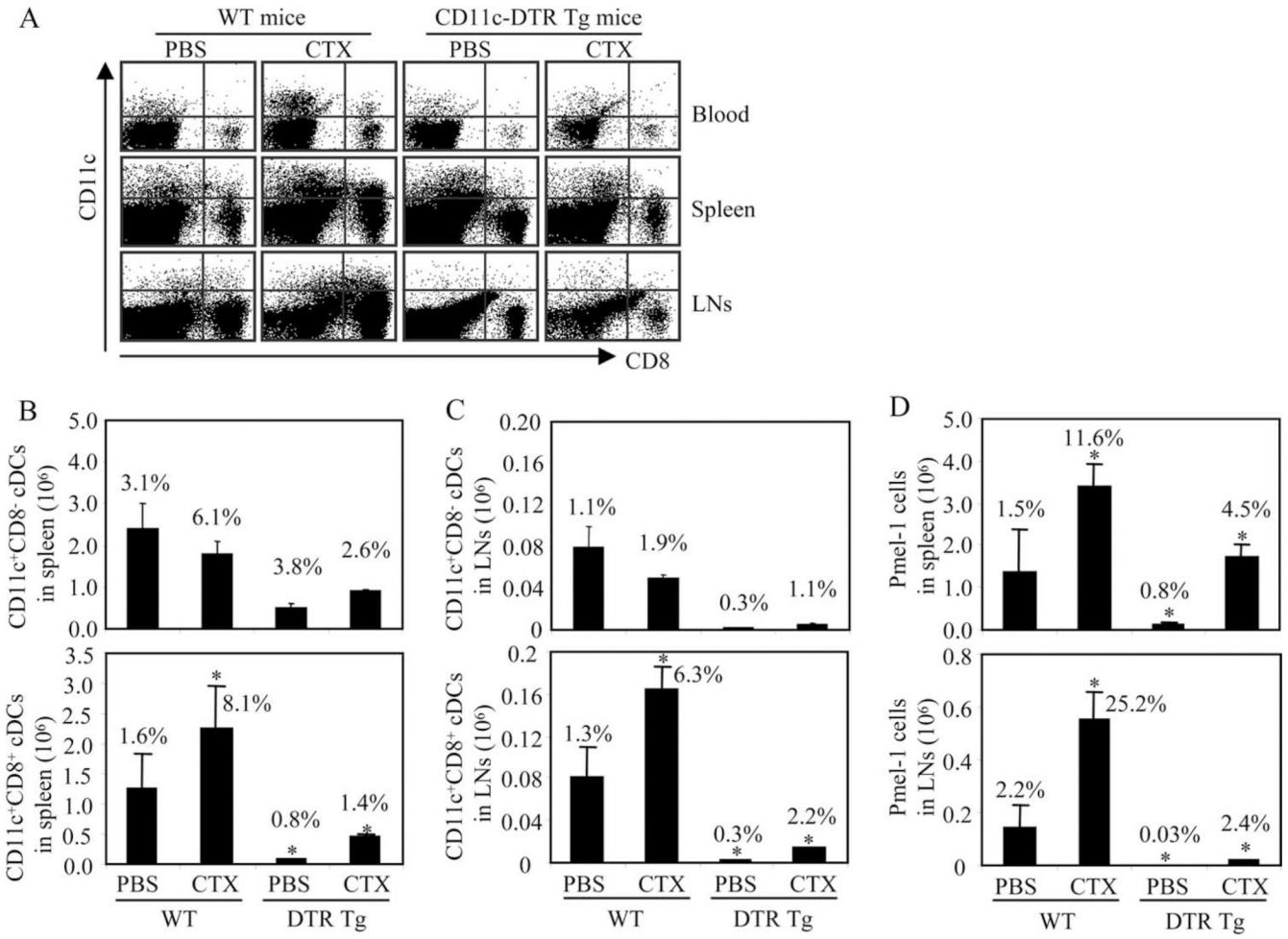

FIGURE 7.

Depletion of DCs before peptide/poly(I:C) boosting at the peak of expansion abrogates the augmented T cell responses post-CTX therapy. Wild type and CD11c-DTR Tg Ly5.2 mice (three mice/group) were treated with PBS or CTX and then adoptively transferred 1 day later with 1×10^6 pmel-1 Ly5.1 cells. The mice were vaccinated with 100 μg/mouse $gp100_{25–33}$ along with 200 μg/mouse poly(I:C) on days 2 and 12 post-PBS and -CTX treatments. On day 11 (1 day before boosting), the CD11c-DTR Tg mice were treated i.p. with 90 ng/mouse DTx to deplete DCs. The mice were killed 2 days after boosting (day 14 post-CTX treatment) to analyze the numbers of DCs and the expansion of pmel-1 cells. *A*, Depletion of $CD11c^+CD8^+$ (*upper right* of the quadrant) and $CD11c^+CD8^-$ (*upper left* of the quadrant) cDCs in the blood, spleen, and LNs of PBS- and CTX-treated CD11c-DTR Tg mice after injection of DTx. *B* and *C*, Percentage and absolute numbers of $CD11c^+CD8^-$ cDCs (*B*, *upper panel*) and $CD11c^+CD8^+$ cDCs (*B*, *lower panel*) in spleen and $CD11c^+CD8^-$ cDCs (*C*, *upper panel*) and $CD11c^+CD8^+$ cDCs (*C*, *lower panel*) in LNs. *D*, Percentage and absolute numbers of pmel-1 cells in spleen (*upper panel*) and LNs (*lower panel*), respectively. The absolute numbers were calculated as the percentage of cells × the total numbers of cells/100. *, $p < 0.05$. These experiments were performed two times.

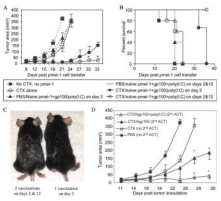

FIGURE 8.

Boosting with $gp100_{25–33}$ peptide/poly(I:C) at the peak of post-CTX DC expansion is essential to establish therapeutic antitumor immunity of naive pmel-1 cells. *A*, B6 mice (n = 5/group) were challenged s.c. with 2.5×10^5 B16 cells and treated with CTX after 10 days. Then mice were primed and/or boosted with $gp100_{25–33}$ peptide/poly(I:C) on day 2 or on both day 2 and day 12 post-CTX treatment. Two extra groups were s.c. challenged with B16 cells and treated with PBS or CTX without further manipulation. Tumor size was recorded at the indicated time points. *B*, Survival of the tumor-bearing mice in *A*. *C*, Vitiligo developed in mice vaccinated once or twice after 30 days of pmel-1 cell transfer. *D*, Prime-boost vaccination with $gp100_{25–33}$/poly(I:C) induces generation of memory pmel-1 cells, which acquire intrinsic antitumor activities. Mice were inoculated with B16 cells, treated 7 days later with CTX, and received one million pmel-1 cells and vaccinated with $gp100_{25–33}$ alone or $gp100_{25–33}$/poly(I:C) on days 2 and 12 post-CTX therapy. Mice were revaccinated 70 days after boosting with the same regimen. Mice were killed 5 days later and spleens and LNs were harvested and pooled. Pooled cells, containing 1×10^5 pmel-1 cells, from each group were adoptively transferred into recipient mice (second recipients) challenged 7 days before with s.c. injection of 2×10^5 B16 cells. These second recipients were injected with CTX 1 day before the second adoptive transfer of pmel-1 cells. PBS- and CTX-treated controls (with no second ACT) were injected with B16 cells as controls. Tumor size was recorded at the indicated time points. *, Statistically significant compared with controls ($p <$ 0.5). These experiments were performed three times.