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Cyclophosphamide Induces Dynamic Alterations in the Host Microenvironments Resulting in a Flt3 Ligand-Dependent Expansion of Dendritic Cells

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Abstract

Preconditioning a recipient host with lymphodepletion can markedly augment adoptive T cell therapy. However, the precise mechanisms involved are poorly understood. In a recent study, we observed a significant increase in the circulating levels of dendritic cells (DCs; $CD11c^+CD11b^+$) during the recovery from cyclophosphamide (CTX)-induced lymphodepletion. Herein, we demonstrate that the CTX-induced DC expansion was not altered by adjuvant chemotherapy or tumor burden but was augmented by coadministration of granulocyte-colony stimulating factor. Although the increase in the number of DCs was preceded by a systemic expansion of a population expressing the phenotype of myeloid-derived suppressor cells ($Gr\text{-}1^+CD11b^+$), depletion of these $Gr\text{-}1^+$ cells had no effect on the noted expansion. Moreover, when $Gr\text{-}1^{high}CD11b^{high}$ cells were sorted from CTX-treated mice and adoptively transferred into control or CTX-treated recipients, they did not differentiate into DCs. Post-CTX expansion of DCs was associated with proliferation of DCs in bone marrow (BM) during the lymphopenic phase and in the blood and spleen during the recovery phase. Furthermore, adoptive transfer of BM cells from CTX-treated mice produced equal numbers of DCs in the blood of either CTX-treated or untreated recipients. CTX induced a dynamic surge in the expression of growth factors and chemokines in BM, where CCR2 and Flt3 signaling pathways were critical for DC expansion. In sum, our data suggest that CTX induces proliferation of DCs in BM prior to their expansion in the periphery. Targeting DCs at these phases would significantly improve their contribution to the clinical application of lymphodepletion to adoptive immunotherapy

Cyclophosphamide (CTX) is a common chemotherapeutic agent used clinically for the treatment of several human malignancies (1). CTX has also been widely used in combination with growth factors such as granulocyte-colony stimulating factor (G-CSF) for the mobilization of hematopoietic stem cells (HSCs) from bone marrow (BM) to circulation (2–4). In addition, recent preclinical studies have shown that a CTX preconditioning

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regimen can be associated with a marked enhancement of antitumor immunity (5–7). In this setting, the CTX preconditioning consisted of treatment of a recipient host with CTX before adoptive T cell transfer alone or followed by vaccination. Recent clinical studies also demonstrated that a CTX preconditioning regimen can induce a marked enhancement in the antitumor responses of adoptively transferred in vitro activated T cells when followed by IL-2 therapy (8–10). The antitumor efficacy of the combination of CTX preconditioning regimen, adoptive T cell therapy and vaccination in the clinical settings remains to be tested.

Although the immunomodulatory effects of CTX are not fully understood, several mechanisms have been proposed, including: 1) an enhanced homeostatic expansion of Ag-specific T cells by the creation of a favorable niche for the immune cells (9,11); 2) the induction of T cell growth and survival factors such as type I IFNs, IL-7, and IL-15 (5,12,13); 3) elimination of T regulatory cells (14–18); and 4) a rapid activation of dendritic cells (DCs) after induction of lymphodepletion by CTX (13,19,20) similar to what has been observed after total body irradiation (TBI) (21). Because recent studies, including ours, have shown that the adjuvant effects of CTX are independent of the creation of a niche and the elimination of regulatory T cells (6,13,22,23), it appears that DCs may play a crucial role in the beneficial effects of CTX preconditioning to adoptive T cell therapy. Exploring the precise mechanisms underlying the role of DCs effects would improve the promising application of CTX preconditioning in the clinical setting.

Most recently, we have been able to show that CTX induced increases in the circulating levels of DCs during the recovery from leukopenia (24), where administration of the TLR3 agonist polyinosinic-polycyctidylic acid (poly-I:C) during the peak of DC expansion induced activation and migration of DCs to lymph nodes (LNs). Furthermore, concomitant administration of poly-I:C with vaccination with the MHC class I melanoma self-tumor gp100 peptide at the peak of post-CTX DC expansion resulted in therapeutically effective antitumor T cell responses against advanced melanoma (24). The role of the post-CTX–expanded DCs on the beneficial effects of CTX was directly confirmed by the abrogation of the augmented T cell responses after depletion of the expanded DCs before vaccination (24).

To further understand the mechanisms underlying the expansion of DCs post-CTX treatment, our focus was to: 1) determine whether post-CTX expansion of DCs is restricted to the peripheral blood; 2) evaluate the impact of tumor burden, adjuvant chemotherapy or G-CSF therapy on post-CTX expansion of DCs; and 3) define the cellular and molecular mechanisms underlying post-CTX expansion of DCs. We found that CTX treatment preferentially induced increases in the numbers of DCs in the spleen in addition to the peripheral blood. This DC expansion was constant despite the effect of tumor burden and adjuvant chemotherapy, but was found to be transiently increased when CTX treatment was followed by administration of G-CSF. Post-CTX expansion of DCs was associated with proliferation of these cells in BM and periphery during lymphopenic and recovery phases, respectively, with a fine-tuned modulation of certain chemokines and growth factor expression in BM, where CCR2 and Flt3 signaling pathways were found to be critical for DCs to show competent expansion. These data shed a light on the mechanisms mediating post-CTX expansion of DCs, and form a foundation to target these cells at specific time points after chemotherapy to potentially improve the clinical application of adoptive immunotherapy.

Materials and Methods

Mice

B6.SJL (Ly5.1), C57BL/6 (Ly5.2; B6), pmel-1 TCR transgenic (on B6 background), BALB/C, and SCID/beige mice (on BALB/C background) were purchased from The Jackson

Laboratory (Bar Harbor, ME). Splenectomized mice (on B6 background) and CCR1, CCR2, CCR5, and CCL3 knockout mice (on B6 background) were purchased from Taconic Farms (Germantown, NY). Pmel-1 (Ly5.2) mice were bred with Ly5.1 wild-type (WT) 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, an $H2K^b$ adherent cell line, was maintained in complete RPMI 1640 medium. BrdU was purchased from Sigma-Aldrich (St. Louis, MO). Anti-CD16/CD32, and FITC-, PE-, and cychrome-conjugated mAbs against Ly5.1, CD11c, CD11b/CD18, Gr-1 (Ly6G/Ly6C), and BrdU were purchased from BD Pharmingen (San Diego, CA). CTX (Sigma-Aldrich) was reconstituted in PBS and frozen until used. G-CSF (Neupogen) was purchased from the local pharmacy at the Medical University of South Carolina (Charleston, SC).

Flow cytometry

Fresh single-cell suspensions of leukocytes from blood, spleen, and BM were prepared. Peripheral blood samples were collected by bleeding (~5 drops) each mouse from retro-orbital plexus at multiple time points after PBS or CTX treatment. We have found that bleeding mice every 3 d does not impact the total blood cell count (data not shown). About 1×10^6 cells were treated with anti-CD16/CD32 for 5 min on ice. Cells were then stained with the indicated conjugated mAb, and incubated for 30 min on ice. The cells were washed twice and resuspended in 0.3 ml 0.5% BSA, 0.02% sodium azide solution. Cells were analyzed by flow cytometry using the Cell Quest software package (Becton Dickinson, San Jose, CA).

Tumor challenge

B16 cells were thawed and expanded in vitro for two cycles, 3 d each, and collected when their confluence reached ~70%. Naive B6 mice (n = 6/group) were challenged with s.c. injection in their right shaved flank with 0.5×10^6 B16 cells. Cell viability was >98% before inoculation as assessed by trypan blue exclusion. Tumor was detectable on day 7 and palpable on day 10 and then grew quickly reaching ~200 mm^2 on day 20. Tumor growth was measured by caliper every 3 d, and mice were sacrificed when the tumor area reached 400 mm^2.

TBI, chemotherapy, and G-CSF treatment

Mice were treated with i.p. injection of PBS or 4 mg/mouse CTX (13,25), 5 mg/kg doxrubicin (DOX) (26), or 120 mg/kg gemcitabine (GEM) (27), or 5 Gy TBI (28). For treatment with G-CSF, mice were treated with s.c. of 5 μg/mouse G-CSF (29) (Neupogen) either from days 1 to 5 or days 5–9 post-CTX treatment.

DC proliferation assay

To determine DC proliferation, BrdU proliferation assay was used as we previously described (30). BrdU uses nucleotide substitution to replace thymidine with uridine in the DNA structure of dividing cells both in vitro and in vivo (31). Mice were treated with i.p. injection of PBS or 4 mg/mouse CTX. These PBS- and CTX-treated mice were injected i.p.

with 1 mg/mouse of BrdU in 300 μl the day before harvesting cells at the indicated time points. Control mice received a sham injection of 300 μl PBS. Twenty-four hours after BrdU injection, mice were sacrificed and the peripheral blood, spleen, and BM were harvested. Cells from these compartments were stained for surface markers as above, fixed, permeablized, and then stained with anti- BrdU mAb to detect its intracellular levels. Cells were analyzed by flow cytometry using the Cell Quest software package (Becton Dickinson). Analysis of BrdU expression was performed on gated $CD11c^+CD11b^+$ cells.

Adoptive transfer of Gr-1^+CD11b^+ cells and BM

For adoptive transfer of Gr-1^+CD11b^+ cells, donor Gr-1^+CD11b^+ cells were sorted from spleens of Ly5.1 mice treated 7 d before with CTX. Cells were sorted by flow cytometry after staining splenocytes with anti-CD11b, anti–Gr-1 (Ly6G), and anti-CD11c mAbs. Freshly sorted cells were washed twice and resuspended in PBS, and 3×10^6 cells were transferred via lateral tail vein injection into recipient Ly5.2 mice treated 7 d previously with PBS or CTX. Then, recipient mice were sacrificed on day 3 after cell transfer (i.e., 12 d after PBS and CTX treatment of the recipients) to analyze the number and phenotype of the donor Gr-1^+CD11b^+ cells in the peripheral blood. The donor $Ly5.1^+$Gr-1^+CD11b^+ cells were defined in the blood of the recipient mice after staining with anti-Ly5.1, anti-CD11b, and anti–Gr-1 mAbs. Their expression of the DC marker CD11c was analyzed after staining with anti-CD11c mAb. All the phenotypic analyses were performed by four-color flow cytometry.

For adoptive transfer of BM, donor unfractionated BM cells were prepared from BM of Ly5.1 mice treated 2 d before with CTX. Fresh BM cells were washed twice and resuspended in PBS, and 5×10^6 cells were transferred via lateral tail vein injection into recipient Ly5.2 mice treated 2 d before with PBS or CTX. Then, the recipient mice were bled at multiple time points after cell transfer to analyze the numbers and phenotype of the donor BM cells in the peripheral blood. Donor cells were defined as $Ly5.1^+$ cells and their phenotype was analyzed after staining with mAbs against CD11c, CD11b, and Gr-1. All the phenotypic analyses were performed by four-color flow cytometry.

Adoptive T cell transfer and peptide vaccination

Recipient WTand Flt3 ligand deficient ($Flt3L^{-/-}$) Ly5.2 mice were treated with PBS or 4 mg/mouse CTX. One day later, the mice were adoptively transferred with 1 million of naive pmel-1 Ly5.1 cells by lateral tail vein injection and then vaccinated with s.c. injection of 100 μg/mouse $gp100_{25–33}$ melanoma peptide along with or without 200 μg/mouse poly-I:C on days 2 and 12 of PBS and CTX treatments. The mice were bled and sacrificed at multiple time points to analyze the expansion and contraction of pmel-1 cells.

Real-time RT-PCR analysis on BM

Alterations in the levels of gene expression of chemokines, growth factors, and metalloproteinase in unfractionated BM cells harvested at multiple time points after PBS and CTX-treated B6 mice were determined by real-time PCR as previously described (30). The sequences of the primers used were designed in our laboratory. Total cellular RNA was isolated using a guanidium thiocynate-phenol-chloroform solution. Real-time RT-PCR was performed on a Gene Amp 5700 Sequence Detection System (PE Biosystems, Foster City, CA) as previously described (32).

Statistical analysis

Numerical data obtained from each experiment were expressed as mean ± SD and the statistical differences between experimental and control groups were assessed using the Student *t* test. The p values <0.05 were considered statistically significant.

Results

CTX induced increases in the circulating levels of DCs after the contraction of Gr-1$^+$CD11b$^+$ cells

Our recent studies showed that CTX treatment induces a rapid leukopenia (days 1–6) in the peripheral blood, spleen, and BM; this leukopenia was restored in the spleen and BM by day 12 and in the peripheral blood by day 18 (24). We were also able to show that CTX treatment induced significant increases in the numbers of DCs (24) and Gr-1$^+$CD11b$^+$ cells (13) in the peripheral blood during the recovery from leukopenia. Therefore, we asked in the current work whether post-CTX expansion of DCs also occurred in other lymphoid and nonlymphoid compartments, and whether it was associated with Gr-1$^+$CD11b$^+$ cell expansion. To this end, we analyzed the sequential changes in the percentage and absolute numbers of DCs (CD11c$^+$ CD11b$^+$Gr-1$^-$) and Gr-1$^+$CD11b$^+$ CD11c$^-$ cells in the peripheral blood leukocytes (PBLks), spleen, and BM during the 2 wk after a single i.p. injection of PBS or 4 mg/mouse CTX. DCs in the peripheral blood in mice can be categorized into conventional DCs (cDCs; CD11c^{high}CD11b^{high} B220^{low}) and plasmacytoid DCs (pDCs; CD11c^{high}CD11b^{low} B220^{high}). In other compartments such as spleen, however, cDCs are categorized into myeloid (CD8α^{low}CD11b^{high}) and lymphoid (CD8α^{high}CD11b^{low}) subtypes. PBS-treated control mice are shown as day 0. Consistent with our previous studies (24), CTX treatment induced significant increases in the percentage and absolute numbers of DCs (CD11c$^+$CD11b$^+$) in the peripheral blood from days 9 to 12 posttreatment (Fig. 1*A*, 1*B*). In the spleen, CTX treatment also induced significant increases in the percentage but not the absolute numbers of DCs, including CD11c$^+$CD11b$^+$ and CD11c$^+$CD11b$^-$, only on day 12 post-CTX treatment (Fig. 1*C*, 1*D*). In BM, the percentage, but not the absolute number, of DCs including CD11c$^+$CD11b$^+$ and CD11c$^+$CD11b$^-$, showed a slight increase only on day 3, and then returned to the pretreatment levels (Fig. 1*E*, 1*F*). Of note, CTX resulted in increases in the levels of DCs in the liver and peritoneal exudates cells, during the lymphopenic and recovery phases, respectively, with no changes in the levels of these cells in LNs (data not shown).

We then analyzed the kinetics of the changes in the percentage and absolute numbers of Gr-1$^+$CD11b$^+$ cells in the peripheral blood, spleen, and BM, after 3, 6, 9, and 12 d of CTX treatment. In PBLks, CTX treatment had no effect on the percentage of Gr-1$^+$ CD11b$^+$ but induced significant decreases in their absolute number measured on day 3. Both the percentage and absolute numbers of Gr-1$^+$CD11b$^+$; however, showed significant increases on days 6, 9, and 12, with the highest level on day 6 (Fig. 1*A*, 1*B*). In the spleen, CTX treatment induced similar effects on the percentage and absolute numbers of Gr-1$^+$CD11b$^+$; the highest increases in their percentage and absolute numbers were observed on days 6 and 9, respectively (Fig. 1*C*, 1*D*). In BM, both the percentage and absolute numbers of Gr-1$^+$CD11b$^+$ were significantly decreased on day 3, followed by significant increases on days 6, 9, and 12 post-CTX treatment (Fig. 1*E*, 1*F*). In the liver, LNs, and peritoneal exudates cells, the numbers of Gr-1$^+$CD11b$^+$ cells were also remarkably increased on day 6 and then gradually declined, reaching the normal levels by day 18 after CTX treatment (data not shown).

The post-CTX expansion of DCs depended on the degree of the induced lymphodepletion

We then asked whether post-CTX expansion of DCs depended on the magnitude of the induced lymphodepletion by CTX. We focused our subsequent studies on the $CD11c^+CD11b^+$ DCs because they constituted the majority of the expanded DCs in the peripheral blood. We found that the CTX-induced leukopenia was dose-dependent as evidenced by the total count of the PBLks 2 d after treating mice with escalating (1, 2, 3, or 4 mg/mouse) doses of CTX (Fig. 2*A*). Analysis of the percentage of $CD11c^+CD11b^+$ DCs in the peripheral blood 12 d post-CTX treatment showed that the fold increase in the number of DCs was maximal in mice treated with 4 mg CTX (Fig. 2*B*), indicating that post-CTX DC expansion was dose dependent. We, then, tested whether other lymphodepletion regimens can also lead to a similar surge in the circulating DCs. Treatment with TBI, DOX, or GEM are known to induce transient leukopenia. Thus, we determined the levels of DCs 12 d after a single i.p. treatment of mice with PBS, 4 mg/mouse CTX (13,25) 5 mg/kg DOX (26), or 120 mg/kg GEM (27), or 5 Gy TBI (28). The doses of DOX, GEM, and TBI are based on their antitumor and immunomodulatory effects. TBI induced a significant increase in the numbers of DCs, with comparable levels to those induced by CTX treatment. In contrast, DOX and GEM induced a lesser expansion of DCs, with more numbers in GEM-treated mice than in DOX-treated mice (Fig. 2*C*). As we found with CTX, the levels of DC expansion in response to TBI, GEM, or DOX were correlated with the degree of leukopenia induced by each lymphodepletion agent (data not shown). Furthermore, SCID/beige mice, which suffer from a permanent state of leukopenia, did not show any significant increases in the numbers of DCs in their peripheral blood (Fig. 2*D*). Taken together, these data would indicate that the magnitude of recovery of the numbers of DCs correlate with the degree of the induced lymphodepletion.

Post-CTX expansion of DCs was not affected by adjuvant chemotherapy or tumor burden

Because CTX therapy is usually coadministered with other adjuvant chemotherapy, we evaluated whether cotreatment with chemotherapeutic drugs, such as DOX or GEM, altered the expansion of DCs by CTX. Combining single administration of DOX or GEM with CTX treatment did not alter their kinetics (data not shown) or the peak of expansion of DCs as compared with treatment with CTX alone (Fig. 2*C*). We then tested the impact of the tumor burden on DC expansion. To this end, mice were challenged with s.c. injection of 2×10^5 B16 melanoma cells. Ten days later, the time point at which the tumors were palpable, the mice were treated with PBS or CTX, and then the tumor growth and DC frequency were monitored. Consistent with our previous studies, the growth of B16 tumor was transiently regressed in CTX-treated mice, and then grew rapidly afterward (Fig. 2*E*). The tumor progression did not affect the kinetics (data not shown) or the peak of DC expansion in CTX-treated mice (Fig. 2*F*). Taken together, these data would suggest that CTX-induced DC expansion is stable even under the effects of adjuvant chemotherapy or tumor growth.

Administration of G-CSF after CTX treatment augmented DC expansion

In the clinical setting, CTX therapy is often followed by treatment with growth factors, in particular G-CSF, to correct the induced leukopenia by mobilizing HSCs (2–4). To determine whether administration of 5 μg/mouse G-CSF after CTX treatment altered the expansion of DCs, mice (n = 4/group) were i.p. injected with PBS or CTX, followed by s.c. treatment with PBS or 5 μg/mouse G-CSF for two different treatment cycles: from days 1 to 5 cycle or days 5 to 9 cycle after PBS or CTX treatment. Mice were bled on days 7 (i.e., 2 d after 1–5 cycle and in the middle of 5–9 cycle), day 9 (i.e., 4 d after 1–5 cycle and on day 9 of 5–9 cycle), and day 12 (i.e., 7 d after 1–5 cycle and 3 d after 5–9 cycle). Although treatment of the control (PBS-treated) mice with G-CSF did not induce a significant increase in the percentage of DCs (Fig. 3*A*), it significantly increased their absolute numbers when it was analyzed 1 d, but not 2 d, after G-CSF treatment (Fig. 3*B*). As expected, CTX-treated

mice showed higher percentage (Fig. 3*A*) and absolute (Fig. 3*B*) numbers of DCs in their blood on days 9 and 12. Administration of G-CSF after CTX treatment induced transient increases in the absolute number (Fig. 3*A*), but not the percentage (Fig. 3*A*), of DCs. This effect appeared at 24 h of the second or the last injection of G-CSF of the 5-d treatment cycle (Fig. 3*B*). The effect of G-CSF on the number of DCs, however, diminished after 48 h of its last injection. This could explain why the increase in the numbers of DCs is shown only when it was analyzed on day 7 or 9 in the groups treated with G-CSF from days 5 to 9 but it was absent on the same days in the mice treated with G-CSF from days 1 to 5. This is further confirmed by the absence of any effects of G-CSF on DC number when it was analyzed on day 12 (i.e., 7 d after "days 1–5" cycle and 3 d after "days 5–9" cycle. Similar effects of the G-CSF treatment on DCs were also observed on the percentage and absolute numbers of Gr-1$^+$CD11b$^+$ cells (data not shown). Taken together, these results suggest that CTX-induced DC expansion could be transiently increased by the administration of G-CSF.

Post-CTX expansion of DCs was independent of Gr-1$^+$CD11b$^+$ cell expansion

The early kinetics of the expansion and contraction of Gr-1$^+$ CD11b$^+$ cells prior to the expansion of DCs in CTX-treated mice may indicate to their contribution to the expansion of DCs. To address this possibility, we tested the impact of systemic depletion of Gr-1$^+$CD11b$^+$ cells on post-CTX expansion of DCs using GEM. Consistent with previous studies (27), single treatment with GEM on day 5 or 8 post-CTX treatment resulted in a complete depletion of Gr-1$^+$CD11b$^+$ cells, but only for 3 d and then cells rapidly recovered to their levels before GEM injection (Fig. 4*A*). We injected GEM 5 or 8 d after PBS or CTX treatment (the peak of Gr-1$^+$CD11b$^+$ cell expansion) and then analyzed the numbers of CD11c$^+$CD11b$^+$ and Gr-1$^+$CD11b$^+$ cells at day 12 post-CTX treatment. Treatment with GEM had no significant effect on the percentage of Gr-1$^+$CD11b$^+$ (Fig. 4*B*, *upper panel*) or CD11c$^+$ CD11b$^+$ cells (Fig. 4*B*, *lower panel*). To further examine the potential contribution of Gr-1$^+$CD11b$^+$ cells to post-CTX expansion of DCs, we adoptively transferred Gr-1$^+$CD11b$^+$ cells sorted from spleens of 7-d CTX-treated mice into either naive or 7-d CTX-treated recipient mice. As shown in Fig. 4*C*, transferred Gr-1$^+$CD11b$^+$ cells appeared in the peripheral blood of the recipient mice. Equal low numbers (~7%) of Gr-1$^+$CD11b$^+$ cells in the peripheral blood of naive and CTX-treated recipients acquired CD11c expression, suggesting that Gr-1$^+$CD11b$^+$ cells are not a direct or precursor source for post-CTX expansion of DCs. Adoptive transfer of Gr-1$^+$ cells had no effect on the increased number of endogenous DCs in CTX-treated mice (data not shown).

CTX induced DCs to proliferate in BM with subsequent proliferation and expansion in the peripheral blood and spleen

To understand whether the expansion of DCs post-CTX therapy was due to proliferation of DC progenitors before and/or after their mobilization from BM to periphery, we analyzed the proliferation of DCs in the blood, spleen, and BM at multiple time points post-CTX therapy by BrdU incorporation assay. As shown in Fig. 5*A*–*F*, both the percentage and the absolute numbers of BrdU$^+$ DCs (CD11c$^+$CD11b$^+$) was higher in BM 3 d post-CTX treatment as compared with their numbers in the control (PBS-treated) mice. Although the percentage of BrdU$^+$ DCs per cell basis in the peripheral blood and spleen at this time point was higher than in the BM, there was no significant difference in the numbers of cycling DCs between CTX- and PBS-treated mice. By day 6, however, the percentage and absolute numbers of BrdU$^+$ DCs abruptly increased in the peripheral blood and spleens, but not in BM, and then slowly decreased (Fig. 5*A*–*F*). These results indicate that DCs may start to proliferate in the periphery 3–4 d before their peak of expansion or that DC expansion might be due to the proliferation of DC progenitors in BM as well as in the periphery after their mobilization.

To directly address this hypothesis, we transferred unfractionated BM cells harvested from BM after 3 d of CTX treatment (i.e., a BM harboring higher numbers of $BrdU^+$ DCs) into either naive recipient (i.e., devoid of post-CTX microenvironment) or into mice treated 2 d before with CTX (i.e., with the same post-CTX microenvironment). Then, we counted the frequency of DCs in the peripheral blood at multiple time points in the recipient mice. Our data indicate that equal numbers of BM cells acquired DC phenotype ($CD11c^+$) in the peripheral blood of PBS- and CTX-treated recipients (Fig. 6*A*). Similar results were obtained when BM harvested from control (PBS-treated) mice were transferred into 3-d CTX-treated mice (data not shown). These data would indicate that DCs expanded after CTX treatment may derive from the mobilized DC progenitors in BM.

Post-CTX expansion of DCs is independent of spleen or the integrin CD11b

As shown in Fig. 1, CTX induced systemic increases in the numbers of $Gr\text{-}1^+CD11b^+$ cells, which occurred prior to the expansion of DCs. Furthermore, the contraction in the numbers of $Gr\text{-}1^+CD11b^+$ cells in the peripheral blood also occurred prior to DC expansion, indicating a possible contribution of these cells to the increased frequency of DCs. Because the spleen harbored the largest pool (>60%) of $Gr\text{-}1^+CD11b^+$ cells and showed splenomegaly (Fig. 6*B*), we wanted to evaluate whether removal of this lymphoid compartment impacted post-CTX expansion of DCs. To this end, the levels of DCs were determined in the peripheral blood of splenectomized mice treated with CTX as compared with PBS- and CTX-treated WT mice. Removal of spleen did not significantly alter the kinetics or the peak of circulating DC expansion induced by CTX treatment (Fig. 6*B*); indicating that spleen is not a potential niche for the differentiation or expansion of post-CTX expanded DCs.

Certain integrins in particular VLA-4 has been found to be involved in the HSC mobilization (4). Similarly, recent studies suggested a role for the integrin CD11b (33). Because post-CTX expanded DC and $Gr\text{-}1^+CD11b^+$ cells showed high levels of CD11b expression, we tested the role of this integrin using $CR3^{-/-}$ mice that lack CD11b. Thus, WT and $CR3^{-/-}$ mice were i.p. treated with PBS or 4 mg/mouse CTX and the percentage of DCs in the peripheral blood was measured on days 2, 6, 10, and 14. Interestingly, CTX treatment of WT and $CR3^{-/-}$ mice induced similar patterns of DC expansion, indicating that expression of CD11b by DCs might not be required for the expansion of DCs after CTX treatment (Fig. 6*C*).

Post-CTX expansion of DCs depends on the induction of CCR2/CCL2 and Flt3/Flt3L signaling pathways

Because BM cells from CTX-treated mice gave rise to DCs (Fig. 6*A*), we analyzed the kinetic expression of different arrays of chemokines and growth factors in BM (data not shown). Among these factors, CTX treatment significantly increased the expression of the chemokines CCL2, CCL3, CCL4, and CCL12 (Fig. 7*A*) and the growth factors M-CSF, GM-CSF, and Flt3L (Fig. 7*B*). A summary of the actual gene expression as well as the fold increases in gene expression of these chemokines and growth factors in BM harvested after 3, 6, 9, 12, and 14 d of CTX treatment is shown in (Fig. 7*C*) relative to those in BM harvested from control mice (PBS-treated; day 0). Taken together, these data would indicate to the importance of these factors in the observed CTX-induced DC expansion. To directly test this possibility, we followed the kinetics of DC expansion in mice lacking CCR1 (binds to CCL3), CCR2 (binds to CCL2 and CCL12), CCR5 (binds to CCL3 and CCL4), CCL3, or Flt3L. Interestingly, DC expansion was significantly abrogated in mice deficient in CCR2, CCL3, and Flt3L (Fig. 8*A*).

Because the deficiency in the Flt3L resulted in an almost an absolute abrogation of the DC expansion, we tested the impact of Flt3L deficiency on postvaccination $CD8^+$ T cell responses. Using pmel-1 adoptive transfer mouse model, we have recently established that post-CTX expanded DCs are required for the prime-boost vaccination with gp100/poly-I:C regimen during the lymphopenic and restoration (i.e., the peak of DC expansion) phases post-CTX treatment to induce robust Ag-specific $CD8^+$ T cells and eradication of established melanoma (24). Therefore, we used this pmel-1 adoptive transfer model and prime-boost vaccination regimen on CTX preconditioned WT and $Flt3L^{-/-}$ recipients. Thus, WT and $Flt3L^{-/-}$ mice were treated with PBS or CTX and adoptively transferred 1 d later with 1×10^6 naive pmel-1 cells and then primed and boosted with $gp100_{25–33}$ ± poly-I:C on days 2 and 12, respectively, posttreatments. The mice were sacrificed 3 d after boosting to analyze the number of pmel-1 cells. Consistent with our recent studies (24), prime-boost vaccination of CTX-treated WT recipient mice significantly augmented the expansion of pmel-1 cells. However, the same regimen of prime-boost vaccination induced a marginal expansion of pmel-1 cells in CTX-treated $Flt3L^{-/-}$ recipient mice, showing ~6-fold decrease in the number of pmel-1 cells (Fig. 8*B*). In contrast, only ~1.5-fold decrease in the pmel-1 cell expansion was observed in PBS-treated WT recipient mice (Fig. 8*B*). Of note, administration of poly-I:C alone into CTX-preconditioned recipients with no vaccination had marginal but not significant increases in the numbers of the transferred pmel-1 cells. Taken together, these data indicate that Flt3L is a critical growth factor required for the expansion of functional DCs expanded after CTX treatment.

Discussion

We recently have reported that a single CTX treatment can induce substantial expansion of DCs in the peripheral blood from days 9 to 12 during the recovery from leukopenia (24). The current study further showed that this post-CTX expansion of DCs occurs in the spleen in addition to the peripheral blood. CTX treatment induced expansion of both $CD11c^+CD11b^+$ and $CD11c^+CD11b^-$ DCs in the blood and spleen. The $CD11c^+CD11b^+$ DCs, however, constituted the majority of the expanded DCs. Interestingly, DC precursors proliferated in BM at early time points during the lymphopenic phase and in the blood and spleen a few days before the peak of their expansion. This post-CTX expansion of DCs was associated with a surge in the BM expression of the growth factors Flt3L, GM-CSF, and M-CSF as well as several chemokines, in particular CCL2 and CCL3. Both CCR2 and Flt3 signaling pathways were found to be critical for DCs to show a competent expansion. Taken together, our data show that CTX treatment induces an expansion of the DCs found in the PBLks through induction of mobilizing growth factors in the BM. Targeting DCs at certain time points during their expansion and proliferation could augment their roles in enhancing adoptive immunotherapy.

CTX is often administered together clinically with other chemotherapeutic agents to enhance its anticancer effects or with growth factors such as G-CSF to enhance HSC mobilization and correction of leukopenia (34–37). Interestingly, in our studies, coadministration of DOX or GEM with CTX had no effect on DC expansion. The presence of a tumor burden of B16 melanoma also had no effect. Administration of G-CSF for 5 consecutive days after CTX treatment, however, induced transient increases in the numbers of expanded DCs (Fig. 3). In line with our results, previous studies also showed increases in the numbers of DCs during the restoration phase in the peripheral blood of cancer patients receiving combinatorial treatment with CTX and G-CSF (38–40). Although it was not clear in these studies whether the increase in the frequency of DCs was solely due to the effects induced by CTX or the growth factors, our results demonstrate that CTX per se is capable of inducing DC expansion in the peripheral blood in a murine model. Because G-CSF is used commonly in clinic to correct CTX-induced leukopenia (2–4), our data indicate that in vivo Ag targeting

of DCs expanded after CTX treatment can be performed even during the correction of leukopenia by G-CSF–based therapy. Although it is not clear in the current study why G-CSF treatment augmented post-CTX expansion of DCs, it could be suggested that G-CSF acts on the residual progenitors in BM after CTX treatment because immature progenitors have been previously shown to reappear in murine BM after 24 h of CTX treatment (29). This notion is consistent with the results of recent clinical studies showing increases in the DC mobilization into the peripheral blood of cancer patients after treatment with CTX plus G-CSF (41).

Consistent with several previous studies (25,42,43), (13), we found significant systemic expansion of Gr-1$^+$CD11b$^+$ cells prior to the surge in DCs. Our data further showed that a contraction of Gr-1$^+$CD11b$^+$ cells in the blood occurs prior to the peak of DC expansion. Moreover, Gr-1$^+$CD11b$^+$ cells from tumor-bearing mice have been found by other investigators to acquire the phenotype characteristic to DCs (CD11c^{high}) on their adoptive transfer into naive, but not tumor-bearing, recipient mice (44,45). Coupling these observations together, it could be suggested that some of Gr-1$^+$CD11b$^+$ cells may directly contribute to DC expansion after their differentiation into DCs. Our data, however, exclude this suggestion because: 1) depletion of Gr-1$^+$CD11b$^+$ cells in CTX-treated mice by GEM at their peak of expansion (from days 5 to 8) did not alter the magnitude of DC expansion; 2) removal of the spleen, which harbored high numbers of Gr-1$^+$ CD11b$^+$ cells even during the peak of DC expansion, from CTX-treated mice did not alter DC expansion; and 3) only 5–7% of Gr-1$^+$CD11b$^+$ cells sorted from CTX-treated mice acquired the expression of the typical phenotype of DCs (CD11c) in the peripheral blood when adoptively transferred into PBS- or CTX-treated recipient mice. Several observations from our studies suggest BM as a potential cellular source for post-CTX expansion of DCs by releasing DC progenitors into the circulation. First, the peak of DCs expansion occurred on day 12. The peak of HSC mobilization by CTX, however, has been established to occur by day 6–8 (2). This prior HSC mobilization to DC expansion would indicate that CTX-expanded DCs might be driven from the BM progenitors. Second, the number and proliferation of DCs increased in BM, but not in the blood or spleen, of CTX-treated mice during the lymphopenic phase (day 3) and promptly declined afterward. Third, proliferation of DCs occurred in the blood and spleen on day 6 (i.e., ~3 d prior to DC expansion in the peripheral blood). Fourth, adoptive transfer of BM cells, harvested 3 d after CTX treatment, resulted in equal numbers of DCs in the peripheral blood regardless of whether the recipients were pretreated with PBS or CTX. This would indicate that post-CTX expansion of DCs might be due to an enhanced intrinsic capacity of BM cells acquired in a lymphopenic microenvironment. In addition, CTX-mobilized HSCs have been found to be cyclic in BM just before their migration to the periphery (46). In the same vein, a recent study showed that the beneficial effects of CTX to the antitumor effects of T cells associate with DC turnover in the spleen, liver, and tumor site. These newly recruited DCs were suggested to be originated from proliferating early DC progenitors and secreted more IL-12 and less IL-10 compared with those from untreated tumor-bearing animals and were fully capable of priming T cell responses and ineffective in inducing expansion of T regulatory cells (47). Taken together, it could be suggested that post-CTX expansion of DCs is due to the migration of the proliferating DC progenitors from BM to the circulation, where they undergo local proliferation and differentiation into DCs.

One of the molecular mechanisms of post-CTX expansion of DCs could be the disruption of the adhesion between HSCs and the matrix components in BM (2,3). One of the key adhesion pairings is the interaction between VLA-4 integrin expressed in the majority of HSCs with VCAM-1 expressed in stromal BM cells. Disruption of VCAM-1/VLA-4 interactions by Abs directed against VCAM-1 or VLA-4 dampened HSC mobilization (48,49). Disruption of this adhesion is mediated by the release of metalloproteinase enzymes (50). Although we have not analyzed the levels of VLA-4 expression on BM cells, we found

a significant upregulation of metalloproteinase 9 enzyme in the BM at early time points post-CTX treatment (data not shown). CR3 is another integrin that has recently suggested to be implicated in various adhesive interactions and trafficking of DCs and macrophages (51). A deficiency in this integrin has been found to dampen HSC mobilization in response to G-CSF (33). Our data, however, showed that CD11b deficiency had no effect on DC expansion, suggesting that CD11b expression in HSCs or DC progenitors is not among the integrins that are required for CTX-induced DC expansion. We cannot exclude, however, the possibility that CD11b play distinct roles in HSC mobilization and DC expansion. Further studies are required to dissociate between these two possibilities.

Another molecular mechanism that might mediate post-CTX expansion of DCs is the modulation of hematopoietic growth factors, in particular G-CSF, GM-CSF, and Flt3L or chemokines, in particular CCL3, CXCL8, and CXCL12, because most of these factors have shown potent mobilizing effects (3,52). Recent murine studies also showed elevated levels of certain chemokines (e.g., CXCL12) (53) and growth factors (e.g., GM-CSF, IL-1β, IL-7) (5) in BM after CTX treatment. Indeed, we found a dynamic upregulation in the gene expression of a wide array of chemokines as well as of M-CSF, GM-CSF, and Flt3L. The kinetics of the induction of these factors appeared to be coordinated with the proliferation of DCs in BM during the lymphopenic phase (e.g., GM-CSF, M-CSF, Flt3L, and CCL2) and in the BM, blood, and spleen during the recovery phase (e.g., CCL2, CCL3, and M-CSF). Of note, certain factors such as CCL2 and M-CSF were induced at both the lymphopenic and the recovery phases, and the peak of induction of certain factors such as GM-CSF and CCL2 occurred at the peak of both DC proliferation in BM and expansion in the periphery. Taken together, these data indicate to a role for certain chemokines (e.g., CCL2 and CCL3) and growth factors (e.g., M-CSF and Flt3L) in mediation of post-CTX DC expansion, by acting at certain time points in a complementary fashion post-CTX therapy to favor the expansion of DCs. Abrogation of post-CTX expansion of DCs in mice deficient in CCR2 (which binds to CCL2 and CCL12) and in CCL3 confirmed the roles of these chemokines. These data provide a baseline for the use of chemokines such as CCL2, CCL12, and CCL3 for in vivo HSC mobilization and DC expansion, in particular in combination with CTX.

In addition to the role of the chemokines discussed previously, deficiency in Flt3L markedly abrogated the expansion of DCs, indicating to the crucial role of this particular growth factor in mediation of DC expansion. This role of Flt3L was confirmed by the abrogation of $CD8^+$ T cell responses to prime-boost vaccination with peptide plus poly-I:C in $Flt3L^{-/-}$-recipient mice. Furthermore, this role of Flt3L would explain why most of the CTX-expanded DCs are with DC with the phenotype of cDCs $CD11c^+CD11b^+$, because Flt3L preferentially expands more myeloid DCs (cDCs) than pDCs (54,55). We do not exclude contribution of other growth factors, in particular G-CSF that has been reported to favors the mobilization of DCs toward pDC rather than cDC phenotype (2–4). Although it is not clear how Flt3L mediated post-CTX DC expansion, it has been shown that stimulation of Flt3 receptor through its cognate Flt3L expands early HSCs and DCs in mice (56,57), primates (58), and humans (59–63). Given that common lymphoid and myeloid progenitors, as well as steady state DCs, in thymus, spleen, and epidermis, express Flt3 (64) and that only $Flt3^+$ progenitor cells and their DC progeny are expanded on in vivo Flt3L administration (65), it is possible that Flt3L drives differentiation of DCs from both the lymphoid and myeloid developmental pathways from $Flt3^+$ progenitors to $Flt3^+$ DCs. Coupling our data showing the role of Flt3L in mediation of post-CTX expansion of DCs with the potent mobilizing effects of Flt3L (57,66), we suggest a superior expansion of DCs after combination of Flt3L with CTX. Of note, CTX induced higher and sustained expression levels of GM-CSF and M-CSF in BM as compared with Flt3L. Therefore, the roles of these growth factors in mediation of post-CTX expansion of DCs as well as their combinatorial treatment with CTX warrant investigation.

Our findings of the significant systemic expansion of DCs after a single dose of CTX present post-CTX environment represent a significant source of DCs in vivo even in the absence of exogenous administration of growth factors. This represents an opportunity for therapeutic manipulation aimed at increasing the efficacy of immune-based therapies.

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Abbreviations used in this paper

BM	bone marrow
cDC	conventional DC
CTX	cyclophosphamide
DC	dendritic cell
DOX	doxrubicin
Flt3L	Flt3 ligand
G-CSF	granulocyte-colony stimulating factor
GEM	gemcitabine
HSC	hematopoietic stem cells
LN	lymph node
PBLk	peripheral blood leukocytes
pDC	plasmacytoid DC
poly-I:C	polyinosinic-polycyctidylic acid
TBI	total body irradiation
WT	wild type

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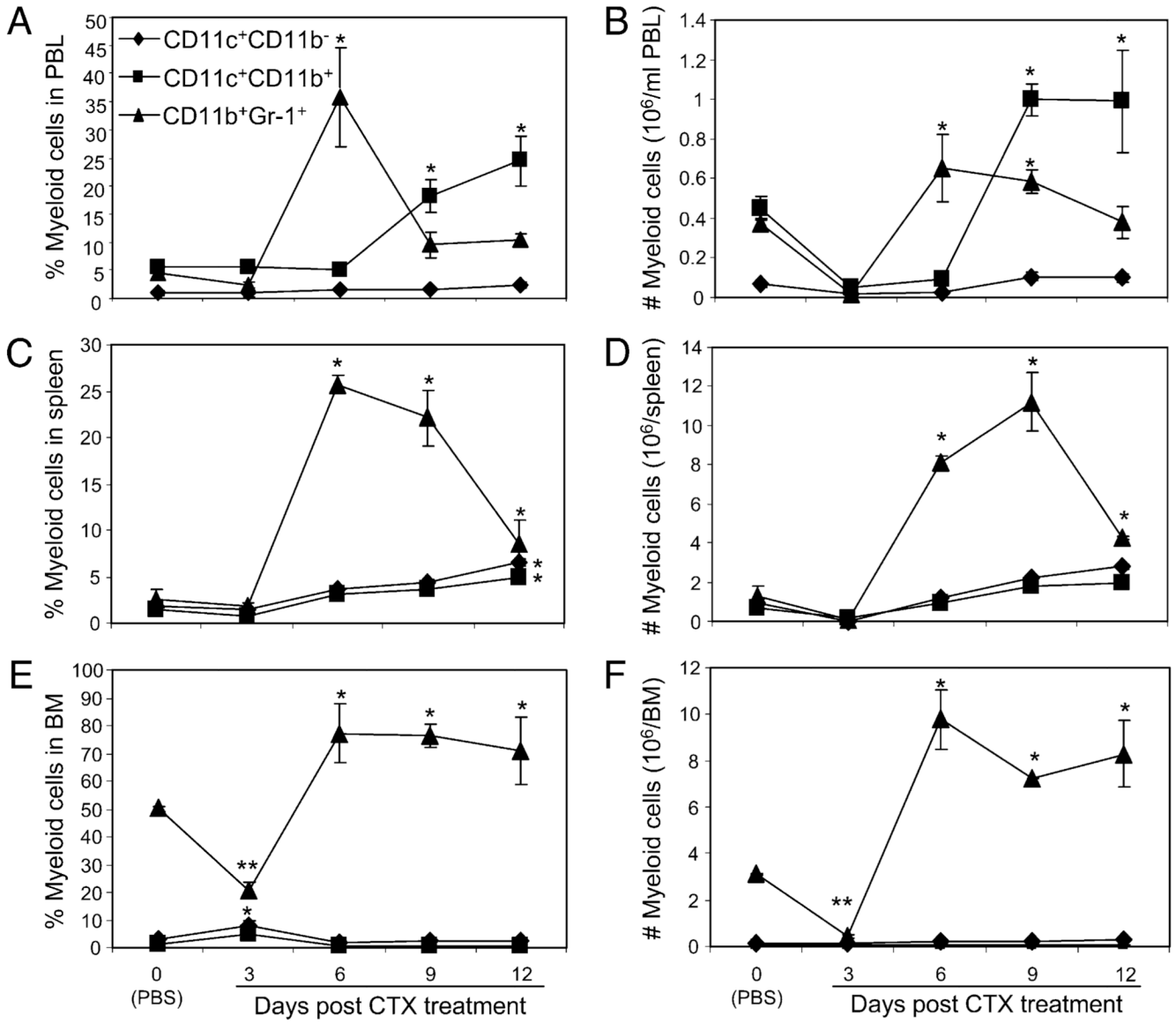


FIGURE 1.

CTX induces systemic expansion of DCs after contraction of Gr-1$^+$CD11b$^+$ cells in peripheral blood. Female C57BL/6 mice (n = 4/group) were i.p. injected with PBS or CTX (4 mg/mouse). Mice were bled to harvest blood and then sacrificed to harvest spleen and BM at the indicated time points. Leukocytes were counted, stained with mAbs against CD11c, CD11b, and Gr-1, and then analyzed by flow cytometry. The percentage and absolute numbers of DCs or Gr-1$^+$ CD11b$^+$ cells in PBLks (*A*, *B*), spleen (*C*, *D*), and BM (*E*, *F*) are shown. The absolute numbers of DCs and Gr-1$^+$CD11b$^+$ cells were calculated as follows: (total cell count × % cells)/100. *A significant increase ($p < 0.05$) as compared with day 0. **A significant decrease ($p < 0.05$) as compared with day 0.

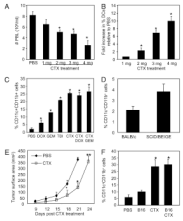


FIGURE 2.

DC expansion by CTX is dose dependent and is not altered by the adjuvant chemotherapy or tumor burden. *A*, Female C57BL/6 mice (*n* = 4/group) were i.p. injected with PBS or CTX (1, 2, 3, or 4 mg/mouse) and the number of PBLks was determined 2 d after treatment. *B*, Shows the fold increase in the number of DCs (relative to PBS) in PBLks on day 12 posttreatment. *C*, Shows the numbers of DCs in PBLks 12 d after single i.p. treatment of female C57BL/6 mice (*n*= 4/group) with PBS, 200 mg/kg CTX, 5 mg/kg DOX, 120 mg/kg GEM, CTX and DOX, CTX and GEM, or 5 Gy TBI. *D*, Shows the numbers of DCs in PBLks of BALB/c WT mice and in SCID mice on BALB/c background. *E*, Mice bearing B16 tumor were treated with PBS or 4 mg/mouse CTX on day 10 posttumor challenge. Tumor growth was then monitored at the indicated time points. *F*, Mice in *E* were bled 12 d posttreatments to analyze the percentage of DCs. *A significant increase ($p < 0.05$) as compared with control (PBS-treatment). **A significant decrease ($p < 0.05$) as compared with control (PBS-treatment).

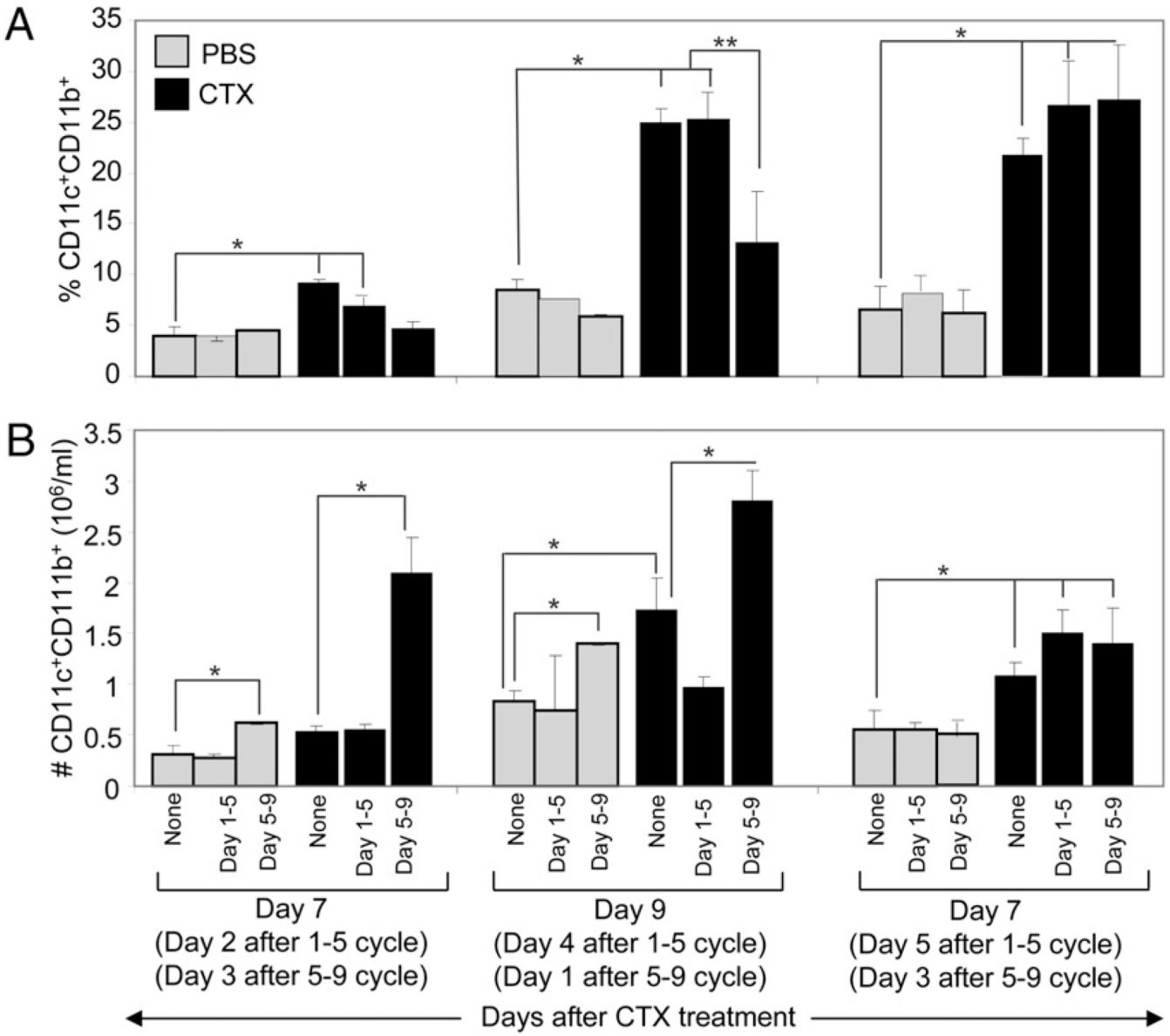


FIGURE 3.

Administration of G-CSF during mobilization of DCs post-CTX treatment induced increases in DC expansion. Female C57BL/6 mice (*n*= 4/group) were i.p. injected with PBS or 4 mg/mouse CTX. Mice were left without further treatment (groups: PBS and CTX) or treated with s.c. injection of 5 μg/mouse G-CSF daily for 5 d from days 1 to 5 (groups: PBS/G-CSF/d1–5 and CTX/G-CSF/d1–5), from days 2 to 5 (groups: PBS/G-CSF/d2–5 and CTX/G-CSF/d2–5) or from days 5 to 9 (groups: PBS/G-CSF/d5–9 and CTX/G-CSF/d5–9) of PBS and CTX treatments. Mice were bled after 7, 9, and 12 d of PBS and CTX treatments to determine the percentage (*A*) and absolute (*B*) numbers of DCs in the peripheral blood. The absolute numbers of DCs were calculated by multiplying the percentage numbers of $CD11c^+CD11b^+$ cells by the total number of the PBMCs. *A significant increase ($p < 0.05$) as compared with day 0. **A significant decrease ($p < 0.05$) as compared with day 0.

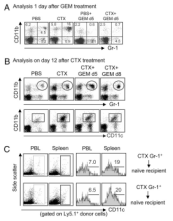


FIGURE 4.

Gr-1$^+$CD11b$^+$ cells do not significantly contribute to CTX-induced DC expansion. *A*, Shows establishment of Gr-1$^+$CD11b$^+$ cell depletion after treatment with GEM. Female C57BL/6 mice (n = 4/group) were treated with PBS or 4 mg/mouse CTX and injected with or without 120 mg/kg GEM on day 5 post-CTX treatment. One day after GEM, mice were bled and the frequencies of Gr-1$^+$CD11b$^+$ cells and DCs were assessed by flow cytometry. *B*, Shows normal expansion of DC after GEM-induced depletion of Gr-1$^+$CD11b$^+$ cells in CTX-treated mice. CTX-treated mice were injected with or without GEM 5 or 8 d post-CTX treatment and bled on day 12 after PBS or CTX treatment to determine the percentage of Gr-1$^+$CD11b$^+$ cells and DCs. *C*, Gr-1$^+$CD11b$^+$ cells sorted from spleens of Ly5.1 mice treated 7 d before with CTX were adoptively transferred into recipient Ly5.2 mice treated 7 d before with PBS or CTX. Four days later, recipient mice were sacrificed and the percentages of CD11c-expressing donor Gr-1$^+$CD11b$^+$ cells were determined in PBLks, spleen, LNs, and liver.

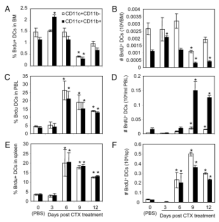


FIGURE 5.

DCs proliferate initially in BM and later in peripheral pool. B6 mice (n = 4/group) were treated with PBS or 4 mg/mouse CTX, bled to harvest blood, and then sacrificed 3, 6, 9, and 12 d posttreatments. Mice were i.p. injected with 1 mg/mouse BrdU 1 d before each time point. The percentage and absolute numbers of $BrdU^+$ $CD11c^+$ $CD11b^-$ and $CD11c^+CD11b^+$ DCs were determined in BM (*A*, *B*), PBLks (*C*, *D*), and spleen (*E*, *F*). The absolute numbers were calculated as (total DC count × % BrdU + DCs)/100. A significant increase ($p < 0.05$) as compared with day 0.

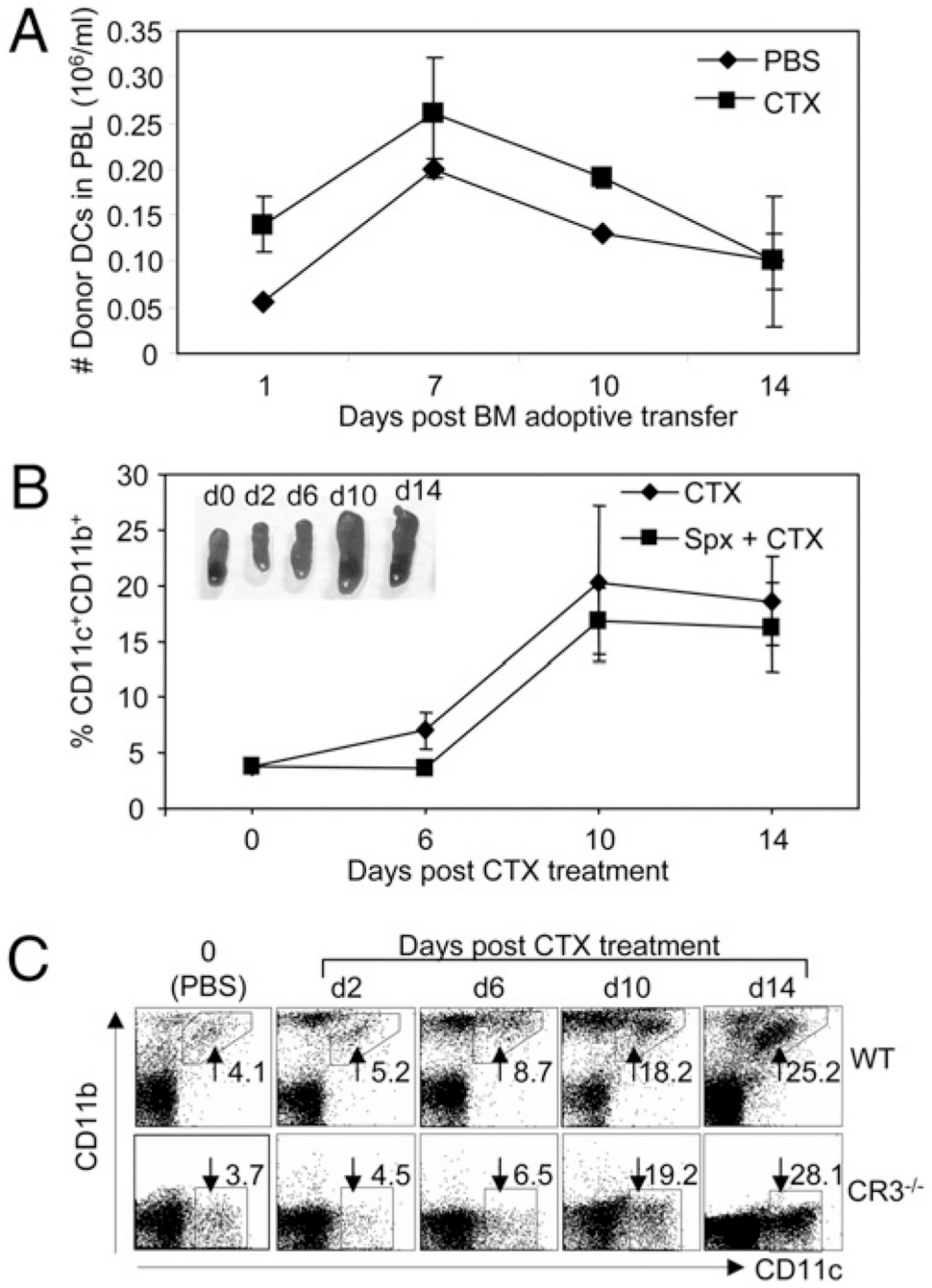
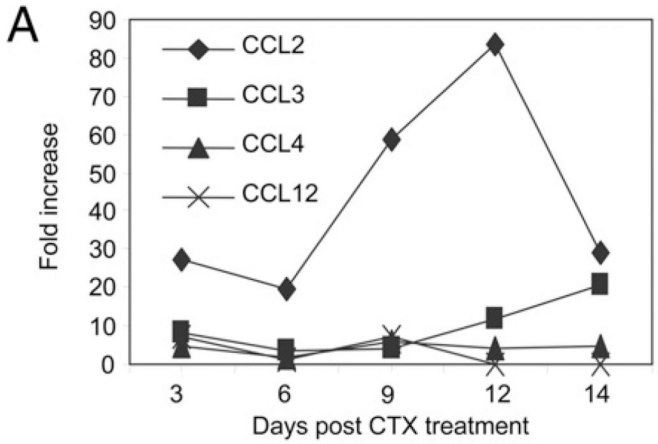
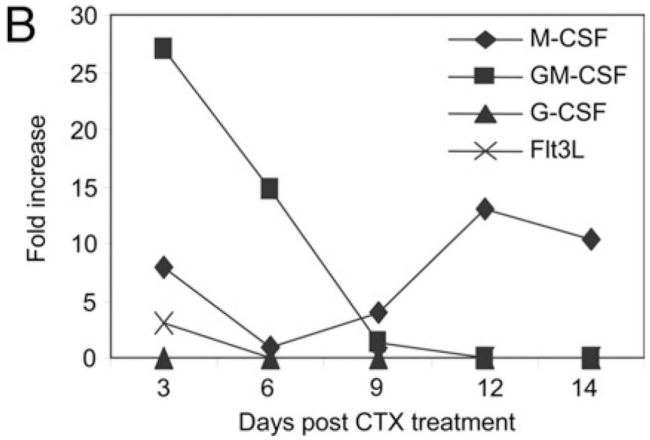


FIGURE 6.

The ability of CTX-derived BM cells to expand into DCs is due to intrinsic mechanisms. *A*, BM cells (Ly5.1) harvested after 3 d of CTX treatment were transferred into Ly5.2 recipient mice treated 3 d before with PBS or CTX. Then, the numbers of donor DCs in the peripheral blood were counted at multiple time points in the recipient mice. The absolute numbers were calculated as (total donor BM cell count × % donor DCs)/100. *B* and *C*, CTX-induced DC expansion is independent of the myeloid integrin CD11b and spleen. WT, splenectomized (*A*), or $CR3^{-/-}$ ($CD11b^{-/-}$) (*B*) mice (n = 4/group) were i.p. treated with PBS or 4 mg/mouse CTX. Mice were then bled at the indicated time points, and the percentages of $CD11c^{+}$ $CD11b^{+}$ DCs in PBLks were determined by flow cytometry.





C

A summary of the gene expression and the fold increase (shown in parentheses) in the gene expression in the BM of CTX-treated mice relative to its expression in the BM of PBS-treated mice.

Days	CCL2	CCL3	CCL4	CCL12	M-CSF	GM-CSF	G-CSF	Flt3L
Day 0	2.33	4.41	8.64	1.86	3.12	2.14	1.08	5.18
Day 3	62.80 (26.95)	35.50 (8.05)	42.40 (4.91)	13.80 (7.42)	24.50 (7.85)	56.70 (26.49)	0.0	15.70 (3.03)
Day 6	45.40 (19.48)	15.80 (3.58)	17.10 (1.98)	2.58 (1.39)	3.19 (1.02)	30.96 (14.47)	0.0	4.30 (-0.83)
Day 9	137.00 (58.80)	18.10 (4.10)	52.00 (6.02)	12.80 (6.88)	12.10 (3.88)	3.05 (1.42)	0.0	3.82 (-0.74)
Day 12	194.00 (83.26)	52.60 (11.93)	35.20 (4.07)	0.00	40.60 (13.01)	0	0.0	1.10 (0.21)
Day 14	67.40 (28.93)	92.30 (20.93)	39.00 (4.51)	0.00	32.40 (10.38)	0	0.0	4.40 (-0.85)

FIGURE 7.

DC expansion post-CTX treatment associates with induction of chemokines and growth factors in BM. C57BL/6 mice (n = 4/group) were treated with CTX and sacrificed at the indicated time points. BM cells were then harvested and the gene expression of chemokines (*A*) and growth factors (*B*) was analyzed by real-time PCR. Data are expressed as the x-fold increase in the gene expression of in the BM of CTX-treated mice relative to its expression in the BM of PBS-treated mice. *C*, A summary of the gene expression and fold increases (shown in parentheses) in the gene expression of the chemokines (CCL2, CCL3, CCL4, and CCL12) and growth factors (M-CSF, G-CSF, GM-CSF, and Flt3L) in fresh BM harvested after 3, 6, 9,12, and 14 of CTX treatment relative to their expression in the BM of PBS-treated mice (day 0).

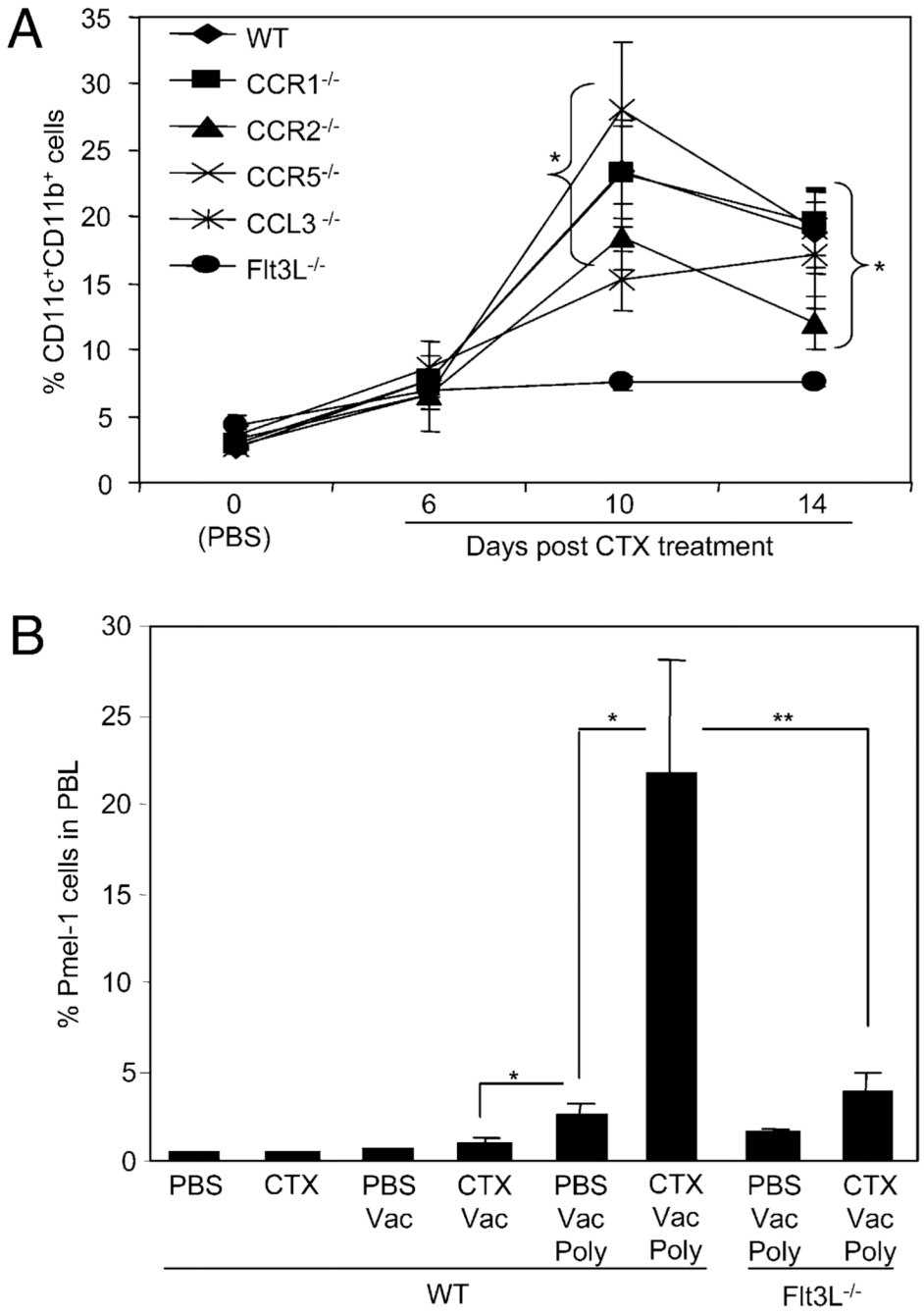


FIGURE 8.

Flt3/Flt3L signaling pathway is required for the enhanced Ag-specific T cell responses to revaccination of CTX-treated mice at the surge of DC expansion. *A*, WT mice or mice deficient in the indicated genes (n = 4/group) were treated with PBS or 4 mg/mouse CTX and bled on days 0, 6, 10, and 14 posttreatment to determine the percentage of DCs. *B*, WT or Flt3L$^{-/-}$ mice (n = 4/group; Ly5.2) were treated with PBS or CTX and adoptively transferred 1 d later with 1 × 106 pmel-1 cells. Recipients were left without further manipulation, or prime-boost vaccination with s.c. injection of 100 μg/mouse gp100$_{25–33}$ ± i.p. injection of 200 μg/mouse poly-I:C on days 2 and 12 posttreatments. Mice were bled on day 3 after boosting (day 15 posttreatments) to assess the % of pmel-1 cells in PBLks by

flow cytometry. *A significant increase ($p < 0.05$) as compared with controls. **A significant decrease ($p < 0.05$) as compared with CTX-vaccinated WT recipients.