Microenvironment and Immunology

Immunosuppressive Myeloid Cells Induced by Chemotherapy Attenuate Antitumor CD4⁺ T-Cell Responses through the PD-1–PD-L1 Axis

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Abstract

In recent years, immune-based therapies have become an increasingly attractive treatment option for patients with cancer. Cancer immunotherapy is often used in combination with conventional chemotherapy for synergistic effects. The alkylating agent cyclophosphamide (CTX) has been included in various chemoimmunotherapy regimens because of its well-known immunostimulatory effects. Paradoxically, cyclophosphamide can also induce suppressor cells that inhibit immune responses. However, the identity and biologic relevance of these suppressor cells are poorly defined. Here we report that cyclophosphamide treatment drives the expansion of inflammatory monocyte myeloid cells (CD11b⁺Ly6ClowCCR2hi) that possess immunosuppressive activities. In mice with advanced lymphoma, adoptive transfer (AT) of tumor-specific CD4⁺ T cells following cyclophosphamide treatment (CTX+CD4 AT) provoked a robust initial antitumor immune response, but also resulted in enhanced expansion of monocyte myeloid cells. These therapy-induced monocytes inhibited long-term tumor control and allowed subsequent relapse by mediating functional tolerization of antitumor CD4⁺ effector cells through the PD-1–PD-L1 axis. PD-1/PD-L1 blockade after CTX+CD4 AT therapy led to persistence of CD4⁺ effector cells and durable antitumor effects. Depleting proliferative monocytes by administering low-dose gemcitabine effectively prevented tumor recurrence after CTX+CD4 AT therapy. Similarly, targeting inflammatory monocytes by disrupting the CCR2 signaling pathway markedly potentiated the efficacy of cyclophosphamide-based therapy. Besides cyclophosphamide, we found that melphalan and doxorubicin can also induce monocyte myeloid suppressor cells. These findings reveal a counter-regulation mechanism elicited by certain chemotherapeutic agents and highlight the importance of overcoming this barrier to prevent late tumor relapse after chemoimmunotherapy. Cancer Res; 74(13); 1–13. ©2014 AACR.

Introduction

Recent advances in therapeutic antibodies, cancer vaccines, and adoptive T-cell therapy (ACT) have manifested the tremendous therapeutic potential of immunotherapy in treating patients with cancer (1). However, the efficacy of cancer immunotherapy is often restricted by various immune suppressive mechanisms preestablished in the tumor microenvironment. The prevalent immunoregulatory mechanisms include immune suppressor cells such as T regulatory cells (Tregs) and myeloid-derived suppressor cells (MDSC), tolerogenic enzyme indoleamine 2,3-dioxygenase (IDO), immunosuppressive soluble factors such as TGFβ, IL10, and prostaglandin E2 (PGE2), and checkpoint molecules such as CTLA-4 and PD-1 (2). To enhance the efficacy of cancer immunotherapy, chemotherapy is often included in treatment regimens to condition an immune milieu conducive to therapeutic immunologic modulations. The notion of achieving synergistic antitumor effects through combined chemoimmunotherapy has been substantiated by the finding that many widely used chemotherapeutic agents have immunomodulatory effects (3). One complicated issue associated with chemoimmunotherapy strategy is that chemotherapy causes substantial cell death and tissue injuries, which often result in dynamic recovery of lymphoid and myeloid cells, along with extensive inflammation in the postchemotherapy setting. How these events shape the ultimate outcome of immunotherapy is not fully understood.

The antineoplastic agent cyclophosphamide (CTX) is frequently used in the treatment of hematopoietic malignancies.

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More importantly, cyclophosphamide represents a well-studied example of a chemotherapeutic agent with strong immunomodulatory effects. At high doses, cyclophosphamide is cytotoxic and lymphoablative, causing severe immune suppression. At lower doses, cyclophosphamide exhibits multifaceted immune-potentiating effects (4, 5). It has been shown that cyclophosphamide can induce immunogenic tumor cell death that enhances antigen presentation (6), and mitigate immunosuppression by reducing the number and activity of T-regulatory cells (7). In addition, cyclophosphamide induces transient lymphopenia, creating niche-“space” and providing growth factors for the expansion and survival of transfused tumor-reactive T lymphocytes (8). Thus, the postchemotherapy period represents a window of opportunity to exploit for immunotherapy, especially adoptive T-cell therapy (9, 10). Despite cyclophosphamide's well-characterized immunomodulatory effects, it has long been documented that cyclophosphamide can induce a population of cells exhibiting immunosuppressive activities (11, 12). Some early studies suggested that cyclophosphamide-induced suppressor cells were a heterogeneous population with CD11b⁺ myeloid lineage cells being most suppressive (13, 14). Recent studies reported that cyclophosphamide-induced suppressor cells were mainly CD11b⁺Gr1⁺ MDSCs (15, 16). However, because MDSCs themselves are a heterogeneous population of immature myeloid cells (17), the cellular identity of cyclophosphamide-induced suppressor cells has not been well defined. More importantly, the biologic relevance of these cells in the context of cyclophosphamide-based cancer therapy has not been addressed.

In this study, we report that certain cytotoxic chemotherapeutic agents, including cyclophosphamide, can drive the expansion of myeloid cells consisting of monocytic and granulocytic populations. In mice with advanced B-cell lymphoma or lung metastasis of colon cancer, therapy-induced CD4⁺CD11b⁺CD86⁺ effector cells could impact tumor cell growth by tolerizing CD4⁺ effector cells through the PD-1/PD-L1 axis. With direct clinical relevance, our study demonstrates that targeting therapy-induced monocytic or granulocytic myeloid cells may aid the design of more efficacious chemoimmunotherapy strategies.

Materials and Methods

Mice

Female BALB/c mice of 4 to 6 weeks old were purchased from the National Cancer Institute (Frederick, MD). Thy1.1⁺/+ HA-TCR Tg mice expressing an αβ TCR specific for amino acids 110 to 120 from influenza hemagglutinin (HA) presented by MHC class II molecule IAd were generous gifts from Dr. H.I. Levitsky (The Johns Hopkins University School of Medicine, Baltimore, MD). The PD-1KO mice on a BALB/c background were purchased from RIKEN BioResource Center (Ibaraki, Japan). Thy1.1⁺/+ PD-1KOHA-TCR Tg mice were obtained by crossing the two transgenic strains. All mice were housed under specific pathogen-free conditions by Laboratory Animal Services (LAS) of the Georgia Regents University (GRU). All animal experiments were approved by the Institutional Animal Care and Use Committee of GRU.

Antibodies and reagents

The detailed information of antibodies used in the study is provided in the Supplementary Data.

Cell preparation and flow cytometry

The detailed descriptions of procedures of flow cytometry analysis, cell quantification, and cell sorting are provided in the Supplementary Data.

Tumor model and in vivo treatments

The generation and maintenance of HA-expressing murine B-cell lymphoma cell line A20 (A20HA) and colon cancer cell line CT26 (CT26HA) were described previously (18, 19). A20HA tumor cells were subcutaneously inoculated to the right flank of mice (5 × 10⁶ per mouse). Tumor growth was monitored by caliper measurement of the tumor area every three days, and expressed as the product of two perpendicular diameters in mm². Mice were euthanized when tumor size reached 400 mm² or when tumor sites ulcerated. For adoptive T-cell transfer, spleens and lymph nodes from HA-TCR Tg mice were harvested to enrich for CD4⁺ T cells by MACS (Miltenyi Biotec). A total of 2.5 to 3 × 10⁶ CD4⁺ TCR⁺ T cells were injected intravenously into each recipient. Cyclophosphamide was dissolved in PBS and intraperitoneally injected to mice at the dose of 150 mg/kg unless otherwise specified. Gencitabine and 5-fluorouracil were dissolved in PBS and intraperitoneally injected to mice at 75 or 40 mg/kg, respectively, following the specified schedule. All chemotherapy solutions were filtered through a 0.22-μm membrane each time before injection. To deplete CD11b⁺Ly6G⁺Ly6ChiCCR2⁺ monocytes or CD11b⁺Ly6Ghi granulocytes, αCCR2 mAb (MC21, 20 μg per injection) or αLy6G mAb (1A8, 100 μg per injection), respectively, was intraperitoneally injected to mice following the specified schedule. CCX872 was given to mice by daily subcutaneous injection for 14 days, starting four days after cyclophosphamide treatment. In vivo PD-1 and PD-L1 antibody blockade was conducted as described previously (19).

In vitro suppression assay

For non-antigen-specific suppression, spleen cells from HA-TCR Tg or normal Balb/c mice were labeled with 0.5 μM/mL carboxyfluorescein diacetate succinimidyl ester (CFSE) and seeded into a round-bottom 96-well plate (1 × 10⁶ cells/well in 200 μL medium), with or without the addition of 1 μg/mL anti-CD3 Ab (145-2C11) and 5 μg/mL anti-CD28 Ab (37.51). Varied numbers of sorted monocytic or granulocytic myeloid cells were added to the culture. When using CFSE dilution as the readout for suppression, cells were harvested on day 3 or day 4 after culture, and stained with CD4 for FACS analysis. When using ³H-thymidine incorporation as the readout, cells were cultured for three days, then pulsed with ³H-thymidine (1 μCi/well) for additional 8 hours before harvest.
uptake was counted using a liquid scintillation counter and expressed as CPM. In some experiments, anti-PD-1 (10 μg/mL, RMP1-14, Bio X Cell) and anti-PD-L1 (10 μg/mL, 10F.9G2, Bio X Cell) mAbs were added. For antigen-specific suppression, CD4+ T cells (5 × 10^5/well) purified from HA-TCR Tg mice were mixed with cognate peptide-pulsed CD11c+ dendritic cells (5 × 10^5/well) purified from a Balb/c mouse by MACS beads (Miltenyi Biotec).

**Statistical analysis**

Data were analyzed using Prism 4.0 (GraphPad Software, Inc.). The statistical significance of the results was determined using the Student t test. Data for mouse survival were analyzed using a log-rank test. P values less than 0.05 were considered statistically significant.

**Results**

**Chemoinmunotherapy with CTX + CD4 AT induces inflammatory myeloid cells consisting of monocytic and granulocytic subsets**

Using a mouse model of B-cell lymphoma, we previously reported that AT of tumor-specific CD4+ T cells after cyclophosphamide treatment gave rise to polyfunctional CD4+ effector cells, which played a critical role in mounting a robust antitumor immune response (18). However, antitumor CD4+ effector cells were susceptible to functional tolerization in the face of persistent residual tumors. Consequently, the antitumor immunity elicited by chemoinmunotherapy was not durable, and most mice succumbed to tumor relapse (19). Because immunosuppression mediated by MDCs is one of the major tumor-induced immunoregulatory mechanisms by which tumors escape immune attacks, we set out to examine the presence and phenotype of myeloid cells in our model system. As shown in Fig. 1A schema, mice with established subcutaneous A20 tumors expressing HA (A20HA) were either untreated (No Tx) or treated with cyclophosphamide followed by AT of HA-specific CD4+ T cells (CTX+CD4 AT). There was only marginal presence of myeloid cells in the spleens and tumors of untreated mice, as demonstrated by both FACS analysis (Fig. 1A, No Tx) and immunofluorescence (IF) staining (Supplementary Fig. S1) of CD11b+ cells. In contrast, the presence of CD11b+ myeloid cells was markedly increased in mice treated with the combination of cyclophosphamide and CD4 AT (Fig. 1A and Supplementary Fig. S1; CTX+CD4 AT). These CD11b+ cells mainly consisted of two subpopulations expressing varied levels of myeloid lineage markers Ly6C, Ly6G, and Gr1 (Fig. 1B, left). Giemsa stain showed that the CD11b+ Ly6C^hiLy6G^-Gr1^hi subset had a mononuclear feature typical of monocytes, whereas the CD11b^+Ly6C^loLy6G^hiGr1^hi subset exhibited a polymorphnuclear feature characteristic of granulocytes/neutrophils (Fig. 1B, right). Additional phenotypic analysis indicated that the two subsets of myeloid cells were distinct from conventional dendritic cells (cDC) and macrophages (MΦ), and that the monocytic myeloid subset expressed higher levels of CD14 and IL4Rα compared with the granulocytic myeloid subset (Fig. 1C).

The robust expansion of myeloid cells suggested that these cells were proliferative. Indeed, both subsets of myeloid cells expanded after CTX+CD4 AT were Ki67 positive (Fig. 1D). Interestingly, the monocytes in both spleen and tumor exhibited higher Ki67 levels than the granulocytes, suggesting that the former population had a proliferative advantage. Moreover, as we reported previously (19), the CTX+CD4 AT regimen resulted in heightened inflammation, as evidenced by markedly elevated levels of IL1β, CSF1, CXCL10, CXCL12, and CCL2 (Supplementary Fig. S2). Corresponding to this inflammatory immune milieu, the monocytes in both spleen and tumor expressed high levels of CCR2 (Fig. 1E), a key chemokine receptor involved in mediating the trafficking of inflammatory monocytes (20).

**Posttherapy expansion of myeloid cells is driven by cyclophosphamide and intensified by tumor-specific CD4+ effector cells**

To determine which component of the treatment regimen (cyclophosphamide or CD4 AT) led to the induction and expansion of myeloid cells, we quantified the two myeloid cell subsets in mice receiving different treatment. We first compared the frequency of each myeloid subset in treated or untreated tumor-bearing mice. The frequencies of monocytes in untreated mice were low in the spleen and tumor, and were not increased in mice receiving tumor-specific CD4+ T cells (Fig. 2A and B, No Tx vs. CD4 AT). In contrast, the frequencies of monocytes in cyclophosphamide-treated mice were significantly increased both in the spleen and tumor, and were further boosted in the presence of tumor-specific CD4+ T cells (Fig. 2A and B, cyclophosphamide vs. CTX+CD4 AT). For granulocytes, their frequencies in the spleen followed similar pattern as monocytes (Fig. 2C, top). Interestingly, granulocytes were rare in tumor, and their presence in tumor became evident only after the combined treatment of CTX+CD4 AT (Fig. 2A and C, bottom). In a colon cancer model (CT26HA), we confirmed that cyclophosphamide can induce the expansion of myeloid cells, and addition of tumor-specific CD4+ T cells can intensify myeloid cell expansion (Supplementary Fig. S3).

Next we conducted time course experiments to determine the kinetics of myeloid cell expansion after treatment. The absolute numbers of the two subsets of myeloid cells were used to reflect the actual cell accumulation in specified tissues. In the spleen, there was an initial reduction in the numbers of monocytes after cyclophosphamide treatment (day 2) followed by a rebound thereafter (Fig. 2D, top, cyclophosphamide). In the tumor, the rebound of monocytes peaked on day 7, and by day 10 reached a stable level that was elevated than the starting point (Fig. 2D, bottom, cyclophosphamide). Notably, CD4 AT following cyclophosphamide intensified the magnitude of monocyte expansion both in the spleen and tumor (Fig. 2D, CTX+CD4 AT). Similarly, CD4 AT following cyclophosphamide also enhanced the expansion of granulocytes in the spleen and tumor (Fig. 2E). Our previous study showed that in mice with established tumors, the transferred tumor-specific CD4+ T cells were destined to become dysfunctional and suppressive, but cyclophosphamide allowed these cells to differentiate into activated effector cells (18). Thus, antigen-driven CD4+ T-cell effector differentiation might be a
prerequisite for enhanced myeloid cell expansion. Consistent with this idea, AT of irrelevant DO11.10 CD4\(^+\) T cells (OVA-specific) after cyclophosphamide did not boost myeloid cell expansion compared with cyclophosphamide only (data not shown). Altogether, the data indicate that cyclophosphamide is necessary and sufficient to drive systemic myeloid cell expansion in tumor-bearing hosts, and that activated tumor-specific CD4\(^+\) effector cells can amplify this expansion.

Figure 1. CTX + CD4 AT therapy induces inflammatory myeloid cells consisting of monocytic and granulocytic subsets. A20HA tumors were subcutaneously inoculated to mice. When tumor sizes reached \(~170\) mm\(^2\), mice were either untreated (NoTx) or treated with cyclophosphamide followed by AT of HA-specific CD4\(^+\) T cells (CTX + CD4 AT). Seven days after cyclophosphamide treatment, spleens and tumor masses were processed for analyses. A, representative dot plots showing the frequencies of CD11b\(^+\) myeloid cells. The numbers represent the percentages of the gated CD11b\(^+\) population in total live cells. B, coexpression pattern of myeloid lineage markers and Giemsa stain. Spleen cells from treated mice were costained for CD11b, Gr1, Ly6C, and Ly6G. Gating on CD11b\(^+\) cells; representative dot plots show the coexpression profiles of CD11b versus Ly6C, CD11b versus Gr1, and Ly6C versus Ly6G, respectively. The two major subpopulations were color matched using the FACSDiva software. Cells were sorted into CD11b\(^+\)Ly6C\(^{hi}\) and CD11b\(^+\)Ly6C\(^{lo}\) subsets, and stained with Giemsa solution (right). Images shown (magnification, \(\times 100\)) are representative of three independent experiments. C, phenotype comparison between the two myeloid subsets. Gating on monocytic or granulocytic myeloid subsets, expression profiles of CD11c, F4/80, CD14, and IL4Ra are shown in histograms. Conventional DCs and macrophages are included for comparison of the expression levels of CD11c and F4/80, respectively. D and E, expression profiles of Ki67 and CCR2 in therapy-induced myeloid cells. Spleen and tumor samples from treated mice were stained for CD11b and Ly6C, and evaluated for Ki67 (C) and CCR2 (D) expression profiles in each myeloid cell subset. Representative histograms of Ki67 and CCR2 expressions are shown. Scatter plots summarize the medium fluorescence intensity (MFI). Data are pooled from three independent experiments. ***, \(P < 0.001\).
Therapy-induced monocytic myeloid cells possess immunosuppressive activities

To determine whether therapy-induced myeloid cells possessed immunosuppressive activities, we conducted in vitro suppression assays to test the ability of each myeloid cell subset to suppress CD4<sup>+</sup> T-cell activation. Using a CFSE dilution assay as the readout for cell proliferation, Fig. 3A shows that CD4<sup>+</sup> T-cell response to nonspecific stimuli (tCD3 and tCD28 mAbs) was inhibited, in a dose-dependent manner, by monocytes isolated from the spleens of mice receiving the CTX+CD4 AT regimen, whereas granulocytes from the same mice were not suppressive. Similarly, monocytes recovered from the tumors of treated mice were immunosuppressive, whereas tumor-infiltrating granulocytes were nonsuppressive (Fig. 3B). The immunosuppressive activities of monocytes were also confirmed by <sup>3</sup>H-thymidine incorporation assays (Supplementary Fig. S4A). Furthermore, therapy-induced monocytes can also suppress CD4<sup>+</sup> T-cell activation in response to antigen-specific stimulation (Supplementary Fig. S4B).

We asked whether the emergence of immunosuppressive monocytes after therapy was peculiar to the tumor microenvironment, or the presence of tumor-specific CD4<sup>+</sup> T cells. To address this, naive mice (tumor-free) were treated with a single dose of cyclophosphamide, and spleen cells were harvested at different time points to enumerate the numbers of monocytic and granulocytic myeloid cells. Figure 3C shows that the

Figure 2. Cyclophosphamide-driven myeloid cell expansion is amplified by antitumor CD4<sup>+</sup> effector cells. Following the experimental time line depicted in Fig. 1A, mice with established tumors were randomly divided into four groups and received the specified treatment. A, frequencies of myeloid cells in spleens and tumors. Seven days after cyclophosphamide treatment, spleens and tumor masses were processed for FACS analyses. Representative dot plots are shown for costaining of Ly6C and CD11b. Numbers in dot plots represent frequencies of the gated populations. The results are summarized in bar graphs for monocytes (B) and granulocytes (C). Data pooled from three independent experiments are shown as mean ± SD. ***, P < 0.001. D and E, kinetics of monocytic (D) and granulocytic (E) myeloid cell expansion after cyclophosphamide in the presence or absence of tumor-specific CD4<sup>+</sup> T cells. Tumor-bearing mice were treated as indicated. At the indicated time points, spleen cells were enumerated and analyzed for CD11b and Ly6C expressions by FACS. Tumor masses were weighed before being processed for FACS-based cell counting and phenotypic analysis. The numbers of myeloid cells are shown as mean ± SD with at least five samples at each time point. The formula for cell number calculation is: total cell number × percent of specific myeloid subset. Cell number in tumor is normalized to the weight of tumor mass.
numbers of monocytic and granulocytic myeloid cells were both reduced shortly after cyclophosphamide, reaching the nadir by day 2 and day 4, respectively; and both populations rebounded thereafter, resulting in net increases in cell number by day 10. Induction of myeloid cell expansion was seen in mice receiving cyclophosphamide treatment in the range of 100 to 300 mg/kg (Supplementary Fig. S5).

In vitro suppression assays showed that the monocytes, but not granulocytes, from cyclophosphamide-treated tumor-free mice can suppress CD4\(^+\) T-cell activation (Fig. 3D right). As controls, neither subset of myeloid cells from the spleens of untreated naïve mice was suppressive (Fig. 3D left), suggesting that immunosuppression is an acquired property of monocytic myeloid cells induced by cyclophosphamide.

We then extended the above assays to some other chemotherapeutic agents to determine whether induction of immunosuppressive monocytes was a unique feature of cyclophosphamide. We found that the frequencies of monocytic myeloid cells also increased in mice treated with melphalan (Mel) or doxorubicin (Dox; Supplementary Fig. S6A), and these cells were equally capable of suppressing CD4\(^+\) T-cell activation as cyclophosphamide-induced monocytes (Supplementary Fig. S6B). Altogether, the data indicate that certain cytotoxic anticancer drugs can induce the expansion of immunosuppressive monocytes, which phenotypically resemble the well-described monocytic myeloid-derived suppressor cells (mMDSC; ref. 17).

**Therapy-induced monocytes inhibit CD4\(^+\) T-cell activation through the PD-1–PD-L1 axis**

We then sought to dissect the mechanism(s) by which therapy-induced inflammatory monocytes mediated immune suppression. A number of molecules, including Arginase 1, iNOS,IDO, IFNγ, IL10, and TGFβ, have been implicated in suppression mediated by inflammatory monocytes or MDSCs (17, 21). We repeated in vitro suppression assays with the addition of inhibitors or neutralizing mAbs targeting these molecules. However, these inhibitors used either individually...
or in combination had no significant effects on monocyte-mediated suppression (data not shown). Because the co-inhibitory molecule PD-L1 was prominently expressed in inflammatory monocytes (Fig. 4A), we hypothesized that the PD-1 pathway might be involved in monocyte-mediated suppression. To test this, we used two complementary approaches to disrupt PD-1/PD-L1 interactions. First, therapy-induced monocytes were cocultured with PD-1–sufficient (WT) CD4+ T-cell responders in the presence of a PD-1/PD-L1 blocking mAb cocktail. PD-1/PD-L1 blockade largely restored proliferation of the responder cells (Fig. 4B, left).

The second approach used PD-1–deficient (PD1KO) CD4+ T cells as responders. Strikingly, therapy-induced monocytes, which efficiently suppressed PD-1–sufficient CD4+ T-cell responders, were unable to suppress the proliferation of PD-1–deficient CD4+ T cells (Fig. 4B, right). Together, the data support the notion that the PD-1/PD-L1 pathway is involved in monocyte-mediated suppression on CD4+ T cells.

**PD-1 blockaúd after CTX+CD4 therapy leads to persistence of CD4+ effector cells and durable antitumor effects**

Our *in vitro* suppression data (Fig. 4B) suggested that disruption of the PD-1/PD-L1 pathway may prevent CD4+ effector cell tolerization *in vivo*. To test this, tumor-bearing mice were treated with cyclophosphamide followed by AT of either PD-1–sufficient (WT) or PD-1–deficient (PD1KO) tumor-specific CD4+ T cells. Figure 5A shows that there were similar levels of monocyte and granulocytic myeloid cells in mice that received PD-1KO CD4+ T cells compared with those that received WT CD4+ T cells after cyclophosphamide. Nonetheless, the transferred PD-1KO CD4+ T cells persisted in mice and maintained a polyclonal effector phenotype, that is Foxp3<sup>hi</sup>CD40L<sup>hi</sup>IL2<sup>hi</sup>IFNγ<sup>hi</sup>TNFα<sup>hi</sup>; by contrast, WT donor CD4+ T cells eventually acquired a tolerized phenotype, characterized by elevated levels of PD-1 and Foxp3, but reduced CD40L and lack of production of pro-inflammatory cytokines (Fig. 5B).

Correspondingly, AT of PD-1KO CD4+ T cells after cyclophosphamide resulted in prolonged survival in the majority of mice, whereas relapse was prevalent in mice receiving WT CD4+ T cells after cyclophosphamide (Fig. 5C). CTX+PD1KOC4D+ AT vs. CTX+WTCD4D+ AT. As an alternative approach, adding αPD-1/αPD-L1 antibody blockade after CTX+WTC4D4 AT recapitulated the beneficial effect in mouse survival achieved by CTX+PD-1KOC4D4 AT. These data support the notion that disrupting the PD-1/PD-L1 axis can prevent CD4+ effector cell tolerization *in vivo*.

**Administration of low-dose gemcitabine reduces inflammatory monocytes and potentiates the efficacy of CTX+CD4 AT therapy**

A20 tumor cells constitutively express PD-L1 (22). Thus, either tumor cells or mononuclear myeloid cells can provide PD-L1 to engage PD-1 on CD4+ effector cells and consequently render them tolerant. We reasoned that if monocytes were the relevant PD-L1–expressing cells, then reducing the presence of these cells should confer significant therapeutic benefits. Our finding that therapy-induced monocytes were highly proliferative (Fig. 1D) suggested that these cells may be more sensitive to low-dose chemotherapy. We chose to test this using gemcitabine (Gem) because it has been shown that gemcitabine can preferentially eliminate CD11b<sup>hi</sup>Gri1<sup>+</sup> MDSCs but largely spare T lymphocytes (23). As expected, gemcitabine given after CD4+ T-cell transfer reduced the frequency and number of therapy-induced monocytes in peripheral blood, spleen, and tumor (Fig. 6A and B). Interestingly, reduction of granulocytes was also evident in blood and spleen, but was insignificant in tumor. Notably, reduction of therapy-induced monocytes correlated with remarkable therapeutic benefits. As shown in Fig. 6C and D, gemcitabine treatment following CTX+CD4 AT (CTX+CD4 AT+Gem) led to complete tumor remission and
levels of CCR2 (Fig. 1E), we administered a CCR2-specific mAb with CCX872, a potent and selective CCR2-speciﬁc mAb with CCX872, a potent and selective CCR2-speciﬁc mAb (MC21) to mice after CTXþCD4 AT therapy. Anti-CCR2 mAb injection led to selective depletion of CD11bLy6Chigh monocytic effector cells and durable antitumor effects. Mice with established A20HA tumors (~170 mm3) were treated with cyclophosphamide followed by AT of either PD-1–sufﬁcient (WT) or PD-1–deﬁcient (PD1KO) HA–speciﬁc CD4+ T cells the next day. A, frequencies of myeloid cell subsets in mice receiving WT or PD1KO CD4+ T cells after cyclophosphamide. Seven days after cyclophosphamide treatment, several mice from each group were killed to collect spleen and tumor samples for FACS analysis. Representative dot plots are shown, and the numbers represent the percentages of the gated population. B, phenotypic analysis of donor CD4+ T cells. Thirty days after cyclophosphamide, spleens were isolated from symptom-free mice that had received PD1KO HA–speciﬁc CD4+ T cells or from relapsed mice that had received WT HA–speciﬁc CD4+ T cells. Spleen cells were stained for CD4 and Thy1.1 to identify the donor CD4+ T cells and evaluated for expressions of PD-1, Foxp3, and CD40L by FACS. Cytokine expression in donor CD4+ T cells were assayed by intracellular cytokine staining after a 4-hour stimulation with the cognate peptide. The results of all samples are summarized in bar graph. Data are shown as mean ± SD with at least three samples per group. **, P < 0.001. C, overall survival of mice receiving WT or PD1KO HA–speciﬁc CD4+ T cells after cyclophosphamide is shown as Kaplan–Meier survival curve indicating the percentage of tumor-free mice as a function of time after cyclophosphamide treatment. Some receiving CTXþWTCD4 AT therapy were subsequently injected with αPD-1 and αPD-L1 mAbs. The number of mice in each group is given.

Disrupting the CCL2–CCR2 axis after CTXþCD4 AT therapy leads to targeted depletion of inﬂammatory monocytes and prevention of relapse

We sought to more speciﬁcally deplete therapy-induced monocytes so as to determine their role in CD4+ effector cell tolerization and tumor relapse. Based on our ﬁnding that therapy-induced monocytes preferentially expressed high levels of CCR2 (Fig. 1E), we administered a CCR2-speciﬁc mAb leading to targeted depletion of inﬂammatory monocytes and prevention of relapse. Long-term survival in the majority of mice. In contrast, the combination of cyclophosphamide and gemcitabine did not differ from cyclophosphamide alone in tumor growth and mouse survival (CTXþGem vs. cyclophosphamide). Moreover, the combination of gemcitabine and CD4 AT had no therapeutic effects (data not shown). Altogether, our results indicate that the effectiveness of gemcitabine in potentiating CTXþCD4 AT therapy is not simply because of the added cytotoxicity on tumor cells by two anticancer drugs, but rather a synergistic effect of the tripartite regimen. To test the idea that the efﬁcacy of the tripartite regimen is not restricted to lymphoma, mice with lung metastasis of CT26HA tumors were treated with different combinations of cyclophosphamide, CD4 AT, and gemcitabine (Supplementary Fig. S7). Again, only the tripartite regimen resulted in substantial improvement in long-term survival in this aggressive tumor model. In addition to gemcitabine, 5-ﬂuorouracil (5-FU) has been shown to deplete MDSCs (24, 25). We demonstrated in the A20HA tumor model that 5-FU can effectively reduce the presence of therapy-induced monocytes (Supplementary Fig. S8), further supporting the notion that therapy-induced immunosuppressive monocytes are sensitive to certain anticancer drugs used at low dose.
antagonist of CCR2. As shown in Fig. 7D, administration of CCX872 following CTX+CD4 therapy significantly improved long-term survival compared with the control mice that received CTX+CCX872. Altogether, our data indicate that disrupting the CCL2–CCR2 axis can effectively relieve tumor-specific CD4+ effector cells from inflammatory monocyte-mediated tolerization and sustain a productive antitumor immunity.

Because many mice had complete tumor remission and survived long term after CTX+CD4 AT therapy in combination with either CCR2-specific mAb (MC21) or gemcitabine, we asked whether these long-term survivors (LTS) had developed immune memory. To this end, mice that became LTS after combinatorial therapy were rechallenged with A20HA. Figure 7E shows that these mice were completely protected from tumor rechallenge, whereas all naive control mice succumbed to rapid tumor growth.

**Discussion**

Induction and expansion of suppressive inflammatory monocytes or MDSCs in the tumor setting has been amply documented. Using tumor cell lines engineered to express proinflammatory cytokines, previous studies have reported that tumor-induced inflammation recruited and expanded inflammatory monocytes/MDSCs capable of suppressing antitumor immune responses (26–28). In addition to mediating immune suppression, tumor-induced inflammatory monocytes were also found to facilitate tumor metastasis in breast cancer (29). Different
from these published studies that focus on cancer-induced inflammation, this study addresses the role of therapy-induced inflammation in regulating ongoing immune responses. Although the tumor-promoting effect of chronic inflammation has been well established (30, 31), the impact of therapy-induced inflammation (often acute) on eventual treatment outcomes has been a subject of debate (32). In our study, it was chemotherapy with cyclophosphamide that drove the expansion of immunosuppressive monocytes, regardless of the presence or absence of tumor. The fact that cyclophosphamide-induced inflammatory monocytes acquired suppressive activities in the absence of tumor growth (Fig. 3D) suggests that this feature may serve as a negative feedback loop to control excessive inflammation.

Surprisingly, this counter-regulation mechanism was reinforced by antitumor CD4⁺ effector cells in a therapeutic setting (Fig. 2). It has been shown that cyclophosphamide induces an inflammatory immune milieu, in which myeloid growth factors and chemotactic factors such as GM-CSF, G-CSF, and CCL2 are abundant (8, 33, 34). We previously showed that this inflammatory milieu was markedly intensified in the presence of CD4⁺ effector cells (19). It is intriguing that cyclophosphamide-induced monocytes shared some similarities with alternatively activated macrophages (M2), such as IL4Rα expression and suppressive activities, raising the question whether M2 cells were included in therapy-induced monocytes. However, IL4 and IL13, which are essential for inducing M2 cells (35), were not

Figure 7. Disrupting the CCL2–CCR2 axis after CTX+CD4 AT therapy leads to targeted depletion of inflammatory monocytes and prevention of relapse. Following the timeline depicted in the schema, mice with established A20HA tumors were treated with CTX+CD4 AT. At the indicated time points, a cohort of mice were injected with CCR2-specific mAb (MC21), and some mice were given Ly6G-specific mAb (1A8). As controls, some tumor-bearing mice were treated with cyclophosphamide only or the combination of cyclophosphamide and αCCR2 mAb. A, selective depletion of myeloid cell subset by specific mAbs. On day 7, two to three mice from the indicated groups were killed and peripheral blood, spleen, and tumor samples were collected for FACS analysis to document the Ab depletion effects. Representative dot plots are shown. Numbers represent the percentages of the gated populations. The remaining mice were monitored for tumor growth kinetics (B) and overall survival (C). D, administration of CCR2 inhibitor CCX872 reduces relapse after CTX+CD4 AT therapy. The treatment procedures are depicted in the schema, and mouse survival curve is shown. E, LTS are resistant to tumor rechallenge. Tumor-bearing mice received CTX+CD4 AT therapy, followed by low-dose gemicitabine or CCR2-specific mAb (MC21). Mice that had complete tumor regression and stayed symptom-free for more than 90 days after the initial cyclophosphamide treatment were considered as LTS. LTS were rechallenged with A20HA tumors on the flank opposite to the initial tumor inoculation site. As controls, naïve mice were inoculated with A20HA tumors. The number of mice in each group is given. The LTS group contained six gemicitabine-treated mice and four MC21-treated mice.

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detected in the immune milieu after CTX+CD4 AT therapy (data not shown), arguing against the emergence of M2 cells in our mouse model. We postulate that CD4<sup>+</sup> effector cell–mediated enhancement of myeloid cell expansion is because of increased myeloid cell homeostasis under heightened inflammation. However, singly neutralizing GM-CSF, IFN<sub>γ</sub>, TNFα, or IL6 after CTX+CD4 AT did not have significant impact on the numbers of myeloid cells (data not shown), suggesting that multiple, overlapping inflammatory cytokines may contribute to myeloid cell expansion in this setting.

In addition to cyclophosphamide, we showed that at least two other anticancer drugs, doxorubicin and melphalan, can induce the expansion of monocytic myeloid suppressor cells (Supplementary Fig S6). This is consistent with the report by Nakasone and colleagues that doxorubicin treatment led to a specific, acute recruitment of CCR2<sup>+</sup> monocytic myeloid cells that contributed to tumor regrowth (36). Intriguingly, Alizadeh and colleagues recently reported that low-dose doxorubicin transiently reduced the number and diminished the suppressive function of MDSCs in the 4T1 mammary cancer model (37). It is important to note that in this study doxorubicin was given to tumor-bearing mice to examine its acute effect on existing tumor-induced MDSCs, whereas in our study the drug was injected to tumor-free naïve mice to investigate therapy-induced myeloid cells. It is possible that doxorubicin can exert cytotxic effect on tumor-induced MDSCs, meanwhile its myeloid depletion effect may result in homeostatic myeloid reconstitution, which gives rise to therapy-induced MDSCs. In future studies it will be of interest to assess the relation between preexisting tumor-induced MDSCs and therapy-induced MDSCs. Chemotherapy-driven MDSC expansion has also been observed in patients with cancer. In a study conducted in patients with breast cancer, circulating MDSC numbers were significantly increased in patients receiving doxorubicin–cyclophosphamide chemotherapy, and correlated with clinical cancer stage and metastatic tumor burden (38).

It is also important to note that induction and expansion of inflammatory myeloid cells with immunoregulatory function is not restricted to chemotherapy. Other cancer treatment modalities, including radiation therapy and surgery, may have similar effects. It was reported in a mouse melanoma model that total body irradiation (TBI) induced rapid reconstitution of MDSCs with enhanced suppressive activity (39). Moreover, a correlative study in patients with pancreatic cancer found that the prevalence of CCR2<sup>+</sup>CD14<sup>+</sup> inflammatory monocytes in the peripheral blood following tumor resection correlated inversely with survival (40). There is accumulating evidence that cancer immunotherapy can also induce immunosuppressive myeloid cells. Mitchell and colleagues reported that therapeutic vaccination induced inflammatory monocytes that counter-regulated vaccine-induced immunity (41). Furthermore, Hosoi and colleagues found that AT of pmel-specific CD8<sup>+</sup> T cells to mice with B16 melanoma led to accumulation of monocytic MDSCs, which acted to temper CTL antitumor activity (42). Altogether, these studies indicate that a broad spectrum of cancer therapies may give rise to inflammatory myeloid suppressor cells that counteract therapy efficacy.

Although this study focused on monocyte-mediated suppression on antitumor CD4<sup>+</sup> T cells, it is worth noting that CD8<sup>+</sup> T-cell activation was also inhibited by cyclophosphamide–induced inflammatory monocytes (data not shown). Therefore, it is reasonable to speculate that targeting therapy-induced myeloid suppressor cells may augment the long-term efficacy of some current cancer immunotherapy strategies, such as cancer vaccines and adoptive T-cell therapy, in which cyclophosphamide is often a component of the treatment regimen. From a therapeutic standpoint, our study outlines multiple targeted approaches that can effectively reduce the number or abolish the suppressive function of inflammatory monocytes, thereby tipping the balance toward unrestrained antitumor immunity. Here we showed that the proliferative nature of inflammatory monocytes rendered them susceptible to low-dose gercitabine and 5-FU. Alternatively, disrupting the relevant CCL2/CCR2 chemotactic pathway represents an attractive approach to reduce the recruitment and accumulation of inflammatory monocytes (Fig. 7). Along this line, the use of CCL2-specific neutralizing mAb or small molecule inhibitors for CCR2 has also shown beneficial effects in several preclinical models (29, 40, 41, 43). A novel finding of our study is that therapy-induced inflammatory monocytes suppress CD4<sup>+</sup> T-cell responses through the PD-1–PD-L1 axis. This provides a mechanistic explanation for our previous observation that the PD-1 pathway is critically involved in CD4<sup>+</sup> effector cell tolerization (19). Although A20 tumor cells constitutively express PD-L1, it has been shown that direct encounter of A20 tumor cells in vivo was stimulatory, rather than tolerogenic, to the activation of tumor-specific CD4<sup>+</sup> T cells (44). Here we showed that targeted depletion of inflammatory monocytes was quite effective in preventing PD-1–dependent CD4<sup>+</sup> T-cell tolerization (Fig. 7), supporting the notion that the relevant ligands for PD-1 primarily come from inflammatory monocytes as oppose to tumor cells.

In summary, our study reveals that inflammation engendered after chemoimmunotherapy may facilitate tumor escape by engaging myeloid suppressor cells that attenuate antitumor immunity, and that mitigating this counter-regulation mechanism can augment the efficacy of chemoimmunotherapy. We have identified multiple clinically applicable approaches that can effectively target chemotherapy-induced myeloid suppressor cells, leading to durable antitumor immunity. These findings imply that modulation of therapy-induced inflammation represents an attractive strategy to augment the efficacy of chemoimmunotherapy and achieve a long-lasting curative effect.

**Disclosure of Potential Conflicts of Interest**

M. Walters has ownership interest (including patents) from Chemocentryx. A.L. Mellor has other commercial research support from NewLink Genetics Inc. No potential conflicts of interest were disclosed by the other authors.

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