REPORT

IMMUNOTHERAPY

Rescue of exhausted CD8 T cells by PD-1–targeted therapies is CD28-dependent

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Programmed cell death–1 (PD-1)–targeted therapies enhance T cell responses and show efficacy in multiple cancers, but the role of costimulatory molecules in this T cell rescue remains elusive. Here, we demonstrate that the CD28/B7 costimulatory pathway is essential for effective PD-1 therapy during chronic viral infection. Conditional gene deletion showed a cell-intrinsic requirement of CD28 for CD8 T cell proliferation after PD-1 blockade. B7-costimulation was also necessary for effective PD-1 therapy in tumor-bearing mice. In addition, we found that CD8 T cells proliferating in blood after PD-1 therapy of lung cancer patients were predominantly CD28-positive. Taken together, these data demonstrate CD28-costimulation requirement for CD8 T cell rescue and suggest an important role for the CD28/B7 pathway in PD-1 therapy of cancer patients.

In recent years, strategies that reinvigorate tumor-specific T cells have uncovered the potential of immunotherapy (*1*). Sustained expression of the inhibitory receptor programmed cell death–1 (PD-1) characterizes exhausted T cells, and PD-1–targeted therapies have shown clinical activity in a wide variety of cancer types (*2*, *3*). However, not all patients experience clinical benefit from PD-1 therapy, and there is a critical need to determine the requirements for optimal T cell rescue not only to improve current therapies but also to identify predictive biomarkers. Blockade of inhibitory molecules improves function of exhausted T cells, but it is not known whether rescue of exhausted CD8 T cells also requires positive costimulation. CD28 is a key T cell costimulatory molecule that binds B7 molecules (*4*). CD28 engagement reduces the T cell receptor signaling threshold required for T cell activation and may provide qualitatively different signals (*5*). Naïve CD8 T cells are more dependent than antigen-experienced cells on CD28, and the requirement for CD28 signaling varies according to strength and duration of antigen exposure (*6*–*8*).

In this study, we address the role of the CD28/B7 pathway for rescue of exhausted CD8 T cells after PD-1 therapy using the mouse model of lifelong chronic lymphocytic choriomeningitis virus (LCMV) infection (*9*, *10*). Expression of CD28 on LCMV-specific exhausted CD8 T cells was similar to naïve T cells but lower than LCMV-specific memory CD8 T cells (fig. S1). CD28 expression did not change significantly on LCMV-specific CD8 T cells after PD-1 therapy of chronically infected mice (fig. S2). To determine the role of CD28-costimulation in PD-1–mediated rescue of exhausted CD8 T cells, we first blocked the CD28/B7 pathway by means of CTLA-4–immunoglobin (Ig) fusion protein administration during anti-PD-L1 therapy of chronically infected mice (Fig. 1A). As reported by Barber *et al.*, PD-1 blockade rescues virus-specific CD8 T cells in LCMV chronically infected mice (*11*). Rescue was evident from the increased frequency and number of LCMV-specific CD8 T cells in anti-PD-L1–treated mice (Fig. 1, B and C). In contrast, CTLA-4-Ig prevented anti-PD-L1–mediated expansion of LCMV-specific CD8 T cells in multiple tissues (Fig. 1, B and C, and fig. S3A). CD8 T cells expanded by PD-L1 blockade also regained effector function, as evidenced by increased interferon-γ (IFN-γ) production (fig. S3, B and C). However, when CTLA-4-Ig was combined with anti-PD-L1, IFN-γ production was similar to that in untreated mice (fig. S3, B and C). Thus, CTLA-4-Ig prevented rescue of LCMV-specific CD8 T cell responses mediated by PD-1 blockade.

To further extend these observations, we administered blocking antibodies to B7-1 (CD80) and B7-2 (CD86) to chronically infected mice during anti-PD-L1 therapy (Fig. 1A). B7 blockade prevented anti-PD-L1–mediated expansion of LCMV-specific CD8 T cells in spleen, lung, and liver (Fig. 1D). Accordingly, B7 blockade precluded cell-cycle progression of LCMV-specific CD8 T cells (Fig. 1E). B7 engagement was also necessary for increase in granzyme B expression on LCMV-specific CD8 T cells upon PD-1 blockade (Fig. 1F). In addition, when anti-PD-L1 was combined to B7 blockade, there was no increase in IFN-γ–producing cells compared with that of untreated mice (Fig. 1, G and H). Last, anti-PD-L1 therapy was unable to reduce the viral load of chronically infected mice that received B7 blockade (Fig. 1, I and J). These data show that B7-costimulation is required for effective PD-1 therapy in LCMV chronically infected mice. To ensure that transient inhibition of the B7 pathway had no meaningful impact on the maintenance of exhausted CD8 T cells, we used an adoptive transfer of transgenic CD8 T cells specific for LCMV-GP33 (P14 cells) (Fig. 1K). The number of P14 cells was increased with PD-L1 therapy but remained similar between mice treated with the anti-PD-L1/anti-B7 combination, mice treated with anti-B7 antibodies alone, or untreated mice (Fig. 1L and fig. S4). Likewise, IFN-γ production by P14 cells was similar between mice receiving anti-PD-L1/anti-B7, mice receiving anti-B7 alone, or untreated mice (Fig. 1M). Hence, transient B7-blockade in mice with established chronic LCMV infection had no major effects on virus-specific CD8 T cells and did not affect viral load (fig. S5).

PD-L1 can also bind B7-1 to deliver an inhibitory signal (*12*, *13*). The anti-PD-L1 antibodies used in this study function by blocking both PD-L1/PD-1 and PD-L1/B7-1 interactions (*14*). To further clarify and confirm the role of B7/CD28-costimulation in rescuing exhausted CD8 T cells during PD-1 therapy, instead of anti-PD-L1, we used two different clones of anti-PD-1 blocking antibodies (figs. S6 and S7) (*10*). Similar to the data obtained with anti-PD-L1, B7-blockade also prevented rescue of LCMV-specific CD8 T cells mediated through administration of anti-PD-1.

To directly determine a cell-intrinsic requirement for CD28 signaling in PD-1–mediated rescue, we examined whether CD28-deficient P14 CD8 T cells could be rescued by anti-PD-L1 blocking antibodies during chronic LCMV infection (fig. S8). PD-1 blockade resulted in expansion of WT P14 cells, whereas P14 cells in which CD28 was knocked out (CD28KO) failed to expand in blood, spleen, or lung. These data show

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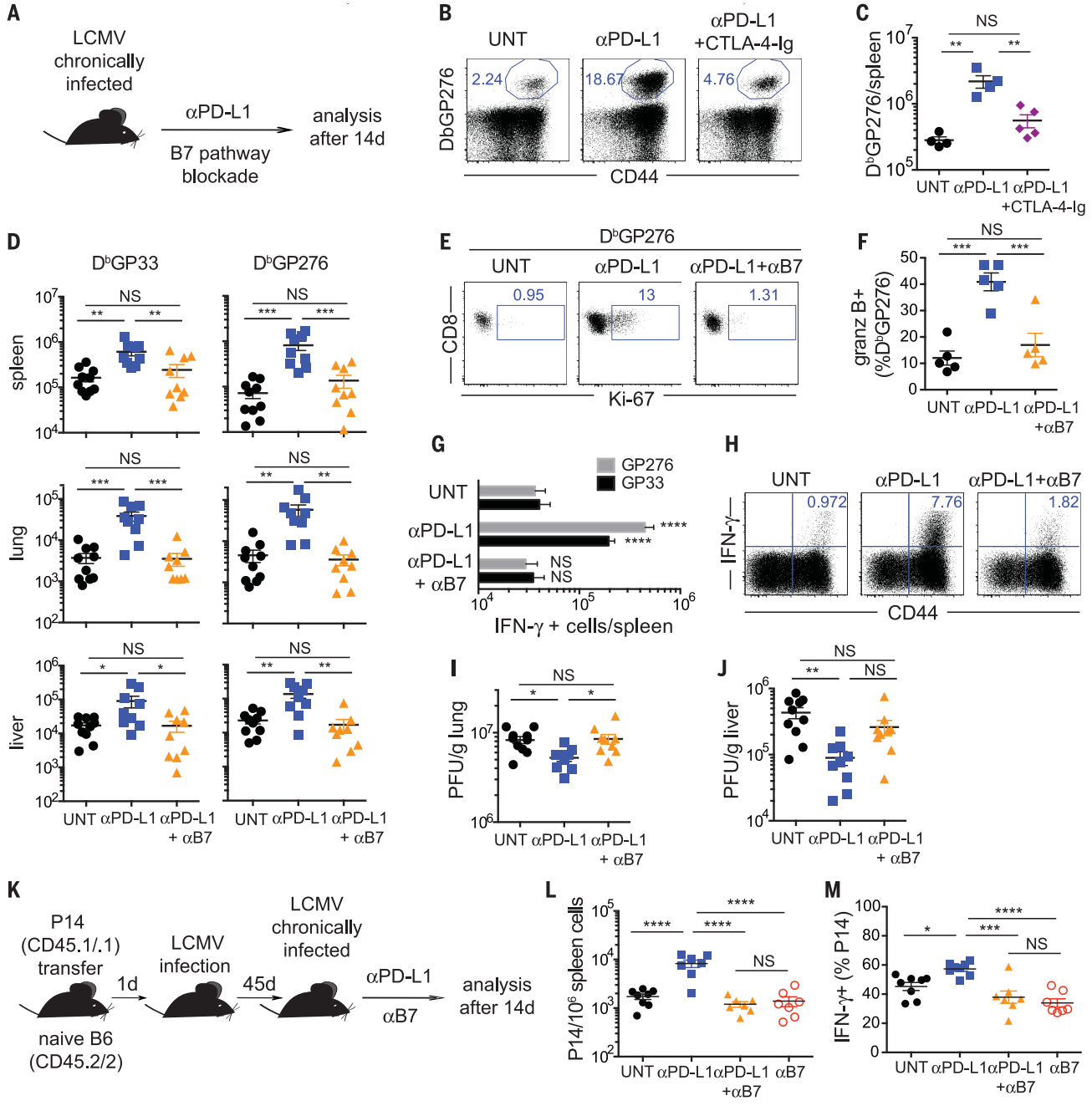

Fig. 1. B7-costimulation is necessary for rescue of virus-specific CD8 T cells after PD-1 blockade during chronic LCMV infection. (**A**) Experimental layout. In (B) and (C), mice received CTLA-4-Ig, and in (D) to (J), mice received anti-B7-1 and anti-B7-2 blocking antibodies during the course of anti-PD-L1 treatment. (**B**) Frequencies of LCMV-D^{b}GP276–specific CD8 T cells in the spleen. Data are representative of three independent experiments, with at least four mice per group. (**C**) Numbers of LCMV-D^{b}GP276–specific CD8 T cells in the spleen. Data show one representative experiment of three independent experiments, with at least four mice per group. Error bars indicate SEM. (**D**) Numbers of LCMV-D^{b}GP33 and LCMV-D^{b}GP276–specific CD8 T cells in different organs. Data show combined data from two of three independent experiments, with three to five mice per group. Error bars indicate SEM. (**E**) K_i-67 expression on LCMV-D^{b}GP276–specific CD8 T cells in the spleen. Data are representative of three independent experiments, with three to five mice per group. (**F**) Frequencies of splenic LCMV-D^{b}GP276–specific CD8 T cells expressing granzyme B. Data show one representative experiment of three independent experiments, with three to five mice per group. Error bars indicate SEM. (**G**) Numbers of IFN-γ–producing CD8 T cells in the spleen after ex vivo restimulation with the indicated peptides. Data show combined data from two of three independent experiments, with three to five mice per group. Comparisons are between treated groups and untreated mice. Error bars indicate SEM. (**H**) Frequencies of CD8 T cells producing IFN-γ in the spleen after ex vivo restimulation with a pool of LCMV peptides. Data are representative of three independent experiments, with three to five mice per group. (**I** and **J**) Viral titer in (I) lung and (J) liver, as quantified by means of plaque assay. PFU, plaque forming units. Data show combined data from two of three independent experiments, with three to five mice per group. Error bars indicate SEM. (**K**) Experiment layout for (L) and (M). (**L**) Frequency of P14 cells in spleen. Data show combined data from two of three independent experiments, with three or four mice per group. Error bars indicate SEM. (**M**) Frequency of P14 cells producing IFN-γ after ex vivo restimulation with LCMV GP33 peptide. Data show combined data from two of three independent experiments, with three or four mice per group. Error bars indicate SEM. Analysis of variance (ANOVA) with Sidak's correction for multiple comparisons; *P < 0.05, **P < 0.01, ***P < 0.001. ****P < 0.0001. NS, not significant.

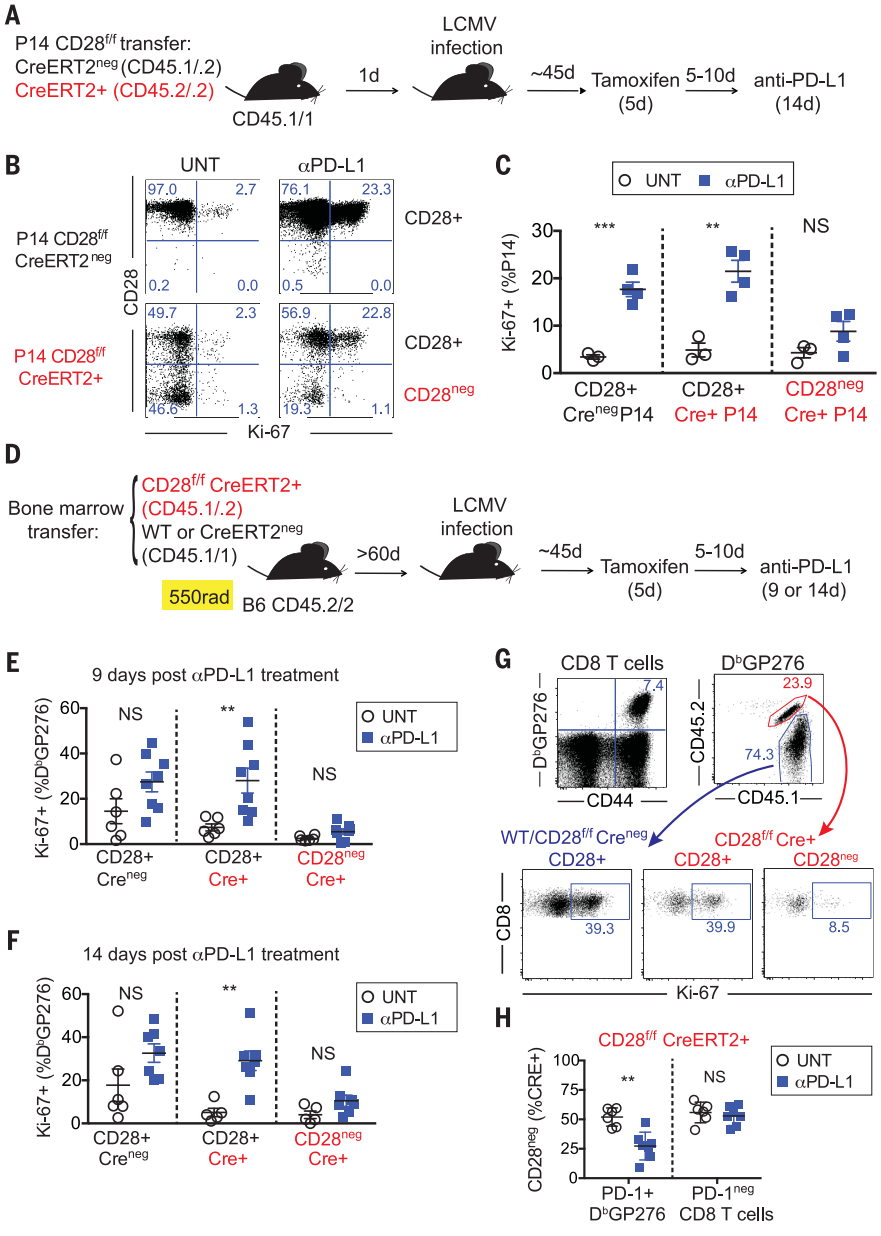

Fig. 2. Cell-intrinsic requirement of CD28 expression for exhausted CD8 T cell expansion upon PD-1 blockade. (**A**) Experimental layout for (B) and (C). (**B**) CD28 and K_i-67 expression on P14 CD28$^{f/f}$ CreERT2^{neg} and P14 CD28$^{f/f}$ CreERT2^{+}, in the spleen of a representative mouse for each group (n = 9 mice). (**C**) Summary of data as in (B). Graph shows frequencies of cells expressing K_i-67 in the spleen among each indicated population. Data show one representative experiment of three independent experiments, with at least three mice per group. Error bars indicate SEM. (**D**) Experimental layout for (E) to (H). (**E**) Frequencies of cells expressing K_i-67 in the spleen among each indicated population, 9 days after anti-PD-L1 treatment. Data show combined data from two independent experiments with at least three mice per group. Error bars indicate SEM. (**F**) Frequencies of cells expressing K_i-67 in the spleen among each indicated population, 14 days after anti-PD-L1 treatment. Data show combined data from two out of three independent experiments with at least three mice per group. Error bars indicate SEM. (**G**) Gating strategy and K_i-67 expression on splenocytes from a representative mouse treated with anti-PD-L1 for 14 days, as in (F). (**H**) Frequency of CD28^{neg} cells among each population of Cre^{+} cells as indicated, 14 days after anti-PD-L1 treatment. Data show combined data from two experiments out of three independent experiments, with at least three mice per group each. Error bars indicate SEM. (C), (E), (F), (H) unpaired t test; $^{**}P < 0.01$, $^{***}P < 0.001$. NS, not significant.

that CD28 deficiency prevents expansion of exhausted CD8 T cells by PD-L1 blockade in a cell-intrinsic manner in both lymphoid and nonlymphoid organs of chronically infected mice. However, activation and differentiation of naïve CD28KO P14 cells into exhausted T cells may not appropriately mirror exhaustion of CD28-expressing CD8 T cells. To overcome this issue, we used an inducible genetic deletion system to investigate whether loss of CD28 expression in already exhausted CD8 T cells would also impair rescue by PD-1 blockade (Fig. 2A). CD28 deletion by tamoxifen was achieved in ~50% of P14 cells expressing CreERT2, and CD28-expressing cells could be easily distinguished from CD28-deficient cells (Fig. 2B). In mice treated with anti-PD-L1 blocking antibodies, K_i-67 expression on P14 cells was largely restricted to CD28-expressing cells (Fig. 2, B and C). Thus, PD-1 blockade was ineffective to induce proliferation of exhausted P14 cells that had lost CD28.

To determine whether cell-intrinsic CD28 expression also affects responsiveness of nontransgenic exhausted CD8 T cells to PD-1 blockade, we performed similar experiments in mixed bone marrow chimeric mice (Fig. 2D). We assessed proliferation of LCMV-specific CD8 T cells by means of K_i-67 expression after anti-PD-L1 treatment and found that proliferation was restricted to CD28^{+} cells (Fig. 2, E to G). Selective proliferation of CD28-expressing cells through PD-1 therapy resulted in decreased frequency of CD28^{neg} cells among PD-1^{+} LCMV-specific Cre^{+} CD8 T cells compared with those of untreated mice. In contrast, we found no differences in the frequency of CD28^{neg} cells among PD-1^{neg} CD8 T cells between untreated and anti-PD-L1–treated mice (Fig. 2H). Proliferation of PD-1^{+} CD8 T cells after blockade of the PD-1 pathway during chronic LCMV infection is contingent on CD28 expression.

To examine whether CD28 signaling would also be necessary for reinvigoration of anti-tumor CD8 T cell responses, we analyzed the role of the CD28/B7 pathway for tumor control of CT26 colon carcinoma through PD-1 therapy. We observed rapid tumor growth in all untreated mice and mice receiving anti-B7, whereas PD-L1–blocking antibodies elicited tumor regression in eight of nine animals. In contrast, 8 of 10 mice receiving anti-PD-L1 in combination with B7-blocking antibodies showed tumor progression (Fig. 3A). The effectiveness of PD-1 therapy for suppressing CT26 tumor growth resulted in a significant improvement in overall survival of anti-PD-L1–treated mice compared with untreated mice ($P < 0.001$), mice treated with anti-B7 alone ($P < 0.001$), or mice receiving both anti-PD-L1 and anti-B7 ($P = 0.0169$) (Fig. 3B). Also, there was no significant improvement in the survival of mice treated with anti-PD-L1 plus anti-B7 compared with untreated mice ($P = 0.1092$). The summary of three independent experiments is shown in Fig. 3C. In accordance to our findings, PD-1 blockade failed to control growth of YUMM2.1 melanoma tumor in mice deficient for CD28 or B7-1/B7-2 (*15*). Our experiments show that CD28-costimulation is necessary for effective PD-1 therapy in a mouse tumor model.

To further explore the role of the CD28/B7 pathway in cancer immunotherapy, we analyzed samples from advanced lung cancer patients receiving PD-1

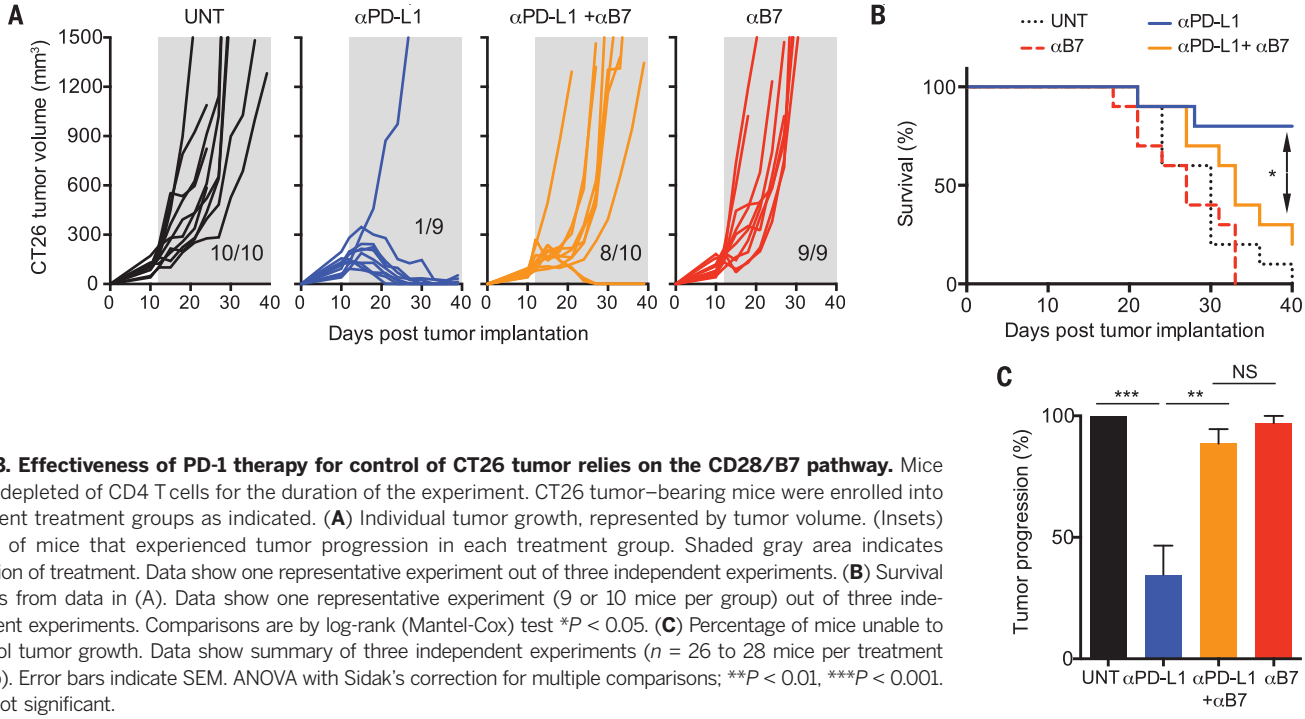

Fig. 3. Effectiveness of PD-1 therapy for control of CT26 tumor relies on the CD28/B7 pathway. Mice were depleted of CD4 T cells for the duration of the experiment. CT26 tumor–bearing mice were enrolled into different treatment groups as indicated. (**A**) Individual tumor growth, represented by tumor volume. (Insets) Ratio of mice that experienced tumor progression in each treatment group. Shaded gray area indicates duration of treatment. Data show one representative experiment out of three independent experiments. (**B**) Survival curves from data in (A). Data show one representative experiment (9 or 10 mice per group) out of three independent experiments. Comparisons are by log-rank (Mantel-Cox) test *$P < 0.05$. (**C**) Percentage of mice unable to control tumor growth. Data show summary of three independent experiments (n = 26 to 28 mice per treatment group). Error bars indicate SEM. ANOVA with Sidak's correction for multiple comparisons; **$P < 0.01$, ***$P < 0.001$. NS, not significant.

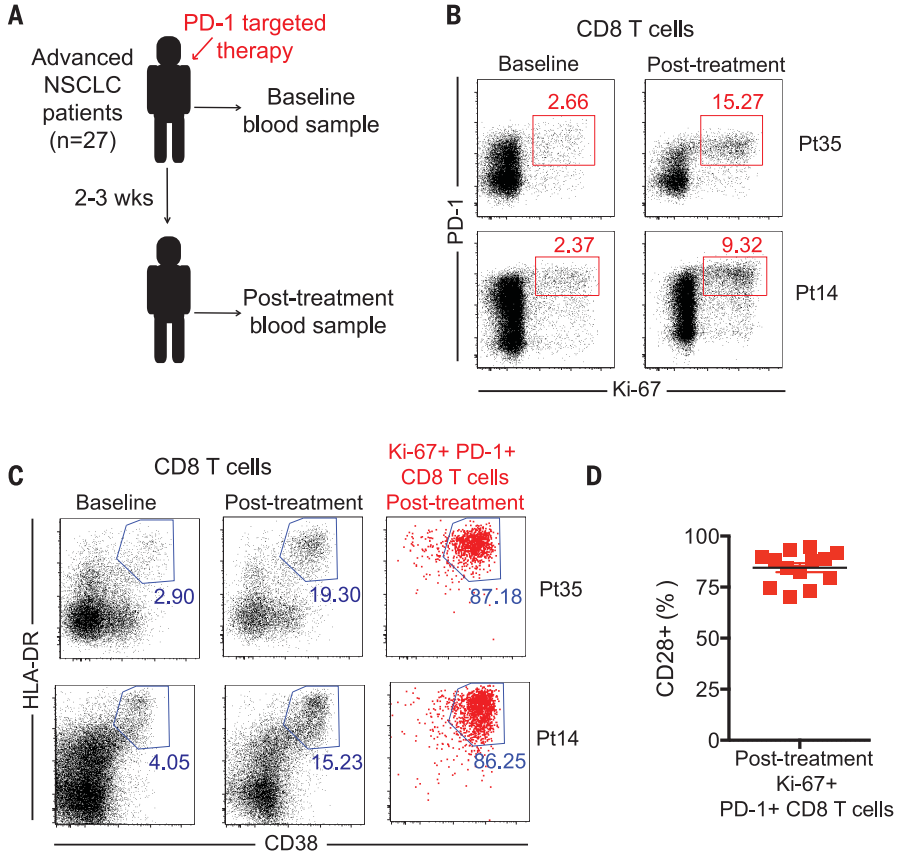

Fig. 4. PD-1$^+$ CD8 T cells that proliferate in the peripheral blood of lung cancer patients receiving PD-1 therapy express CD28. (**A**) Overview of study design. (**B**) K_i-67 and PD-1 expression on CD8 T cells from two representative patients (Pt) out of 13 patients with increased CD8 T cell responses after PD-1–targeted therapy. (**C**) As in (B), but showing HLA-DR and CD38 expression. Dot plots at the far right were gated on posttreatment K_i-67$^+$ PD-1$^+$ CD8 T cells, as indicated in (B). (**D**) CD28 expression on K_i-67$^+$ PD-1$^+$ CD8 T cells in posttreatment samples, as gated in (B) (n = 13 patients with least a twofold increase from baseline in the frequency of K_i-67$^+$PD-1$^+$ CD8 T cells).

therapies. We hypothesized that human PD-1$^+$ CD8 T cells would need to express CD28 in order to efficiently expand on PD-1 therapy. Human effector CD8 T cells induced by vaccination have been identified through human lymphocyte antigen–antigen D related (HLA-DR), CD38, and K_i-67 expression (*16*). Hence, we analyzed proliferation (K_i-67) and activation (HLA-DR and CD38) of peripheral blood PD-1$^+$ CD8 T cells during PD-1 therapy in non–small cell lung cancer (NSCLC) patients (Fig. 4A). After therapy initiation, in about half of patients we observed an increase in K_i-67–expressing CD8 T cells, largely restricted to PD-1–positive cells (Fig. 4B) (*17*). These CD8 T cells also expressed high levels of CD38 and HLA-DR (Fig. 4 C). To assess expression of CD28 on CD8 T cells responding to PD-1 therapy, we focused our analysis on patients that had at least a twofold increase in the frequency of K_i-67$^+$ PD-1$^+$ CD8 T cells (Fig. 4D). In accordance with our predictions, PD-1$^+$ CD8 T cells activated by PD-1 therapy in NSCLC patients were mostly CD28$^+$. These data suggest that CD28 signals may also be important for proliferation of PD-1$^+$ CD8 T cells during PD-1 therapy in cancer patients.

Many studies have assessed expression of inhibitory receptors on exhausted CD8 T cells, but positive costimulatory molecules have not been a major focus. In humans, antigen-experienced CD8 T cells can lose CD28 expression, and loss of CD28 has been associated with chronic stimulation (*18*). We obtained tumor samples from early-stage NSCLC patients and examined CD28 expression on freshly isolated CD8 T cells (fig. S9A). Confirming previous reports (*19*, *20*), we found variable CD28 expression, ranging from 20 to 90% of the CD8 tumor-infiltrating lymphocyte (TIL) population, in individual NSCLC patient samples (fig. S9, B and C). Among NSCLC TILs, TIM-3^{neg} PD1$^+$ CD8 T cells contained

a higher proportion of CD28-positive cells when compared with TIM-3$^+$ PD-1$^+$ TILs in the same tumor (fig. S9, D and E). Thus, many human CD8 TILs do not express CD28 and—according to our data and hypothesis, as well as with previous studies on T cell senescence—therefore may be less responsive to proliferate upon PD-1 blockade (*18*, *19*).

Our laboratory recently identified in chronically infected mice the LCMV-specific CD8 T cell subpopulation that provides the proliferative burst after PD-1 therapy (*21*). These stem-cell–like TIM-3^{neg} PD-1$^+$ TCF-1$^+$ virus-specific CD8 T cells express higher levels of positive costimulatory molecules (CD28, ICOS, OX40, and LIGHT) and lower levels of inhibitory receptors as compared with TIM-3$^+$ PD-1$^+$ TCF-1^{neg} counterparts that do not expand after PD-1 therapy. Yet in spite of the expression of several positive costimulatory molecules by TIM-3^{neg} PD-1$^+$ TCF-1$^+$ virus-specific CD8 T cells, we show that CD28-signaling plays a major and nonredundant role for response to PD-1 blockade. This highlights the dominant role of CD28 signaling in the proliferative response to PD-1 blockade.

Because most tumors (and many virus-infected cells) do not express B7 molecules, our model implicates participation of B7-expressing antigen-presenting cells in the efficacy of PD-1 therapy. Indeed, recent studies found associations between dendritic cell infiltration and maturation and response to PD-1 blockade (*22*, *23*). The PD-1 pathway can modulate T cell responses at two different levels: (i) reducing T cell activation by antigen-presenting cells and (ii) inhibiting target cell elimination (infected cells or tumors) (*24*). Because most target cells do not express B7, it is conceivable that local elimination of target cells may be enhanced by PD-1 blockade in a CD28-independent manner. In contrast, we show that T cell expansion that follows PD-1 therapy requires CD28-costimulation. Efficacy of PD-1 therapy in cancer patients has been associated with proliferation of CD8 TILs (*25*). Therefore, systemic effects of PD-1 blockade that result in clinical benefit most likely require amplification of T cell responses through the proliferation of PD-1$^+$ CD8 T cells and thus CD28-costimulation.

We show a cell-intrinsic requirement for CD28-costimulation in the expansion of PD-1$^+$ CD8 T cells and effectiveness of PD-1 therapy in mouse models of chronic viral infection and cancer. In lung cancer patients, PD-1$^+$ CD8 T cells that proliferate in the peripheral blood after PD-1 blockade express CD28. Our data imply selective proliferation of CD28$^+$ cells through PD-1 therapy and suggest further evaluation of CD28 as a potential biomarker to predict CD8 T cell responses in cancer patients. In addition, Hui *et. al.* show that PD-1 directly targets CD28 cytoplasmic tail, with higher affinity than that of T cell receptor downstream molecules, further emphasizing the interplay between the CD28 and the PD-1 pathway (*26*). Taken together, these data provide greater insight into the molecules and interactions involved in T cell exhaustion and PD-1–directed immunotherapy.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
Figs. S1 to S9
References (*27*–*30*)

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Rescue of exhausted CD8 T cells by PD-1–targeted therapies is CD28-dependent

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CD28 is a critical target for PD-1 blockade

PD-1–targeted therapies have been a breakthrough for treating certain tumors and can rejuvenate T cells to unleash the anticancer immune response (see the Perspective by Clouthier and Ohashi). It is widely believed that PD-1 suppresses signaling through the T cell receptor (TCR). However, Hui *et al.* find instead that the TCR costimulatory receptor, CD28, is the primary target of PD-1 signaling. Independently, Kamphorst *et al.* show that CD28 is required for PD-1 therapies to kill cancer cells efficiently and eliminate chronic viral infections in mice. Lung cancer patients that responded to PD-1 therapy had more $CD28^+$ T cells, which suggests that CD28 may predict treatment response.

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