Published OnlineFirst April 4, 2016; DOI: 10.1158/2326-6066.CIR-15-0250

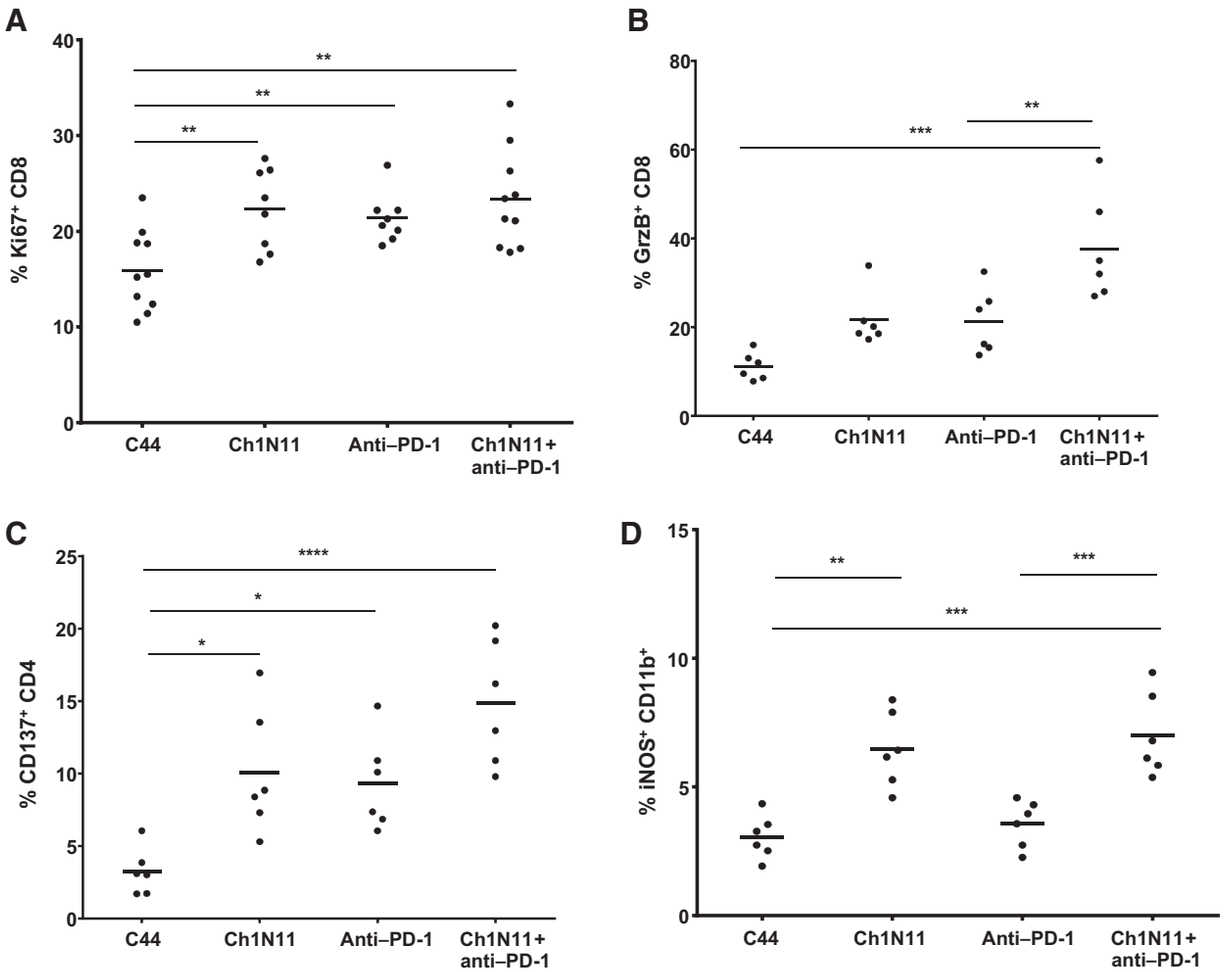

Figure 4.

Activation of TILs in B16 and K1735 melanoma following combination therapy with antibodies targeting PS and PD-1. K1735 tumors from mice treated weekly with a single antibody or a combination of ch1N11 and anti–PD-1 were excised when tumors reached a size of 800 to 1,000 mm^3. TILs were stained for coexpression of CD8 and Ki67 (A), CD8 and granzyme B (B), CD4 and CD137 (C), and CD11b and iNOS (D). Data are expressed as the group mean and individual animal TILs positive for a specific surface marker by FACS analysis. Statistically significant differences between treatment groups were determined by the Student *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; and ****, $P < 0.001$.

Discussion

An underlying challenge to virtually all cytotoxic cancer therapies is therapy-induced exposure of PS that exacerbates immunosuppression in the tumor microenvironment (38, 49, 50). We have found that PS-driven immune suppression can be inhibited by antibody-mediated PS blockade (38, 51). In the present study, we demonstrate that inhibition of upstream PS-mediated immune suppression enhances the efficacy of downstream checkpoint blockade in two murine melanoma models. Blockade of PS combined with anti–CTLA-4 or anti–PD-1 improved tumor control and antitumor immune activation as determined by multiple parameters, including the following: (i) an increase in the activated $CD4^+$ and $CD8^+$ TILs and peripheral immune cells; (ii) a reduction in immunosuppressive intratumoral and peripheral MDSCs and Tregs; and (iii) induction of proinflammatory cytokines IL2, IFNγ, and TNFα from tumor-reactive immune cells. These data are consistent with results from prior studies that found antibody-mediated PS blockade dramatically increased the ratio of M1:M2 macrophages and increased the number of mature dendritic cells in tumors while reducing MDSCs (51). In this study, we have evaluated the effect of PS-targeting antibodies in combination with anti–CTLA-4 and anti–PD-1 in two melanoma models with different sensitivities to therapy. B16 tumors are less immunogenic than K1735 tumors, grow rapidly, and are moderate responders to treatment by immune checkpoint inhibitors (52). In contrast, K1735 tumors are more immunogenic than B16, grow more slowly, and are able to better respond to immune checkpoint blockade. In addition, our observations on the numbers of tumor and peripheral MDSCs and Tregs, and sensitivity to reducing these suppressive cell populations in K1735 versus B16 tumors, may further contribute to the relative responsiveness of these tumors to therapy. In summary, blockade of PS signaling in combination with PD-1 checkpoint inhibition increases the frequency and activation state of $CD8^+$ TILs and enhances the ratio of $CD8^+$ cells to immunosuppressive MDSCs and Tregs.

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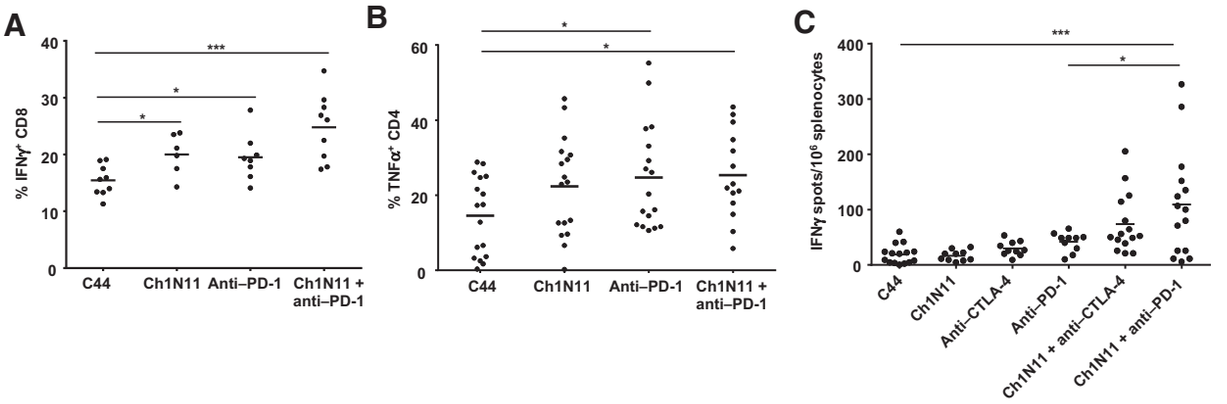

Figure 5.
Cytokine production in splenocytes in B16 and K1735 melanoma following combination therapy with antibodies targeting PS and PD-1. A and B, mice with K1735 tumors were treated weekly with single-agent therapy as indicated, or a combination of ch1N11 and anti–PD-1 and spleens were excised when tumors reached a size of 800 to 1,000 mm^3. Splenocytes from each treatment group were stained for surface CD4, CD8, and CD45 followed by intracellular staining of IFNγ and IL2. C, mice with B16 tumors were treated on days 3, 7, and 10 after tumor implantation with single-agent therapy as indicated or a combination of ch1N11 and anti–PD-1 or anti–CTLA-4, and spleens were excised on day 12. Splenocytes from each treatment group were analyzed for IFNγ secretion by ELISpot. A–C, data are expressed as the group mean and individual animal TILs positive for a specific surface marker by FACS analysis or ELISpot count. Statistically significant differences between treatment groups were determined by the Student *t* test. *, $P < 0.05$ and ***, $P < 0.005$.

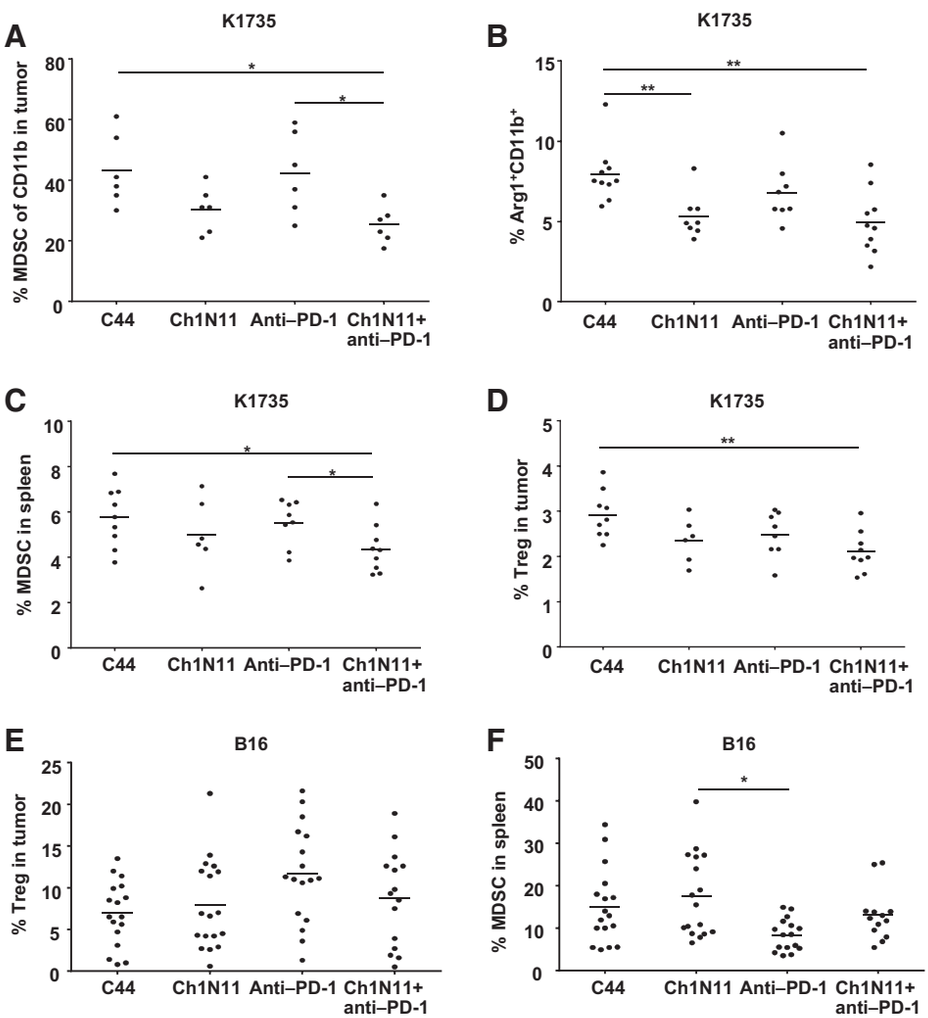

Figure 6.
Reduction in MDSCs and Tregs following combination therapy of antibodies targeting PS and PD-1. Mice with B16 tumors were treated on days 3, 7, and 10 after tumor implantation with a single antibody or a combination of ch1N11 and anti–PD-1 and spleens, and tumors were excised on day 12. K1735 spleens and tumors from mice treated with a single antibody or combinations of antibodies were excised when tumors reached a size of 800 to 1,000 mm^3. Single-cell preparations of tumors were stained with antibodies specific for CD45, MDSC ($CD11b^+$, $GR\text{-}1^+$), Treg ($CD4^+$, $CD25^+$, $FoxP3^+$), and Arg-1. A, summary graph of the percentage of $CD45^+CD11b^+$ in K1735 tumors. B, the percentage of Arg-1^+ $CD11b^+$ in K1735 tumor. C, the percentage of MDSC in spleen in K1735 tumor-bearing mice. D, the percentage of Tregs in spleen of K1735 tumor-bearing mice. E, the percentage of Tregs in B16 tumors. F, the percentage of MDSCs in B16 tumors. Data are presented as results for individual animals and mean per treatment group. Statistically significant differences between treatment groups were determined by the Student *t* test. *, $P < 0.05$ and **, $P < 0.01$.

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The sustained antitumor effect of passive antibody treatment is mediated by specific targeting and/or blocking of suppressive ligands and the interaction of Fc-receptors on myeloid effector cells. Data from previous studies indicate that the Fc portion of PS-targeting antibodies contributes to differentiation of mature dendritic cells because the $F(ab')_2$ fragment alone has lower potential to differentiate splenic monocytic MDSCs from macrophages and dendritic cells (51). Studies show that antibodies to CD20 ligate FcγRIIA receptors on $CD11c^+$ dendritic cells generate a vaccinal effect by stimulating long-term cellular immune responses (53). Thus, the induction of IFNγ secretion from TILs and peripheral immune cells of tumor-bearing animals treated with ch1N11 and anti–PD-1 antibodies may be due to cross-presentation of tumor antigens via Fc receptors on dendritic cells or macrophages. The combination of ch1N11 and anti–CTLA-4 or anti–PD-1 in the B16 tumor model significantly increased the frequency of IFNγ-producing splenocytes compared with single-agent therapy. In contrast, few IFNγ-producing splenocytes were observed from naïve tumor-free animals dosed with the same antibody combinations, suggesting that tumor antigens were required for stimulation (see Supplementary Fig. S3). The activated immune response bias after PS therapy is likely the result of several mechanisms, including the following: (i) opsonization of tumor-derived, PS-expressing microvesicles or cell fragments and subsequent activation of Fc receptors on splenic macrophages; (ii) blockade of immunosuppressive signaling mediated by TIM (especially TIM-3) and TAM PS receptors on immune cells; and (iii) enhanced cross-presentation of tumor antigens by mature dendritic cells due to proinflammatory cytokine upregulation of MHC class II and costimulatory molecules CD80 and CD86.

In summary, we have shown that PS-targeting antibodies improve the efficacy of anti–CTLA-4 or anti–PD-1 therapy in murine models of melanoma, suggesting that these combinations have the potential to improve outcome in patients with advanced-stage melanoma. Bavituximab, a chimeric PS-targeting antibody, is currently being evaluated in late-stage clinical trials for the treatment of cancer patients with solid tumors (46, 54), and strong antitumor activity has been demonstrated in melanoma clinical trials using checkpoint inhibitor antibodies targeting CTLA-4 (10), PD-1 (12, 13), and PD-L1 (55). It is increasingly apparent that successful immunotherapy requires tumor cell killing, induction of proinflammatory immune responses, and concomitant reduction of immunosuppressive signals leading to increased tumor infiltration by activated T cells. Based on the distinct mechanism of action and multiple points of blockade, PS-targeting antibodies such as bavituximab may enhance the antitumor responses of immune checkpoint inhibitors by further blocking suppressive signals in the tumor microenvironment.

Disclosure of Potential Conflicts of Interest

C.C.W. Hughes is consultant at, and is a consultant/advisory board member for, Peregrine Pharmaceuticals. R.A. Brekken reports receiving commercial research support, has ownership interest (including patents), and is a consultant/advisory board member for Peregrine Pharmaceuticals. X. Huang reports receiving commercial research support from Peregrine Pharmaceuticals, for which he also serves as a consultant/advisory board member. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.D. Freimark, J. Gong, V. Nguyen, S. Yin, M.M.S. Hatch, C.C.W. Hughes, X. Huang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.D. Freimark, J. Gong, M.J. Gray, V. Nguyen, S. Yin, C.C.W. Hughes, A.J. Schroit, R.A. Brekken, X. Huang

Writing, review, and/or revision of the manuscript: B.D. Freimark, J. Gong, C.C.W. Hughes, A.J. Schroit, J.T. Hutchins, R.A. Brekken, X. Huang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Ye, R.A. Brekken

Study supervision: B.D. Freimark, J. Gong, J.T. Hutchins, R.A. Brekken, X. Huang

Acknowledgments

The authors thank Jason Toombs and Janie Iglehart for technical assistance.

Grant Support

This work was supported in part by a sponsored research agreement from Peregrine Pharmaceuticals Inc. (to R.A. Brekken).

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Received October 2, 2015; revised December 29, 2015; accepted January 20, 2016; published OnlineFirst April 4, 2016.

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Cancer Immunology Research

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Cancer Immunol Res 2016;4:531-540. Published OnlineFirst April 4, 2016.