FcγRs Modulate the Anti-tumor Activity of Antibodies Targeting the PD-1/PD-L1 Axis

Graphical Abstract

Highlights

- Anti-PD-1/PD-L1 Abs differ in their FcγRs requirements for optimal activity
- The activities of anti-PD-1 Abs are FcγR independent
- Engagement of activating FcγRs by anti-PD-L1 Abs augments the in vivo activity
- Anti-PD-L1, if bound to FcγR, alters myeloid subset composition within the TME

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In Brief
Dahan et al. report that Fcγ receptor engagement augments the anti-tumor activity of anti-PD-L1 antibodies (Abs) but compromises the anti-tumor activity of anti-PD-1 Abs. These findings provide rationale for Fc engineering of these Abs to optimize anti-tumor efficacy.
FcγRs Modulate the Anti-tumor Activity of Antibodies Targeting the PD-1/PD-L1 Axis

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SUMMARY

Immune checkpoint blockade of the programmed cell death protein 1 (PD-1) pathway by monoclonal antibodies (Abs) has shown promising clinical benefit in the treatment of multiple cancer types. We elucidated the contribution of the fragment crystallizable (Fc) domains of anti-PD-1 and anti-PD-ligand 1 (L1) Abs for their optimal anti-tumor activity. We revealed that distinct Fcγ receptor (FcγRs) dependency and mechanisms account for the in vivo activity of anti-PD-1 versus anti-PD-L1 Abs. Anti-PD-1 Abs were found to be FcγR independent in vivo; the presence of FcγR-binding capacity compromises their anti-tumor activity. In contrast, the anti-PD-L1 Abs show augmented anti-tumor activity when activating FcγR binding is introduced into the molecules, altering myeloid subsets within the tumor microenvironment.

INTRODUCTION

The programmed cell death protein 1 (PD-1)/PD-ligand 1 (L1) axis has a central role in the suppression of anti-tumor immunity and has been actively studied as a potential therapeutic target in cancer immunotherapy (Page et al., 2014). The immune checkpoint receptor PD-1 is expressed mainly on activated lymphocytes (Nishimura et al., 1999) and is widely upregulated by tumor-infiltrated T lymphocytes (TILs) (Ahmadzadeh et al., 2009; Chapon et al., 2011). Many tumor types exploit the PD-1 pathway to escape immune surveillance by upregulating the expression of PD-L1 (Dong et al., 2002; Konishi et al., 2004). Enhanced anti-tumor immunity is obtained by antibody (Ab)-mediated blockade of the PD-1/PD-L1 axis. The therapeutic potential of both anti-PD-1 and -PD-L1 Abs were demonstrated in multiple mouse models of cancer (Blank et al., 2004; Dong et al., 2002; Iwai et al., 2002) and in human clinical trials (Brahmer et al., 2012; Hamid et al., 2013; Topalian et al., 2012, 2014; Wolchok et al., 2013). Recently, the anti-PD-1 Abs pembrolizumab and nivolumab were approved by the US Food and Drug Administration (FDA) for the treatment of metastatic melanoma and non-small cell lung carcinoma, whereas other antibodies targeting this pathway are in advanced stages of clinical development.

The interaction of the Fc portion of many therapeutic anti-tumor immunoglobulin Gs (IgGs) with Fcγ receptors has been found to be crucial for their therapeutic activities, resulting from the induction of tumor cytotoxicity (Clynes et al., 2000). These activities are induced through Ab-dependent cellular cytotoxicity (ADCC) by type I FcγR-expressing effector cells, such as natural killer cells and macrophages (Musolino et al., 2008; Nimmerjahn and Ravetch, 2012). There are two classes of type I FcγRs that can be distinguished functionally: the activating receptors (FcγRI, FcγRIII, and FcγRIV in mice; FcγRIA, FcγRIIA, and FcγRIIIA in humans) and the inhibitory receptor (FcγRIIB in both mice and humans). Effector responses mediated by different IgG subclasses are dependent on their differential affinities for activating or inhibitory FcγRs. A ratio of activating-to-inhibitory receptor binding (A/I) can therefore predict the in vivo cytotoxic activity of IgGs (Nimmerjahn and Ravetch, 2005). Optimizing the effector function induced by the Fc portion of therapeutic IgGs by increasing their A/I ratio has, as a consequence, resulted in enhanced therapeutic responses (Goede et al., 2014; Mössner et al., 2010).

Significance

Therapeutic Abs targeting the PD-1/PD-L1 immune checkpoint pathway are drugs recently approved as monotherapies for advanced melanoma and are currently being evaluated for the treatment of various human malignancies. However, the contribution of the Fc domain to the activity of these Abs has not been critically evaluated. Because Fc variants are being used for anti-PD1/PD-L1 Abs under clinical development, a detailed understanding of Fc-mediated effector activities that may contribute to their therapeutic efficacy is necessary. Here we report that antibodies to PD-1 and PD-L1 have different requirements for FcγR engagement for optimal anti-tumor activity. This study provides a rationale for Fc engineering of these immunomodulatory Abs for optimal clinical efficacy.
In contrast to the cytotoxic class of anti-tumor Abs described above, immunomodulatory Abs targeting molecules expressed by immune cells have emerged as a promising approach to induce therapeutic anti-tumor response. Immunomodulatory Abs can act as either agonists to stimulate anti-tumor immune responses or antagonists to dampen regulatory mechanisms (generally referred to as immune checkpoint blocking Abs; Pardoll, 2012). Initially, these classes of immunomodulatory Abs were designed with the target antigen in mind, with emphasis placed on identifying ideal fragment antigen-binding (Fab) regions of Abs to engage the target appropriately. However, recent findings highlight the importance of Fc-Fc receptor interactions for the in vivo activities mediated by immunomodulatory Abs. Antagonistic anti-CTLA-4 Abs have been shown to mediate their in vivo effects, in part, by interacting with activating FcγRs overexpressed by intratumoral Tregs, such as GITR and OX40 (Bulliard et al., 2013, 2014). In this study, we investigate the role of Fc-FcγR interactions for the immunomodulatory Abs targeting the PD-1/PD-L1 pathway for their therapeutic anti-tumor activities.

RESULTS

FcyγR Engagement by Anti-PD-1 or -PD-L1 Abs Results in Distinct Anti-tumor Responses In Vivo

To determine the contribution of Fc-FcγR interactions to the in vivo anti-tumor activities mediated by rat anti-mouse anti-PD-1 and anti-PD-L1 Abs, we modified existing rat Ab clones to create chimeric antibodies containing rat Fab and mouse Fc domains. The mouse Fc domains were selected to vary the selective engagement of mouse FcγRs, with IgG2a binding preferentially to activating FcγRs and IgG1 binding preferentially to inhibitory FcγRIIB (A/I = 0.1) and a null variant the IgG1-D265A Fc that lacks detectable FcγR binding (Nimmerjahn and Ravetch, 2005). We verified that the different Fc domains introduced into the chimeric PD-1/PD-L1 targeting Abs did not alter either the binding specificity or the affinity of their variable domains to PD-L1 (clone 14D8) and PD-1 (clones 4H2 and RMP1-14) (Figure S1A), nor their pharmacokinetic (PK) properties (Figure S1B). While there are no changes in the PK and antigen-binding properties between the different subclasses of each Ab clone, their differential capacity to engage mouse FcγRs provides a means to explore the contributions of FcγR engagement to their in vivo activity.

We compared the anti-tumor activity of the different subclasses of chimeric anti-PD-L1 Ab (clone 14D8) administered as a monotherapy to mice bearing MC38 colon adenocarcinomas. Anti-PD-L1 Ab exhibited significant reduction in tumor volume only when administered as IgG2a isotype (Figure 1A). The respective isotype controls were found to have no effect on tumor growth, eliminating the possibility of Fc-mediated effect not related to its cognate Fab domain (Figure S1C). A similar
trend was observed when these constructs were tested in the B16 melanoma model in a combinatorial treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-transfected B16 tumor cell vaccine (GVAX) (Figure 1B). Because similar results were obtained in both tumor models, for consistency in the following experiments we used anti-PD-1/L1 as monotherapies in the MC38 tumor model unless otherwise noted. The enhanced activity of IgG2a compared to the IgG1 and IgG1-D265A subclasses of this Ab indicated that engagement of activating FcγRs by this anti-PD-L1 Ab is required for its optimal anti-tumor activity.

In contrast, a different trend was observed when we evaluated the anti-tumor activity of chimeric anti-PD-1 Abs with the different mouse Fc domains described above. The IgG1-D265A Fc variant of the two different chimeric anti-PD-1 Ab exhibited superior efficacy for the treatment of MC38 tumors (Figures 1C and 1D). The optimal activity of anti-PD-1 chimeric antibodies with an IgG1-D265A Fc indicated that FcγR engagement was not required for the optimal activity of anti-PD-1 Abs. Furthermore, when FcγR-binding capabilities were introduced into these chimeric anti-PD-1 Abs we observed reduced anti-tumor activity. Although both of the anti-PD-1 mAbs that we tested had greater activity in the absence of FcγRs engagement, these chimeric mAbs were distinguished by the specific FcγR pathways that interfered with their optimal activity. The IgG2a Fc subclass of chimeric anti-PD-1 clone 4H2 did not result in significant anti-tumor responses, as compared to the activity seen for this antibody when delivered as a chimera with either mouse IgG1 or IgG1-D265A, indicating that the reduced potency of this Ab clone resulted from the engagement of activating FcγRs (Figure 1C). However, another rat anti-PD-1 Ab clone, RMP1-14, which recognizes an epitope distinct from 4H2 (Figure S1D), exhibited diminished activity when administered as a chimera antibody with either an IgG2a or IgG1 Fc (Figure 1D). Thus, distinct FcγR-dependent mechanisms (e.g., engagement of activating versus inhibitory FcγRs) may account for reduced anti-tumor activity of anti-PD-1 Abs. Collectively, our data indicate that although anti-PD-1 and -PD-L1 Abs were rationally designed to block the PD-1/PD-L1 axis, distinct FcγR requirements contribute to their activity, and therefore differentiate their in vivo mechanisms of action.

**Engagement of Activating FcγRs Enhances the Anti-tumor Activity of an Anti-PD-L1 Antibody and Correlates with Modulation of Myeloid Cell Subsets**

To characterize the mechanistic basis for the activating FcγR requirement for the anti-tumor activity of anti-PD-L1 Ab, we compared the activity of an anti-PD-L1 antibody Ab (clone 10F.9G2) in wild-type and mice that lack all activating FcγRs but retain the expression of inhibitory FcγRIIb (FcεR1) (Clynes et al., 1998; Takai et al., 1994). 10F.9G2 is a rat IgG2b isotype that interacts with all mouse FcγRs and has a relatively high A/I ratio of 40 (Figure 2A). Treatment of MC38 tumors with the 10F.9G2 anti-PD-L1 rat IgG2b clone as a monotherapy significantly reduced the tumor volume in wild-type mice. However, this therapeutic effect was attenuated in Fcer1g−/− mice (Figure 2B). Similarity, a dependency on activating FcγR engagement for anti-tumor activity of this anti-PD-L1 clone was also observed in the B16 melanoma models (Figure S2A). Thus, blocking of the PD-1/PD-L1 interaction by anti-PD-L1 clone 10F.9G2 was not sufficient to induce its anti-tumor therapeutic activity. Instead, an activating FcγR-dependent mechanism was required for optimal in vivo activity, confirming the observations in Figure 1A.

To determine if the levels of PD-L1 expression in different splenic and tumor-infiltrating myeloid populations from mice bearing MC38 tumors could account for this activating FcγR requirement, we characterized myeloid cells in the TME for PD-L1 expression. CD11b+F4/80−, CD11b+Gr-1−, and CD11b+CD11c+ exhibited elevated PD-L1 expression levels in the TME, as compared to the spleen (Figure 2C). PD-L1 expression was slightly increased on the MC38 tumor cells, but at significantly lower levels when compared to the infiltrating immune cells. These differences in PD-L1 density could result in selective depletion of TME myeloid cells. We evaluated the effect of anti-PD-L1 clone 10F.9G2 on the percentages of these myeloid populations in MC38-treated mice (Figure 2D). Anti-PD-L1 treatment of wild-type mice resulted in the overall reduction in the percentages of tumor-infiltrating CD11b+ cells, with significant reduction observed for CD11b+F4/80−, CD11b+Gr-1−, and CD11b+CD11c+ cells. The decrease of CD11b+Gr-1− cells is attributed to myeloid derived suppressor cells (MDSC) and not neutrophils (CD11b+Ly6G−Ly6Chi/MHC II−F4/80−), whose presence was not affected by the treatment (Figure S2B). In contrast, CD8+ TILs percentages were increased (Figure S2B). This decrease in the myeloid cell populations was lost in FcγRnull mice only for CD11b+F4/80+, but not for the other populations tested, indicating that both FcγR-dependent and independent mechanisms are responsible for reduction of TME myeloid populations. In contrast, there was no change observed in the different myeloid cell populations in the spleen from these anti-PD-L1 treated tumor-bearing mice. To determine whether activating FcγR engagement by the anti-PD-L1 antibody is responsible for the attenuation of CD11b+F4/80+, we compared the percentages of these cells within the overall infiltrated immune cells after treatment of MC38 tumor-bearing mice with anti-PD-L1 10F.9G2 in wild-type and Fcer1g−/− mice (Figure 2E). The reduced percentages of CD11b+F4/80+ observed in wild-type mice was abrogated in Fcer1g−/−, indicating a role for activating FcγRs in mediating the depletion of these immunosuppressive cells and thereby contributing to the enhanced anti-tumor activity observed for this anti-PD-L1 antibody when it engages activating FcγRs. Of clinical importance, the increase in CD3+ TILs observed after anti-PD-L1 treatment is not affected by engagement of activating FcγRs (Figure S2C).

We compared the effect of IgG2a and IgG1-D265A subclasses for another anti-PD-L1 Ab clone, 14D8, on the overall percentages and absolute numbers of myeloid and lymphocyte subpopulations in the TME. Treatment with the IgG2a subclass of 14D8 Ab decreases the percentages (Figure 2F) and absolute number (Figure S2D) of monocytes, but increases the presence of immature myeloid cells. These changes were not observed in the TME of mice treated with the Fc null (D265A mutant) subclass. Other myeloid cells including macrophages, dendritic cells, and neutrophils were not altered by either subclass (Figures 2F and S2D). An increase of CD8 TILs is observed at similar levels after treatment with both subclasses (Figure S2E), indicating that the
A therapeutic increase in CD8 TILs is mediated by the Fab domain and is not affected by the Fc domain of 14D8 Ab. However, when activating FcγRs are engaged by this Ab, distinct mechanisms lead to modulation of the myeloid cell composition in the TME. The combination of both FcγR engagement and PD-1/PD-L1 blocking by PD-L1 Abs thus results in optimized treatment efficacy.

Activating FcγR Engagement Results in Diminished Anti-tumor Response for Anti-PD-1 Abs
In contrast to the results shown above for an anti-PD-L1 Ab, anti-PD-1 Abs demonstrated reduced anti-tumor activity when their Fc domain was able to engage FcγRs. To characterize the involvement of FcγRs in the diminished activity of the chimeric anti-PD-1 IgG2a Fc (clone 4H2), we compared the activity of

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Figure 2. Activating FcγR Engagement Enhances Anti-PD-L1 Activity through Alteration of Myeloid Subset Percentages
(A) Relative mouse Fcγ-R-binding profile for rat IgG2b. Binding affinities based on SPR analysis. A/I were calculated as the ratios of KD values of FcγRIIb and FcγRIIa.
(B) Wild-type and Fcer1g−/− mice with established MC38 tumors were treated with rat IgG2a anti-PD-L1 clone 10F.9G2 or isotype control. Data are represented as mean ± SEM (n = 6). One of two experiments is shown.
(C) PD-L1 expression pattern of the indicated cell populations from spleens and tumors from MC38-bearing mice. Representative data from two experiments conducted with five mice.
(D) Wild-type and FcγRα−/− mice with established MC38 tumors were treated with anti-PD-L1 clone 10F.9G2 or isotype control. Data are represented as mean ± SEM of two experiments that were pooled (n > 10).
(E) Tumors from mice with genotypes and treatments as described in (B) were harvested and analyzed for the percentages of CD11b+ F4/80+ cells. Dots are individual mice. Bars represent mean ± SEM.
(F) Mice with established MC38 tumors were treated with the indicated chimeric versions of anti-PD-L1 clone 14D8 or mIgG1 isotype control. Tumors were harvested and analyzed for the percentages of monocytes (CD11b+ MHCII− Ly6C+ F4/80+ CD11c− Ly6G−), macrophages (CD11b+ MHC II+ F4/80+ CD11c+ Ly6C+ Ly6G−), and immature myeloid cells (CD11b+ MHC II+ F4/80+ Ly6C− Ly6G−). Dots are individual mice. Bars represent mean ± SEM.

See also Figure S2.
this chimeric antibody in wild-type mice, mice that lack all activating FcγRs (Fcer1g−/−), and mice deficient in the inhibitory FcγRIlb (Fcgr2b−/−) (Figure 3A). No significant difference in anti-tumor activity was observed in wild-type or Fcgr2b−/− mice. However, significantly higher reduction in tumor volume was observed in Fcer1g−/− mice, indicating the dominant role of activating FcγRs for diminished activity of 4H2 anti-PD-1 IgG2a. This is consistent with the preferential binding of this mouse Fc subclass to activating FcγRs and its reduced anti-tumor activity compared to Fc subclass with reduced or absence FcγR binding described above. This role of FcγRs was further supported by evaluating the activity of chimeric 4H2 anti-PD-1 IgG2a subclass in FcγRαnull mice lacking mouse FcγR α chain-encoding genes (Fcgr1, Fcgr2b, Fcgr3, and Fcgr4) (Smith et al., 2012); significant anti-tumor response of this chimeric antibody was observed in these mice, similar to that seen Fcer1g−/− mice (Figure S3A). The mouse IgG2a subclass binds to the activating Fc receptors FcγRI, III, and IV. We sought to determine the contribution of these different activating FcγRs to the diminished anti-tumor response. Similar to Fcer1g−/− mice, improved activity of the chimeric anti-PD-1 IgG2a Fc antibody was observed in Fcgr1−/− mice (Figure 3B). This effect was unique to Fcgr1−/− mice and was not observed in mice with deficiency in other activating FcγRs, Fcgr3−/−, or Fcgr4−/− (Figure S3B), indicating that FcγRI is necessary and sufficient for the reduced efficacy of anti-PD-1 treatment of this chimeric anti-PD-1 Ab/Fc configuration.
Engagement of Activating FcγRs by Anti-PD-1 Eliminates Activated Intratumoral CD8 T Cells

The involvement of the activating FcγRI in the decreased efficacy of the chimeric 4H2 anti-PD-1 IgG2aFc Ab suggested that ADCC-mediated depletion of PD-1-expressing cell populations by this Ab correlated with its diminished anti-tumor activity. To identify potential targets for ADCC we determined PD-1 expression levels on T cell subsets in the TME and the periphery (Figure 3C). PD-1 expression levels on both effector CD8+ and CD4+Foxp3+ T cells and regulatory CD4+Foxp3+ T cells were relatively low in the spleen but upregulated within the TME. CD8+ and CD4+ effector TILs were found to have greater PD-1 expression than intratumoral regulatory T cells. Within the effector subsets in the TME, CD8+ cells tended to have higher levels of surface PD-1 as compared to effector CD4+ and have a higher percentage of PD-1+ cells. Similar PD-1 expression patterns were observed in the B16 melanoma microenvironment (data not shown).

Because PD-1 is highly expressed on CD8 TILs and anti-PD-1 treatment is correlated with increased T-cell-mediated anti-tumor responses, we sought to determine whether CD8 TILs are targeted through an ADCC mechanism by anti-PD-1 Ab, thereby contributing to its reduced efficacy. First, we tested how the presence of CD8+ TILs was affected by treatment with the different anti-PD-1 mouse IgG subclasses (Figure S3C). The relative percentages of CD8+ TILs were found to be correlated with the binding affinities to activating FcγRs by the anti-PD-1 mouse IgG subclass used for treatment. 4H2 anti-PD-1 IgG1 and IgG1-D265A treatments resulted in a therapeutic increase of CD8+ TILs, whereas the IgG2a treated group exhibited a significant reduction of these cells. Alternation in CD8 T cell percentages was specific to the tumor site and did not occur in splenic T cells, consistent with their lower PD-1 expression. Moreover, when we tested the effect of the anti-PD-1 mouse Fc subclasses on the relative proportion of the PD-1 expressing CD8+ TILs we observed direct correlation between the A/I ratio of the IgG subclass and the percentages of PD-1-positive target CD8 TILs (Figure S3D). Because PD-1 is upregulated by activated T lymphocytes, we observed similar correlation between the IgG A/I ratio and the percentages of activated CD8 TILs (Figure S3E). Thus, FcγRI mediates the elimination of intratumoral activated CD8+PD-1+ by anti-PD-1 IgGs. Such increased elimination of target cells by IgG2a was consistent with previous observations in a variety of in vivo experimental systems (Bulliard et al., 2013; Clynes et al., 1998; Selby et al., 2013; Simpson et al., 2013).

We wished to elucidate the contribution of activating FcγRs for the attenuation in intratumoral T cell populations. Because of the improved activity of 4H2 anti-PD-1 IgG2a in FcεRIg−/− and FcγRIIg−/− mice we evaluated the effect of anti-PD-1 treatment on different T cell subsets in these mice (Figure 3D). While the chimeric 4H2 anti-PD-1 IgG2aFc antibody resulted in 48% reduction in the percentages of CD8+ TILs in wild-type mice, their relative presence within the overall immune cells in the TME after treatment was increased by 4.5- and 2.6-fold in FcεRIg−/− and FcγRIIg−/− mice, respectively. This confirmed a role for activating FcγRs in anti-PD-1-mediated CD8+ elimination from the tumor site, and therefore in the abrogation of their activation and proliferation needed for effective immunotherapy. The effect of activating FcγRs was specific to intratumoral CD8+ cells since the percentages of effector and regulatory CD4 T cells were not altered by this chimeric anti-PD-1 antibody. Although a slight reduction in the percentages of effector CD4 T cells was observed in tumors from FcεRIg−/− and FcγRIIg−/− mice compared to untreated mice, this trend was not statistically significant when compared to anti-PD-1-treated wild-type mice or when total numbers of CD4 T cells per tumor mass were evaluated (Figure S3F). No changes in the splenic T cell subset populations were observed after anti-PD-1 treatment. Thus, the elevated PD-1 expression levels on CD8+ TILs were consistent with the CD8+ specific elimination observed by 4H2 anti-PD-1-IgG2a in the TME. Collectively, our data support the conclusion that in the absence of FcγRI engagement, anti-PD-1 treatment leads to increased CD8+ T cell percentages within the TME, presumably by blocking their PD-1 inhibitory signal. FcγRI engagement by anti-PD-1 targets CD8 TILs by ADCC and therefore reduces their percentages. This decrease of CD8+ TILs correlates with the poor anti-tumor activity induced by 4H2 anti-PD-1 IgG2a.

Decreased Efficacy of Anti-PD-1 Treatment by Engagement of FcγRIIB

In contrast to the chimeric anti-PD-1 antibody derived from clone 4H2 with a mouse IgG1Fc, the chimeric anti-PD-1 clone RMP1-14 with a mouse IgG1Fc had significantly diminished anti-tumor activity (Figure 1), implying that engagement of the FcγRIIB pathway may limit the optimal activity of this Ab clone. The anti-PD-1 Ab produced by the hybridoma clone RMP1-14 is a rat IgG2a subclass (Yamazaki et al., 2005), and had shown anti-tumor activity in the treatment of variety murine tumor models (Curran et al., 2010; Lu et al., 2014; Peng et al., 2012). ELISA+ (Figure S4A) and surface plasmon resonance (SPR)- (Figure 4A) based analysis of the rat IgG2a binding profile to mouse FcγRs revealed that this subclass bound only the low-affinity mouse FcγRIIB and FcγRIII with K D of 6.1 × 10−6 and 2.3 × 10−5, respectively. (In contrast, the mouse IgG2a subclass binds mouse high-affinity FcγRI and IV preferentially). We therefore assessed the anti-tumor activity of this rat antibody in wild-type, FcγRIIB−/−, and FcγRIIB−/− mice. Although treatment of wild-type mice with rat anti-PD-1 RMP1-14 clone (rat IgG2a) resulted in reduction in MC38 tumor volume, significant improvement in the anti-tumor response was achieved in FcγRIIB−/− mice (Figure 4B). In contrast, FcγRIIB deficiency did not affect the therapeutic efficacy of this rat anti-PD-1 clone (Figure S4B), implying FcγRIIB-mediated diminished efficacy of RMP1-14 anti-PD-1. This is consistent with the diminished activity observed for chimeric RMP1-14 anti-PD-1 mouse IgG1Fc, which preferentially engages FcγRIIB, compared to the Fc null IgG1-D265A subclass. Next, we analyzed the effect of FcγRIIB engagement on the expansion of intratumoral CD8 T cells during RMP1-14 anti-PD-1 treatment (Figure 4C). The enhanced effect of RMP1-14 anti-PD-1 (rat IgG2a) treatment in FcγRIIB−/− resulted in complete tumor rejection in 60% of the treated mice (compared to 0% in wild-type mice) at day 7 after treatment onset. We therefore analyzed tumors from mice at earlier time points than in the previous experiments and prior to the completion of our Ab treatment protocol. Similar percentages of CD8 TILs were observed in tumors from control and...
RMP1-14 anti-PD-1 (rat IgG2a)-treated wild-type mice at day 4 (after two Ab injections), while in Fcgr2b−/− mice with established MC38 tumors were treated with anti-PD-1 clone RMP1-14 or isotype control. Data are represented as mean ± SEM (n > 8).

Several humanized anti-PD-1 Abs, including nivolumab and pembrolizumab, are of the human IgG4 subclass, a subclass that has relatively low binding affinities to human FcγRs. Rationally designed as blocking reagents, these Abs were developed as human IgG4 to avoid Fc- mediated cytotoxic effects on T cells. Compared to human IgG1 and IgG3, IgG4 had significantly reduced binding affinity to human activating FcγRs, but retain binding to the human inhibitory FcγRIIB receptor, resulting in a relative low A/I ratio of 1 (Bruhns et al., 2009) (Figure 4A). We therefore sought to test whether administration of a chimeric rat anti-PD-1 RMP1-14 clone with a human IgG4 Fc would alter its activity compared to the chimeric antibody with the FcγRIIb null binding human IgG1-N297A Fc. We generated two human IgG Fc variants of the rat anti-mouse PD-1 clone RMP1-14: either with a human IgG4 Fc immunoglobulin with a stabilizing S228P mutation (Lewis et al., 2009) or with the human IgG1-N297A FcγRI null binding variant (Figure 4A). Because murine systems are not suitable to study human IgG Fc effector functions in vivo, we used a mouse model we developed recently in which the mouse FcγRs had been deleted and all the human FcγRs were expressed as transgenes, faithfully recapitulating the human-specific FcγR expression pattern and diversity (Smith et al., 2012). We compared the anti-tumor response of these two chimeric versions of anti-PD-1 clone RMP1-14 with human Fc in humanized FcγRs mice bearing MC38 tumors (Figure 4D). Treatments with both chimeras of anti-PD-1 containing human IgG4 Fcs, resulted in a similar significant tumor growth inhibition and in a total of 70% (16 of 23) and 63% (15 of 24) tumor-free mice, respectively. Thus, in contrast to our observation for the mouse IgG1 Fc subclass, adding FcγRIIB-binding capabilities in the form of human IgG4 Fc does not alter the anti-tumor activity of this anti-PD-1 clone.
DISCUSSION

The emergence of immunomodulatory antibodies for the treatment of neoplastic diseases is a milestone in therapeutic approaches to cancer. It has become apparent, however, that the in vivo activity of these antibodies result from both Fab- and Fc-mediated pathways (Bulliard et al., 2014; Li and Ravetch, 2011; White et al., 2011). We now report on the evaluation of the role of Fc-FcyR interactions in the activity of the Abs targeting the PD-1 immune checkpoint pathway. Optimal anti-tumor activity of Abs targeting PD-1 was achieved by blocking of the inhibitory PD-1 signal in the absence of Fc-FcyR engagement; FcyR engagement by anti-PD-1 Abs resulted in diminished in vivo activity. Although this negative effect of FcyR engagement was observed for two distinct antibodies recognizing distinct epitopes on PD-1, the mechanism by which FcyR engagement resulted in reduced efficacy differed. The 4H2 anti-PD-1 antibody engagement of activating FcyR resulted in the elimination of effector CD8+ in the TME by ADCC. However, for another anti-PD-1 Ab recognizing a different epitope on PD-1 (clone RMP1-14), engagement of the inhibitory FcyR resulted in diminished in vivo anti-tumor activity, implicating that epitope-specific mechanisms governed the impact of FcyR engagement by this class of immune checkpoint Abs. In contrast to anti-PD-1 Abs, blocking of this axis by disrupting anti-PD-L1 binding by anti-PD-L1 was enhanced by engagement of activating FcyRs. This effect was correlated to elimination of monocytes and modulation of myeloid cells within the TME by anti-PD-L1.

We identified FcyRI as the Fc receptor that was responsible for triggering the elimination of CD8 TILs by the anti-PD-1/IgG2a. This dependence on FcyRI was unexpected considering previous observations in multiple experimental settings, indicating that although FcyRI is capable of binding IgG2a with high affinity, it does not contribute to the in vivo activity of this subclass of IgGs, presumably as a consequence of the saturation of the receptor by circulating IgG. FcyRIV, an intermediate affinity activating FcyR, is the primary activating FcyR that mediates ADCC by the IgG2a subclass (DiLillo and Ravetch, 2015; Hamaguchi et al., 2006; Nimmerjahn et al., 2010). For example, in the B16 melanoma model, direct killing of tumor cells by the anti-gp75 Ab TA99 and elimination of intratumoral Tregs by anti-CTLA4 were mediated through FcyRIV (Nimmerjahn et al., 2010; Simpson et al., 2013). Similar observations had been made in a variety of solid and liquid tumor models, supporting the conclusion that FcyRI, by virtue of its nanomolar affinity for IgG2a, insures its saturation by normal serum concentrations of IgG2a and is unavailable to be recruited by therapeutic Abs. The finding that in the MC38 tumor model mouse FcyRI was responsible for depletion of CD8 effector cells by an IgG2a subclass Fc suggests that the TME may modulate FcyRs differentially than observed on circulating or tissue macrophages.

Two distinct FcyR-mediated mechanisms were recently described for enhancing the potency of immunomodulatory Abs: the engagement of activating FcyR for ADCC-mediated depletion of intratumoral regulatory lymphocytes that overexpress the target antigen (e.g., CTLA-4,OX-40, and GITR), or the engagement of the inhibitory FcyRIIb to provide a scaffold for Ab cross-linking, and therefore enhance the agonistic activity of Abs targeting TNFR family members such as anti-CD40 and DR5. We observed that anti-PD-1 Abs may engage either of these two distinct FcyR-dependent mechanisms to reduce the potency of this class of antibodies, depending on both their Fc and Fab domains. When engaged, both pathways diminished the therapeutic potential of these immunomodulatory Abs. Thus, for the anti-PD-1 clone, RMP1-14, engagement of both activating (when administered as IgG2a) and inhibitory FcyRs (when administered as IgG1) resulted in reduced activity when compared to an FcyR null variant. The reduced activity upon engagement of activating FcyR was shared by the other anti-PD-1 clone tested. However, the FcyRIIb pathway was unique to this clone. We speculate that FcyRIIb engagement by clone RMP1-14 may contribute partial agonistic activity to this clone and therefore deliver augmented inhibitory PD-1 signals that may interfere with its therapeutic PD-1 blocking activity. Alternatively, engagement of FcyRIIb may directly influence FcyRIIb-expressing cells in the tumor microenvironment by altering their activation state and phenotype, thus suppressing T cells in a PD-1 independent manner. Studies elucidating the mechanistic basis for the FcyRIIb-mediated reduced efficacy by anti-PD-1 are undergoing.

CTLA-4 and PD-1 are the only targets for immune checkpoint blocking Abs approved to date by the FDA for the treatment of cancer. The observation that anti-CTLA-4 Abs mediate their anti-tumor activity, in part, through depletion of intratumoral Tregs (Bulliard et al., 2013; Romano et al., 2014; Selby et al., 2013; Simpson et al., 2013) highlights the potential role of FcyRs in the activity of immune checkpoint blocking Abs. However, we observed that not only are anti-PD-1 Abs FcyR independent but also that FcyR engagement reduced their therapeutic potency. Whereas Abs targeting both CTLA-4 and PD-1 aim to mediate therapeutic increases in the intratumoral Teff/Treg ratio, the distinct expression pattern of these immune receptors distinguishes the FcyR requirement and in vivo mechanism of these blocking Abs. CTLA-4 expression density is higher on the regulatory TIL compartment, whereas PD-1 is relatively overexpressed on effector TILs. Therefore, preferential depletion of regulatory versus effector T cells by anti-CTLA-4 or PD-1, respectively, results in correspondingly enhanced or diminished Teff/Treg ratios. With the understanding that many of the immunomodulatory Abs now being tested as therapies will mediate their in vivo effects through different mechanisms depending on their target molecule, target cell, and TME, each class of immunomodulatory Abs will need to be individually analyzed to determine their in vivo FcyR requirements based on their mechanisms of action.

In contrast to the FcyR-independent mechanisms of anti-PD-1 Abs, an anti-PD-L1 Ab displayed significantly enhanced anti-tumor activity only when activating FcyR engagement was optimized. This enhanced activity was correlated with diminished F4/80-positive cells in the MC38 tumor model. The therapeutic potential of targeting TAMs has been demonstrated for a variety of tumors (Fritz et al., 2014; Noy and Pollard, 2014). In many tumor types, TAMs are polarized to a tumor-promoting phenotype, and stimulate tumor initiation and progression, including angiogenesis, tumor invasion, motility, and metastasis (Qian and Pollard, 2010). In addition, these TAMs are immunoregulatory and suppress the T cell-mediated anti-tumor immune response in the TME (Coussens et al., 2013). Direct targeting of PD-L1...
expressing myeloid cells may account for the enhanced activity of anti-PD-L1 Abs upon engagement of activating FcγRs. This pathway seems to synergize with the FcγR-independent blocking activity of anti-PD-L1 thereby augmenting the anti-tumor activity of effector T cells.

Whereas the proportion of different myeloid cell populations are reduced in a similar degree after anti-PD-L1 10F.9G2 clone treatment, we observed that only F4/80-positive cells reduction is FcγR-dependent, whereas MDSC reduction is not. MDSC reduction after anti-PD-L1 treatment has been reported previously in different tumor models (Curran et al., 2010). This attenuation in MDSC localization in the TME is also associated with reduction in their suppressive phenotype (Duraiswamy et al., 2013), and is mediated through TNF, which is secreted by CD8+ TILs upon their stimulation by anti-PD-L1 (Deng et al., 2014). We observed similar PD-L1 expression levels on both F4/80-positive cells and MDSC. The basis for this differential dependence on FcγR engagement remains to be elucidated.

Although our studies provide evidence for a role for PD-L1 expression on myeloid cells for the enhanced therapeutic effect of anti-PD-L1 upon engagement of activating FcγRs, they do not address the relative contribution of PD-L1 expressed by tumor cells. The importance of PD-L1 expression by tumor cells and infiltrated immune cells has been recently demonstrated in clinical settings (Tumeh et al., 2014). Indeed, PD-L1 expression by infiltrating leukocytes correlates with patients’ response to anti-PD-L1 treatment (Herbst et al., 2014). Further clinical studies elucidating the contribution of PD-L1 on tumor cells, tumor stroma, and the different infiltrating leukocytes are warranted to highlight potential predictive markers for anti-PD-1/PD-L1 treatments.

This study has significant implications for the selection of the optimal Fc for anti-PD-1/PD-L1 Abs. Both anti-PD-1 mAbs approved for the treatment of advanced melanoma, nivolumab (Topalian et al., 2012) and pembrolizumab (Hamid et al., 2013), are human IgG4 isoforms. This isotype was selected due to its low affinity binding of IgG4 to human FcγRIs to avoid ADCC-mediated depletion of PD-1 expressing cells. We have shown that, depending on the epitope recognized, inhibitory FcγRIIB engagement may diminish the activity of anti-PD-1 Abs. By using mice humanized for FcγRs, we have demonstrated that IgG4 isoforms of an anti-PD-1 Ab may not alter the anti-tumor activity compared to FcγR null mutant. However, the reduced activity of mouse IgG1 through interaction with FcγRIIB suggest that interactions with FcγRIIB should also be taken into consideration when selecting human IgG Fc scaffolds for anti-PD-1 Abs. Pidilizumab is a humanized IgG1 anti-PD-1 mAb in clinical development. Based on the relatively high-affinity interaction of FcγRs and our preclinical studies that demonstrate FcγR-mediated depletion of intratumoral Teff, this Ab may result with unwanted depletion of effector TILs and therefore display reduced anti-tumor activity. Therefore an FcγR null IgG variant of anti-PD-1 is predicted to be the optimal candidate for therapeutic blocking of PD-1 by avoiding the unwanted engagement of FcγR pathways.

Four different Abs targeting PD-L1 are currently being investigated in clinical trials: BMS-936559 (IgG4), MPDL3280A and MEDI4736 (IgG1s engineered to eliminate Fc-FcγR interactions), and MSB0010718C (IgG1). The variety of IgG isoatypes with differential FcγR-binding properties developed by the different companies can provide valuable insight into the contribution of FcγRs for anti-PD-L1 treatment of human malignancies. Further evaluation of the effector cells expressing FcγRs in different human tumor types, PD-L1 expression levels by different myeloid cells, and the efficacy of the different anti-PD-L1 isoatypes should provide valuable information regarding the involvement of FcγR pathways in the activity of anti-PD-L1 Ab treatment in cancer patients, and may provide rational for the design of IgG construct for optimal clinical anti-tumor activity.

EXPERIMENTAL PROCEDURES

A detailed description of the experimental procedures is provided in the Supplemental Experimental Procedures.

Mice

Mice 7–10 weeks of age were used in all experiments. All mice were maintained in The Rockefeller University Comparative Biosciences Center. All experiments were performed in compliance with federal laws and institutional guidelines and had been approved by The Rockefeller University IACUC.

Tumor Challenge and Treatment

MC38 cells (2 × 10⁶) were implanted subcutaneously (s.c), and tumor volumes were measured every 2–3 days with an electronic caliper and reported as volume using the formula (L₁² × L₂)/2, where L₁ is the shortest diameter and L₂ is the longest diameter. Five to seven days after tumor inoculation, mice were randomized by tumor size (day 0) and received intraperitoneal (i.p) injection of 200 μg anti-PD-1, anti-PD-L1, or control IgG. Mice received an additional 200 μg of IgG treatment at days 3 and 6. For the B16 model, mice were challenged with 2 × 10⁵ B16-F10 cells s.c, and after 3 days (day 0), were treated with 10⁶ irradiated B16-GM-CSF-secreting cells (GVAX) s.c and the first IgG treatment i.p. Additional IgGs were injected at days 3 and 6.

Tissue Processing and Flow Cytometry

For functional experiments, mice were challenged and treated as described above, and were killed at day 8, unless otherwise indicated. Spleens were dissected through a 70 μm nylon cell strainer, incubated with red blood cells lysis buffer (Sigma), and washed. Tumors were mechanically dissected and, in most cases, incubated with DNase and Liberase TL (Roche) before dispersed through a 70 μm nylon cell strainer. Different cell populations were identified after excluding dead cells using live/dead fixable aqua dead dye (Life Technologies). For intracellular staining, cells were fixed and permeabilized with Foxp3 Fix/Perm buffer kit (Biolegend).

CountBright Absolute Counting Beads (Life Technologies) were added prior to acquisition. Cell populations were defined by the following markers: monocytes (CD11b”MHC II” Ly6C”F4/80”CD11c”), macrophages (CD11b”MHC II”F4/80”CD11c”Ly6C”Ly6G”), immature myeloid cells (CD11b”MHC II” F4/80”Ly6C”Ly6G”), neutrophils (CD11b”Ly6G”Ly6C”MHC II”F4/80”), dendritic cells (CD11b”CD11c”MHC II”F4/80”), CD8 T cells (CD3”CD8”), CD4 effector T cells (CD3”CD4”Foxp3”), and CD4 regulatory T cells (CD3”CD4”Foxp3”). Data were acquired on Fortessa flow cytometers (BD) and analyzed using FlowJo software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.ccell.2015.08.004.

AUTHOR CONTRIBUTIONS

R.D. designed and performed experiments, analyzed data, and wrote the manuscript. E.S., J.E., and M.S. designed and performed experiments and
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