**FcγR interaction is not required for effective anti-PD-L1 immunotherapy but can add additional benefit depending on the tumor model**

Heng Sheng Sow, Hreinn Benonisson, Cor Breukel, Remco Visser, Onno J.H.M. Verhagen, Arthur E.H. Bentlage, Conny Brouwers, Jill W.C. Claassens, Margot M. Linssen, Marcel Camps, Thorbald van Hall, Ferry Ossendorp, Marieke F. Fransen, Gestur Vidarsson and J. Sjef Verbeek

1Department of Human Genetics, Leiden University Medical Center, Leiden, the Netherlands
2Department of Experimental Immunohematology, Sanquin Research and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands
3Department of Immunohematology, Leiden University Medical Center, Leiden, the Netherlands
4Department of Medical Oncology, Leiden University Medical Center, Leiden, the Netherlands

Immunomodulatory antibodies blocking interactions of coinhibitory receptors to their ligands such as CTLA-4, PD1 and PD-L1 on immune cells have shown impressive therapeutic efficacy in clinical studies. The therapeutic effect of these antibodies is mainly mediated by reactivating antitumor T cell immune responses. Detailed analysis of anti-CTLA4 antibody therapy revealed that an optimal therapeutic efficacy also requires binding to Fc receptors for IgG, FcγR, mediating depletion of intratumoral regulatory T cells. Here, we investigated the role of Fc binding in anti-PD-L1 antibody therapy in the MC38 C57BL/6 and CT26 BALB/c colon adenocarcinoma tumor models. In the MC38 tumor model, all IgG subclasses anti-PD-L1 showed similar therapeutic efficacy when compared to each other in either wild-type mice or in mice deficient for all FcγR. In contrast, in the CT26 tumor model, anti-PD-L1 mlgG2a, the IgG subclass with the highest affinity for activating FcγR, showed stronger therapeutic efficacy than other IgG subclasses. This was associated with a reduction of a myeloid cell subset with high expression of PD-L1 in the tumor microenvironment. This subclass preference for mlgG2a was lost in C57BL/6 × BALB/c F1 mice, indicating that the genetic background of the host may determine the additional clinical benefit of the high affinity antibody subclasses. Based on these data, we conclude that FcγR are not crucial for anti-PD-L1 antibody therapy but might play a role in some tumor models.

**Introduction**

Expression of programmed death ligand 1 (PD-L1), the ligand for programmed death 1 (PD-1), plays a paramount role in suppressing antitumor responses in human cancer patients. Because of the clinical success of PD-L1 blocking antibodies,1–6 Atezolizumab, Avelumab and Durvalumab have been approved by the U.S. Food and Drug administration (FDA) for the treatment of various advanced cancers.

Several lines of evidence suggest that the main effect of anti-PD-L1 mAb is abolishing suppression of on-going antitumor T cell responses through blocking PD-L1 on the tumor or tumor infiltrating myeloid cells from interacting with PD-1 on T cells.5,7 Theoretically, this mechanism should be FcγR independent. Nevertheless, it has been suggested that antibody subclass, and subsequent FcγR binding might contribute to the therapeutic efficacy.10 For example, many studies have demonstrated an essential role for activating FcγR in the antitumor activity of mAbs targeting the coinhibitory receptor C TLA-4.11–13 Binding of the Fc part of anti-CTLA4 mAb to FcγR resulted in the depletion of regulatory T cells by antibody-dependent effector mechanisms within the tumor.

In the clinic, Atezolizumab and Durvalumab are engineered human IgG1 anti-PD-L1 mAbs, with a modification in their Fc-domain to prevent antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).14,15 On the other hand, Avelumab has a wild-type human IgG1 Fc region which has been shown in vitro to...
FcyR interaction is not required for effective anti-PD-L1 immunotherapy

What's new?
Monoclonal antibodies against T cell “exhaustion”-inducing molecules show striking success in reactivating the antitumor immune response, but it is unclear if this success involves the so-called Fc region of the antibody that activates the immune system. Here the authors systematically swapped constant regions of a known mouse antibody against programmed death-ligand-1 (PD-L1) to alter interactions with the Fc receptor. The outcome was nuanced with different results obtained in different mouse tumor models leading the authors to conclude that in some tumors anti-PD-L1 immunotherapies might be enhanced with proper Fc receptor interactions.

engages FcyR expressing cells to induce ADCC. Since PD-L1 is expressed on tumor cells and tumor infiltrating immune cells, it might be that anti-PD-L1 mAb with the capability to engage FcyR influences the antitumor responses not only by blocking PD1-PD-L1 signaling pathway but also by depleting PD-L1 expressing tumor cells and/or tumor associated myeloid cells. Conversely, Fc-mediated effector mechanisms might be detrimental to the immune response due to the depletion of PD-L1+ immune effector cells.

In our study, we investigated the effect of the use of different mAb IgG subclasses on the efficacy of anti-PD-L1 in two mouse models of colorectal cancer (MC38 on C57Bl/6 and CT26 on BALB/c background). For this, we replaced the constant regions of anti-mouse PD-L1 mAb (rat IgG2a; clone MIH5) with different mouse constant regions to vary the binding to mouse FcyR. Our data suggest that FcyR are not critical for anti-PD-L1 mAb therapy but depending on the genetic background, the therapeutic efficacy of anti-PD-L1 could be enhanced by using the IgG subclass with the highest affinity for activating FcyR.

Materials and Methods

Generation of murinized anti-PD-L1 mAb
From the antimouse PD-L1 rat, IgG2a-producing Hybridoma line MIH-5, mRNA was isolated. This RNA was used as a template with the Clonetech SMARTer Ethnic group cDNA 5’Ethnic group kit to synthesize total cDNA. In the next step, the heavy and light chain variable regions encoding DNA sequences of the anti-mouse PD-L1 rat IgG2a cDNA were amplified using the after primers: as a template with the : gccaagttggtgcaaaaaattt (m&e_CH1IgG1_2a_& mlG2b), Heavy chain CH2: aacctgtgtgtgtgtgtgtgcttagtacg (Rat_IgG21_GSP1), Hinge region: ccaagggattgatctt (Rat_IgG2a Hinge R), Light chain: gtcgaagctatgatccctccaca ctaa (Rat lambda R2); gtggacctgcaatacctcaca (Rat Lambda R1); gtggagacctgcaaccttca (Rat Lambda R1 diff); tcggccaaagctcctcaca (Rat Lambda R2).

The resulting single PCR fragments of the variable regions of both the heavy and light chain were analyzed by DNA sequencing. This sequence was codon optimized for expression in human cells. Based on this information, the heavy and light chain variable region encoding DNA fragments were assembled from GBLOCKS (Integrated DNA Technologies, Leuven, Belgium). The anti-mouse PD-L1 heavy chain variable region encoding DNA fragment was cloned in the expression vectors pFUSE.CH1g_mG1, pFUSE.CH1g_mG2a and pFUSE.CH1g_mG3 to express the anti-mouse PDL1 mIgG1, mIgG2a and mIgG3 heavy chain respectively. The light chain variable region encoding DNA fragment was cloned in pFUSECLIG mk to express the anti-mouse PD-L1 Kapa Light chain. Unfortunately, these vectors showed low expression of the proteins when transfected into HEK293 cells and therefore the antibody encoding sequences were cloned into the expression vector pCDNA3.1(A) which showed good transient expression in these human cells. In addition, an anti-PD-L1 mIgG2a antibody was generated with a D256A mutation in the Fc domain of mIgG2a engineered according to the protocol as described in Liu et al.16

Mice
C57BL/6 and BALB/c mice were purchased from Charles River (L’Arbresle, France) and housed in the animal facility of the Leiden University Medical Center (LUMC). FcyRII/III/IV deficient C57BL6 mice were generated in the transgenic mouse facility of the LUMC.17 These transgenic mice were bred in house and routinely checked for their genotype by PCR. All mice were housed in individually-ventilated-cage (IVC) systems under specific pathogen-free conditions and used at 8–12 weeks of age.

The health status of the animals was monitored over time. Animals tested negative for all agents listed in the FELASA (Federation of European Laboratory Animal Science Associations) guidelines for SPF mouse colonies.18 All mouse studies were approved by the animal ethics committee of the LUMC. Experiments were performed in accordance with the Dutch Act on Animal Experimentation and EU Directive 2010/63/EU (‘On the protection of animals used for scientific purposes’).

Syngeneic tumor models and tumor therapy
CT26 (kindly provided by Mario Colombo, Milano) and MC38 tumor cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM) (Lonza) supplemented with 8% Fetal Calf Serum (Greiner), 25 μM 2-mercaptoethanol and 100 IU/mL penicillin/streptomycin (Gibco). Cell lines were mycoplasma and MAP-tested before the start of experiments. The tumor cells (2.5 × 105 for MC38/MC38 PD-L1−/− and 1 × 106 for CT26/CT26 PD-L1−/−) were injected subcutaneously into 8–12 week-old mice in 100 μL of PBS. PD-L1 deficient MC38 and CT26 cell lines were generated using
CRISPR/Cas9 technology as described in Kleinovink et al. 2017.9 Two hundred micrograms of therapeutic antibody was injected intraperitoneally at day 6, 9 and 12 after tumor inoculation. Once palpable tumors were present, tumor size was measured twice per week, using a caliper. Mice were sacrificed when tumors reached a size of 100 mm² because of ethical reasons.

Rechallenge tumor studies
After an 80 days tumor-free period, mice with complete regression of MC38 and CT26 tumors, as well as naïve control mice, were reinoculated subcutaneously in the left flank with either 2.5 × 10⁵ MC38 or 1 × 10⁵ CT26 tumor cells. Once palpable tumors were present, tumor size was measured twice per week, using a caliper. Mice were sacrificed when tumors reached a size of 100 mm² because of ethical reasons.

Flow cytometry
Tumors were harvested into 1 mL of non-supplemented IMDM media in 24-well plates and manually minced into small pieces with scalpels, incubated with 2.5 mg/mL Liberase TL (Roche) for 20 min at 37 °C and single-cell suspensions were made using 70-μm cell strainers (BD Biosciences). FcR were blocked with 10% normal mouse serum and anti-mouse CD16/CD32 antibody (2.4G2). Cell surface markers were stained using the after antibodies: CD8α (clone 53–6.7), CD4 (clone L3 T4), CD3e (clone 145-2c11), CD11b (clone M1/70), F4/80 (clone BM8), CD45.2 (clone 104), Ly6G (clone 1A8), Ly6C (clone HK1.4), PD-L1 (clone MIH5). Dead cells were excluded based on 7-AAD staining (Invitrogen).

For assessing the binding of murinized anti-PD-L1 mAb to mouse PD-L1, PD-L1 expressing MC38 or B16F10 tumor cells (pretreated with 20 U/mL mIFNγ for 24 h) were preincubated for 15 min with titrated murinized anti-PD-L1 mAb or serum collected from anti-PD-L1 mAb treated mice prior to staining with PE-labeled anti-PD-L1 mAb (clone MIH5). Cells were analyzed by flow cytometry for PE labeling, which was blocked by the presence of unlabelled murinized anti-PD-L1 mAb. Analysis were performed using LSRII cytometer (BD) using FacsDIVA software (BD) and FlowJo Software (Tree Star).

Surface plasmon resonance
Surface plasmon resonance (SPR) measurements were carried out on a IBIS MX96 (IBIS technologies) as described.19 The biotinylated FcγRI were spotted in duplicate and in three-fold dilutions, ranging from 30 to 1 nM for FcγRI, FcγRIIb and FcγRIV in PBS 0.075% Tween-80 (VWR, M126–100 mL), pH 7.4. For the His-tagged FcγRIIb, biotinylated anti-His IgG1 (GenScript, A00613) was spotted in duplicate and three-fold dilution onto the sensor and 100 nM his-FcγRIIb (equally diluted in PBS 0.075% Tween-80, pH 7.4) was loaded onto the sensor before each antibody injection. Antibodies were then injected over the IBIS at 1.5 dilution series starting at 8.8 nM until 795.915 nM in PBS in 0.075% Tween-80. Regeneration after every sample was carried out with acid buffer (100 nM H3PO4, 0.075% Tween 80, pH 1.5). Calculation of the dissociation constant (KD) was done by equilibrium fitting to Rmax = 500. In the case of FcγRIIb, anti-His association and dissociation curves were subtracted before calculation of IgG-binding affinity using SPRINT 1.9.4.4 software (IBIS technologies). Analysis and calculation of all binding data was carried out with Scrubber software version 2 (Biologic Software, Campbell, Australia).

Statistical analyses
Data was analyzed using Prism 7.0 (GraphPad Software). Statistical significance was calculated using the two-way ANOVA and Mann Whitney nonparametric test. Statistical significance was defined as p < 0.05. Tumor survival data was analyzed with the Kaplan–Meier method and the log-rank (Mantel-Cox) test.

Results
Characterization of murinized anti-mouse PD-L1 IgG monoclonal antibody
To determine the role of FcγR in anti-PD-L1 mAb therapy, the variable region of the rat anti-mouse PD-L1 mAb (clone MIH5, rat IgG2a) was fused to the different mouse immunoglobulin heavy chain constant regions by molecular cloning and protein was purified from HEK293 cells transfected with the expression vectors encoding the recombinant murinized immunoglobulins. These included mlgG1, mlgG2a, mlgG3 and a mlgG2a containing the D265A mutation (IgG2a D265A) which has been reported to abrogate binding to FcγR and strongly reduces complement activity.20,21 We also assessed whether the murinized mAbs bound to the same epitope on PD-L1 as the parental anti-PDL1 rat IgG2a mAb. PD-L1 expressing MC38 tumor cells were preincubated with respective concentrations of purified murinized anti-PD-L1 mAb and then stained with fluorescently labeled anti-PD-L1 rat IgG2a. Each of these murinized mAbs showed equivalent binding to PD-L1 expressing MC38 tumor cells (Fig. 1a), suggesting that the murinized anti-PD-L1 and parental anti-PD-L1 mAbs recognize the same epitope on PD-L1 and that the different IgG subclasses bind with the same affinity. In addition, the binding affinity of the Fc portion of each mAb to mouse FcγRI, II, III, and IV was measured using surface
plasmon resonance (Fig. 1b). Consistent with literature, mIgG1 bound with low affinity to FcγRIII and FcγRII, mIgG2a bound with high affinity to FcγRI, intermediate affinity to FcγRIV and low affinity to FcγRII, mIgG3 did not bind to any FcγR. The mIgG2a D265A showed only residual binding to FcγRI (Fig. 1b). The calculated $K_D$ values are shown in Figure 1c.

**Therapeutic efficacy of murinized anti-PD-L1 IgG monoclonal ab is FcγR independent in MC38 tumor model**

The MC38 colon adenocarcinoma syngeneic model on C57BL/6 background is highly immunogenic and it has been demonstrated to be sensitive to anti-PD-L1 mAb monotherapy.9,24 The therapeutic efficacy of the murinized anti-PD-L1 mAbs was analyzed in this tumor model. Strong inhibition of tumor growth (Fig. 2a), increased survival rates (Fig. 2b) as well as long-term immune memory (Fig.S1A, Supporting Information) were observed after i.p. administration of each of the murinized anti-PD-L1 mAbs (Figs. 2a and 2b). We did not observe significant differences in delay of tumor outgrowth and long-term survival between mice treated with anti-PD-L1 mAb of different IgG subclasses including the D265A mIgG2a, indicating that FcγR engagement was not required for the optimal therapeutic efficacy of anti-PD-L1 mAb. To confirm this, we studied the therapeutic efficacy of IgG1 and IgG2a anti-PD-L1 in MC38 tumor bearing C57BL/6 mice deficient for the ligand binding chains of all four FcγR (FcγRI/II/II/IV$^{-/-}$ mice) which show normal innate and adaptive immunity.17 In MC38 tumor bearing FcγR$^{-/-}$ mice, the therapeutic efficacy of the murinized anti-PD-L1 IgG1 and IgG2a anti-PD-L1 antibodies was similar, resulting in cure in most of the treated mice (Figs. 2c and 2d) as well as strong memory response (Fig. S1B, Supporting Information).
Figure 2. Antitumor activity of anti-PD-L1 antibodies in MC38 tumor model is Fc independent. (a) Wild-type C57BL/6 mice with established MC38 tumors were treated with the designated antibody. Data are presented as mean of tumor size mm² ± SEM. (b) Same as A except data are presented as Kaplan–Meier survival curve. (c) and (d) Same as A and B respectively, except FcγRI/II/III/IV−/− C57BL/6 mice were used. For the mean of tumor size, statistical significance between groups was determined by two-way ANOVA (*** p < 0.001; ** p < 0.01; * p < 0.05). Logrank test was used to determine the statistical significance of the survival. Pooled data of two independent experiments, 15 to 16 mice per group.

Information). Collectively, our data demonstrate that interactions with FcγR are dispensable for the therapeutic efficacy of anti-PD-L1 IgG in the MC38 tumor model.

Anti-PD-L1 IgG2a is more effective than anti-PD-L1 IgG1 and D265A mlgG2a in CT26 tumor model

As FcγR were dispensable for the therapeutic efficacy of anti-PD-L1 antibody in MC38 tumor model, we aimed to test if this is a general feature of this treatment modality, by verifying this in another tumor model. For this, we selected the CT26 colon adenocarcinoma syngeneic model on BALB/c background which is also known to be immunogenic and hence highly responsive to anti-PD-L1 mAb monotherapy. When CT26 tumor bearing BALB/c mice were treated with murinized anti-PD-L1 mAb, all mlgG subclasses including D265A mlgG2a again effectively delayed tumor outgrowth resulting in prolonged survival (Figs. 3a and 3b) and formation of antitumor memory response (Fig. S1C, Supporting Information). However, in contrast to the MC38 tumor model, overall survival rate of mice treated with anti-PD-L1 mlgG2a was significantly higher compared to mice treated with anti-PD-L1 mlgG1 or D265A IgG2a (Fig. 3b). To establish that this was not caused by differences in antibody clearance we measured the serum levels of circulating murinized anti-PD-L1 mAb in CT26 tumor bearing BALB/c mice using flow cytometry. We found that serum levels of the four murinized anti-PD-L1 mAb were similar. Thus, the higher efficacy of the murinized anti-PD-L1 mlgG2a in the CT26 tumor model cannot be explained by differences in drug exposure (Fig. S2, Supporting Information).

Additional therapeutic effect of anti-PDL1 mlgG2a in a CT26 PD-L1−/− tumor model

CT26 tumor expresses PD-L1, suggesting that the effect of anti-PD-L1 therapy may be a direct elimination of tumor cells mediated by FcγR on innate effector cells. Compared to
mIgG1, mIgG2a binds with higher affinity to activating FcγRs resulting in more effective induction of downstream effector mechanisms such as antibody-dependent cellular phagocytosis (ADCP) or ADCC. To examine whether the difference in efficacy between IgG1 and IgG2a was solely due to Fc-mediated effector function of anti-PD-L1 mIgG2a directed against PD-L1 expressing tumor cells, we made use of a PD-L1<sup>−/−</sup> CT26 tumor model to compare the efficacy of anti-PD-L1 mIgG2a and mIgG1. Although PD-L1<sup>−/−</sup> CT26 tumor bearing mice treated with anti-PD-L1 mIgG1 or mIgG2a showed significant inhibition of tumor growth (Fig. 4a), improved long-term survival was observed only in mice treated with anti-PD-L1 mIgG2a but not mIgG1 (Fig. 4b). Our result suggests that the treatment of PD-L1<sup>−/−</sup> tumor can also benefit from the use of...

Figure 3. Anti-PD-L1 IgG2a is more effective than anti-PD-L1 IgG1 and D265A mIgG2a in CT26 tumor model. (a) Wild type BALB/c mice with established CT26 tumors were treated with the designated antibody. Data are represented as mean of tumor size mm<sup>2</sup> ± SEM. (b) Same as A except data are represented in Kaplan–Meier survival curve. For the mean of tumor size, statistical significance between groups was determined by two-way ANOVA (*p < 0.05; **p < 0.01; ***p < 0.001). Logrank test was used to determine the statistical significance of the survival. Pooled data of two independent experiments, total 15 to 16 mice per group.

Figure 4. Antitumor activity of anti-PD-L1 mIgG1 and mIgG2a antibodies in CT26 PD-L1<sup>−/−</sup> tumor bearing mice. (a) Wild-type BALB/c mice with established PD-L1<sup>−/−</sup> CT26 tumors were treated with the designated antibody. Data are represented as mean of tumor size mm<sup>2</sup> ± SEM. (b) Same as (a) except data are represented as Kaplan–Meier survival curve. Statistical significance of the mean tumor size was determined by two-way ANOVA (*p < 0.05; **p < 0.01; ***p < 0.001). Logrank test was used to determine the statistical significance of the survival. Pooled data of two independent experiments, 12 mice per group.
the mIgG2a subclass of anti-PD-L1 antibody. The additional therapeutic effect might be mediated through ADCC or ADCP of PD-L1 expressing nontumor cells.

**Anti-PD-L1 mIgG2a but not mIgG1 modulates the tumor infiltrating myeloid cell subsets in the CT26 tumor microenvironment**

Because PD-L1 is not only expressed on tumor cells but also on tumor infiltrating immune cells, we evaluated the relative expression of PD-L1 on multiple immune cell populations within the MC38 and CT26 tumor microenvironment. High levels of PD-L1 expression on F4/80hi Ly6Clo myeloid cells was observed as compared to F4/80int Ly6Chi, F4/80hi Ly6Clo, and F4/80low Ly6Clow myeloid cells, CD3 T lymphocytes, granulocytes (Ly6G+) and CD45-negative cells (Fig. 5a). Based on the differences in PD-L1 density, we hypothesized that anti-PD-L1 mAb therapy could result in selective modulation of myeloid cell subsets. Therefore, we compared the impact of murinized anti-PD-L1
mIgG1 and mIgG2a on the frequency of immune cells in the tumor microenvironment of both tumor models. Administration of anti-PD-L1 mAb resulted in an increased frequency of CD4$^+$ and CD8$^+$ T cells in both CT26 and MC38 tumor (Fig. 5b). In MC38 tumors, the frequency of myeloid cell subsets between mice treated with murinized IgG1 or IgG2a anti-PD-L1 mAb and untreated mice was similar. In contrast, in CT26 tumors, anti-PD-L1 mIgG2a treatment resulted in reduction of F480$^{hi}$ Ly6Clo cells, whereas the mIgG1 subclass did not affect this myeloid subset (Fig. 5c). Therefore, we hypothesized that the additional antitumor effect of anti-PD-L1 mIgG2a in the CT26 tumor model is due to its potential capability to directly target tumor-associated myeloid cells.

**Similar therapeutic efficacy of IgG1 and IgG2a murinized anti-PD-L1 antibodies in C57BL/6 x BALB/c F1 mice**

We next analyzed whether the genetic background (BALB/c versus C57BL/6) of the mice or tumor cell intrinsic differences (MC38 versus CT26) determined the difference in therapeutic efficacy of anti-PD-L1 mIgG1 and mIgG2a. For this, C57Bl/6 x BALB/c F1 mice (strain CB6F1/J) were inoculated with either MC38 or CT26 tumor cells and treated with anti-PD-L1 mIgG1 or mIgG2a. In both tumor models, no difference in survival was observed between mice treated with anti-PD-L1 mIgG2a or mIgG1 (Figs. 6a and 6b) indicating that the genetic background of the mice but not tumor cell intrinsic differences played a paramount role in the therapeutic efficacy of these antibodies.

**Discussion**

It has been proposed that the underlying mechanism of tumor rejection in anti-PD-L1 antibody therapy is reactivation and increase of intratumoral T cells as the consequence of blocking PD1-PD-L1 inhibitory activity. It has recently become apparent, however, that the therapeutic efficacy of several other immunomodulatory antibodies such as anti-OX40, GITR, and CTLA4 mAb can also be attributed to the activation of Fc mediated pathways. In contrast, two
preclinical studies have revealed that interaction with its Fc part is detrimental to the therapeutic efficacy of anti-PD-1 mAb, by facilitating macrophages to deplete PD1+ effector T cells10 or to remove this mAb from T cells.26 This illustrates that it is important to understand the impact of the interaction with FcγR on the therapeutic efficacy of any immunomodulatory antibody. Here, we investigated the role of FcγR in the antitumor effect of PD-L1 blocking antibody. From our studies with the MC38 and CT26 syngeneic colon adenocarcinoma models, we conclude that the therapeutic effect of anti-PD-L1 mAb is predominantly based on FcγR independent blocking of PD-L1. This is conflicting with a recent study showing a minor enhancement of therapeutic efficacy of anti-PD-L1 mIgG2a over other IgG subclasses in MC38 tumor model.10 This might be caused by differences in experimental conditions as some of the genetically modified mouse strains and anti-PD-L1 mAb used in their study are different from ours.

On the other hand, in the subcutaneous CT26 tumor model, anti-PD-L1 mlgG2a elicited stronger therapeutic effect and survival compared to anti-PD-L1 mlgG1, mlgG3 and mlgG2a D265A. We observed that this enhanced antitumor efficacy correlated with the reduction of a tumor-infiltrated myeloid subset in CT26 tumor. For this tumor model, an association between an elevated number of myeloid cells and the increased magnitude of their immunosuppressive tumor microenvironment was reported while elimination of these cells can lead to strong antitumor responses.27,28 Preferential reduction of this myeloid subset by anti-PD-L1 mlgG2a may be due to its high levels of surface PD-L1 expression compared to other immune and nonimmune cells, similar to that proposed for anti-CTLA4 mAb, which preferentially depletes high CTLA4 expressing regulatory T cells.12 As mlgG2a (but not IgG1) can also efficiently activate complement,29,30 we cannot exclude that the higher efficacy of IgG2a anti-PD-L1 in the CT26 tumor model is caused by the activation of complement pathways. The potential involvement of various Fc-mediated effector functions toward myeloid cell eradication and how the eradication of a myeloid subset in the CT26 tumor microenvironment augment subsequent adaptive immune responses will need further investigation.

We observed a comparable therapeutic efficacy of anti-PD-L1 mlgG1 and mlgG2a in CT26 tumor bearing C57B/6 mice, offspring from a cross between BALB/c and C57B/6 mice, suggesting that the stronger antitumor efficacy of anti-PD-L1 mlgG2a is strain-dependent. Although multiple in vitro and in vivo data indicate that human Fc polymorphisms can influence the therapeutic activity of anti-CD20 (rituximab), anti-CD52 (alemtuzumab), anti-Her2 (trastuzumab) and anti-EGFR (cetuximab), in mice two polymorphisms are reported, one in FcγRII and one in FcγRIII, but no difference in IgG binding was observed.31 Nevertheless, polymorphism in the Fc region of IgG has been found in mice. Whereas most inbred mouse strains including BALB/c express IgG2a, C57Bl/6 mice express an allelic variant of that, named IgG2c.32 To the best of our knowledge, it is not known whether there is any difference in functionality between IgG2c and IgG2a. However, we cannot exclude the possibility that IgG2a is immunogenic in C57Bl/6 mice and induces an immune response decreasing its efficacy over time.

The objective response rate with approved anti-PD-L1 mAb as monotherapy is ~20% in uterine carcinomas,53–34 ~15% in nonsmall-cell lung cancer (NSCLC),35–36 and ~30% in Merkel cell carcinoma.2,37 Anti-PD-L1 mAb therapy is very effective but so far has only been tested in a restricted number of specific cancer types. However, in animal studies, antitumor activity of anti-PD-L1 treatment varies across a range of syngeneic tumor models.38 Thus, evaluation of the role of different tumor types and genetic background in the additional effect of anti-PD-L1 mlgG2a may have clinical implications, because the results might guide design and development of more effective anti-PD-L1 mAb for cancer therapy. To date, antibodies targeting PD-L1, including Atezolizumab, Avelumab and Durvalumab have been approved by FDA for treating various cancers. Unlike Atezolizumab and Durvalumab which are engineered human IgG1 mAbs to avoid Fc interaction, Avelumab has a wild-type human IgG1 Fc region. Although results of in vitro studies have suggested that Avelumab is capable of inducing ADCC of tumor cells,39–41 as far as we know, no evidence from clinical results has been published to support this. As previously reported, PD-L1 expression on immune but not tumor cells in the tumor microenvironment was significantly associated with higher response to PD-L1 blocking antibodies,3,6 indicating that blocking of PD-L1 on immune cells with antibodies (Atezolizumab and Durvalumab) is critical for the effectiveness of this therapy. Hence, it remains to be evaluated whether Avelumab has an additional therapeutic benefit in patients whose tumors are infiltrated by PD-L1+ myeloid cells as our study in the CT26 model suggests. A study by Boyerinas et al.41 showed very low levels of Avelumab mediated in vitro lysis of PD-L1+ PBMC subsets of patients receiving Avelumab, but this does not rule out the possibility that PD-L1+ immune effector cell subsets in the tumor microenvironment would be subject to some degree of depletion. The concerns related to toxicity versus clinical benefits of Avelumab have to be defined in larger clinical studies. Overall, our work suggests that anti-PD-L1 mAbs work primarily by blocking PD-L1 and binding to FcγR is no prerequisite for their antitumor activity. However, depending on the tumor model, interaction with FcγR can potentially enhance the therapeutic efficacy of anti-PD-L1 mAb.

Acknowledgements
The authors thank the staff of the Central Animal Facility (PDC) of the Leiden University Medical Center (LUMC) for excellent animal care.
References

1. Antonia SJ, Villegas A, Daniel D, et al. Durvalumab after Chemoradiotherapy in stage III non-small-cell lung cancer. N Engl J Med 2017;377:1919–29.

2. Kaufman HL, Russell J, Hamid O, et al. Avelumab in patients with chemotherapy-refractory metastatic Merkel cell carcinoma: a multicentre, single-group, open-label, phase 2 trial. Lancet Oncol 2016;17:1374–85.

3. Powles T, Edze JP, Fine GD, et al. MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. Nature 2014;515: 558–62.

4. Massard C, Gordon MS, Sharma S, et al. Safety and efficacy of Durvalumab (MEDI4736), an anti-programmed cell death Ligand-1 immune checkpoint inhibitor, in patients with advanced urothelial bladder cancer. J Clin Oncol 2016;34:3119–25.

5. Herbst RS, Soria JC, Kowanetz M, et al. Predictive biomarkers of response to nivolumab for advanced NSCLC. J Thorac Oncol 2015;10:104–13.

6. Rosenberg JE, Hoffman-Censits J, Powles T, et al. Avelumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. Lancet (London, England) 2016;387:1909–20.

7. Ribas A, Hu-Lieskovan S. What does PD-L1 positive or negative mean? J Exp Med 2016;213:2835–40.

8. Zhao T, Li C, Wu Y, et al. Prognostic value of FcγRIII, FcγRIIB and FcγRIIA expression in gastric cancer. Clin Cancer Res 2015;21:451–67.

9. Kleinovink JW, Marijt KA, Schoonderwoerd MJA, et al. FcγRs contribute to tumor activity through reduction of intratumoral CD8+ T cells co-depleting of tumor-infiltrating myeloid cells. J Exp Med 2017;214:2615–26.

10. Fransen MF, Benonisson H, van Maren WW, et al. Importance of IgG2c isotype in the immune response to beta-amyloid in amyloid precursor protein/transgenic mice. Neurosci Lett 2003;338:5–8.

11. Balar AV, Galisky MD, Rosenberg JE, et al. Atezolizumab as first-line treatment in cisplatin-eligible patients with locally advanced and metastatic urothelial carcinoma: a single-arm, multicentre, phase 2 trial. Lancet (London, England) 2017;389:67–76.

12. Powles T, O’Donnell PH, Massard C, et al. Efficacy and safety of Durvalumab in locally advanced or metastatic urothelial carcinoma: updated results from a phase 1/2 open-label study. JAMA Oncol 2017;3:e172411.

13. Rittmeyer A, Barlesi F, Waterkamp D, et al. Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. Lancet (London, England) 2017;389:255–65.

14. Fehrenbacher L, Spira A, Ballinger M, et al. Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial. Lancet (London, England) 2016;387:1837–46.

15. Kaufman HL, Russell JS, Hamid O, et al. Updated efficacy of avelumab in patients with previously treated metastatic Merkel cell carcinoma after >1 year of follow-up: JAVELIN Merkel 200, a phase 1–2 clinical trial. J Immunother Cancer 2018;6.

16. Mosely SI, Prime JL, Sainson RC, et al. Rational selection of syngeneic preclinical tumor models for immunotherapeutic drug discovery. Cancer Immunol Res 2017;5:29–41.

17. Fuji R, Friedman ER, Richards J, et al. Enhanced killing of chordoma cells by antibody-dependent cell-mediated cytotoxicity employing the novel anti-PD-L1 antibody avelumab. Oncotarget 2016;7:33498–511.

18. Khanna S, Thomas A, Abate-Daga D, et al. Mitochondrial respiratory chain dysfunction in metastatic melanoma. Cancer Immunol Res 2015;3:1193–205.

19. Boyerinas B, Jochems C, Fantini M, et al. Antibody-dependent cellular cytotoxicity activity of a novel anti-PD-L1 antibody Avelumab (MSB0010718C) on human tumor cells. Cancer Immunol Res 2015;3:1148–57.