ORIGINAL ARTICLE

The anti-lymphoma activities of anti-CD137 monoclonal antibodies are enhanced in FcγRIII$^{-/-}$ mice

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Abstract Agonistic monoclonal antibodies (mAbs) directed against the co-signaling molecule CD137 (4-1BB) elicit potent anti-tumor immunity in mice. This anti-tumor immunity has traditionally been thought to result from the ability of the Fab portion of anti-CD137 to function as an analog for CD137L. Although binding of CD137 by anti-CD137 mAbs has the potential to cross-link the Fc fragments, enabling Fc engagement of low to moderate affinity Fc gamma receptors (FcγR), the relative import of such Fc–FcγR interactions in mediating anti-CD137 associated anti-tumor immunity is unknown. We studied the ability of a rat anti-mouse CD137 mAb (2A) to mediate the anti-tumor response against the EL4E7 lymphoma in WT and FcγR$^{-/-}$ strains. 2A-treated FcRγ$^{-/-}$ mice had improved anti-tumor immunity against EL4E7, which could be completely recapitulated in FcγRIII$^{-/-}$ animals. These improved anti-tumor responses were associated with increased splenic CD8β T cell and dendritic cell (DC) populations. Furthermore, there was an increase in the number of DCs expressing high levels of the CD40, CD80, and CD86 molecules that are associated with more effective antigen presentation. Our results demonstrate an unexpected inhibitory role for FcγRIII in the anti-tumor function of anti-CD137 and underscore the need to consider antibody isotype when engineering therapeutic mAbs.

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Abbreviations

2A	Rat anti-mouse CD137 mAb
ADCC	Antibody-dependent cellular cytotoxicity
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DC	Dendritic cell
FcRγ	Fc common γ-chain
FcγR	Fc gamma receptor
GITR	Glucocorticoid-induced TNFR-related protein
ITAM	Immune receptor tyrosine-based activating motif
ITIM	Immune receptor tyrosine-based inhibitory motif
MHCII	Major histocompatibility II
mAbs	Monoclonal antibodies
NK	Natural killer
Tregs	T regulatory cells
WT	Wild type

Introduction

CD137 (4-1BB) is an inducible co-signaling molecule expressed by T cells, natural killer (NK) cells, dendritic cells (DC), B cells, and other cells of the immune system [1]. CD137 ligation, by either its ligand (CD137L) or agonistic monoclonal antibodies (mAbs), mediates cellular activation and prevents activation-induced cell death [1]. Administration of agonistic antibodies to CD137 results in tumor resolution in a CD8$^+$ T cell-dependent fashion, with additional contributions from CD4$^+$ T cells, NK cells, and/or DCs dependent upon the tumor model [2–5]. To date, the majority of the functional responses to anti-CD137 stimulation have been attributed to Fab-CD137 interactions. However, the contribution of interactions between the Fc fragments of anti-CD137 mAbs with Fc gamma receptors (FcγRs) to the anti-tumor activity of anti-CD137 mAbs remains unknown.

Mice have three activating and one inhibitory FcγR, which are expressed alone or in combination on individual cell populations [6]. The activating receptors, FcγRI, FcγRIII, and FcγRIV, each consists of a unique α-chain and a common Fcγ-chain, containing the immune receptor tyrosine-based activating motif (ITAM) in the cytoplasmic tail. In contrast, the cytoplasmic tail of the inhibitory receptor, FcγRIIB, is comprised of an immune receptor tyrosine-based inhibitory motif (ITIM) [6]. ITAM signaling results in the secretion of pro-inflammatory cytokines, initiation of phagocytosis, and other effector functions, whereas ITIM signaling abrogates these effector responses [6]. Importantly, FcγRIIB and FcγRIII have limited ability to bind to naturally occurring circulating IgG homodimers, but are able to bind aggregated IgG complexes, like those formed after Fab engagement of polyvalent targets [6]. Therefore, Fab-CD137 interactions have the potential to enhance and localize Fc-mediated signaling through low-affinity FcγRs, and possibly alter the functional outcomes of CD137 stimulation.

Recent studies have characterized the import of Fc–FcγR interactions on the function of immunomodulatory mAbs. Collectively, these studies indicate that the relative functional import of Fc interactions with individual FcRs is dependent upon the co-signaling moiety targeted by the Fab fragment. For example, anti-CD40 mAbs require engagement of FcγRIIB for therapeutic efficacy [7, 8]. For other immune receptor targets, such as CD152 (CTLA-4) and the anti-glucocorticoid-induced TNFR-related protein (GITR), engagement of FcγRIV and FcγRIII, respectively, are functionally relevant. Specifically, these interactions mediate depletion of tumor-infiltrating T regulatory cells (Tregs), thus increasing the ratio of CD8$^+$ T cells to Tregs, with a resultant improvement in the anti-tumor immune response [9, 10]. These data underscore the need to define how Fc interactions with each of the cognate FcγRs contribute to the biologic function of mAbs targeting co-signaling pathways.

We have previously demonstrated that ligation of FcγRIII on human NK cells results in the up-regulation of CD137 with loss of FcγRIII [11]. Based on these findings, we hypothesized that Fc–FcγR interactions would augment anti-CD137-mediated anti-tumor immunity. Using an anti-CD137 mAb-sensitive EL4E7 lymphoma tumor model, in mice deficient for individual FcγRs, we now show that anti-CD137-mediated anti-tumor immunity against EL4E7 is enhanced in FcγRIII$^{-/-}$ mice [2]. This effect is associated with increases in CD8β$^+$ T cells and CD11c$^+$ major histocompatibility II (MHCII)$^+$ populations. These data suggest that FcγRIII stimulation impairs anti-CD137-mediated anti-tumor immunity against EL4E7.

Methods

Mice, cell lines, and therapeutic antibodies

C57BL/6 and B6.129P2-*Fcgr3^{tm1Sjv}*/SjvJ (FcγRIII$^{-/-}$) were purchased from Jackson Laboratory. Fcer1g (FcRγ$^{-/-}$) and Fcgr2b (FcγRIIB$^{-/-}$) were purchased from Taconic Farms. CD137$^{-/-}$ and FcγRIV$^{-/-}$ mice were generous gifts from Dr. Lieping Chen and Dr. Jeffery Ravetch, respectively. Breeding colonies for FcγRIII$^{-/-}$, FcγRIV$^{-/-}$, and CD137$^{-/-}$ were maintained in the animal facility at the University of Maryland School of Medicine. Female mice from eight to twelve weeks of age were used in the experiments. The Institutional Animal Care and Use Committee at the University of Maryland School of Medicine approved all experiments.

The EL4E7 cell line, a gift from Dr. Chen, was passaged in vivo four times to generate a cell line for the tumor experiments. B16-F10 cells were a gift from Dr. Paul Antony. The EL4E7 and B16-F10 cells were maintained in RPMI 1640 medium supplemented with 10 % FBS, 1 % L-glutamine (Invitrogen), 1 % penicillin–streptomycin (Invitrogen), and 1 % HEPES buffer (Corning).

Rat anti-mouse CD137 mAb clone 2A (IgG2a) and clone 3H3 (IgG2a) were gifts from Dr. Chen and Dr. Robert Mittler, respectively. The antibodies were purified from hybridoma supernatant using affinity chromatography on a protein G Sepharose column (GE Healthcare). Antibodies were verified to have <1 IU/ml of endotoxin using the limulus amebocyte lysate test (Charles Rivers Laboratories). Rat IgG control was purchased from Sigma or Innovative Research.

Flow cytometry and antibodies

Antibodies against CD8β FITC (ebioH35-17.2), CD103 PE (2E7), CD40 APC (1C10), CD80 APC (16-10A1), CD86 APC (GL1), CD137L (TKS-1) PE, Armenian hamster

IgG PE, rat IgG2a PE, and rat IgG2a APC were purchased from eBioscience. Antibodies against CD11c PercP-Cy5.5 (HL3), hamster IgG PercP-Cy5.5, and mouse IgG APC were purchased from BD Biosciences. Antibodies against MHCII/I-A^b APC (AF6-120.1), CD137 biotin (17B5), Syrian hamster biotin, and TruStain fcX were purchased from Biolegend. Anti-polyhistidine PE was purchased from R&D systems. Recombinant mouse CD137L containing a His tag was purchased from R&D systems. Data were acquired using an LSRII instrument (BD Biosciences) and were analyzed using FlowJo (Tree Star).

Measurement of mouse FcγRs binding to rat IgG2a Surface Plasmon Resonance (SPR)

Antibodies (2A, rat IgG2a (eBioscience), and mouse IgG2a (eBioscience)) at 10 μg/ml in acetate pH 4.5 were immobilized to a CM5 chip in a BIAcore 3000 (GE Healthcare). A response of 600RU was the immobilization target level. Actual immobilization levels were 680RU, 670RU, and 640RU, respectively, on flow cell (FC) 2, FC3 and FC4. FC1 was used as a blank reference. Mouse FcγRI, FcγRIIB, FcγRIII, and FcγRIV were serially diluted from 1 μM to less than 1 nM with running buffer (HBS-EP) and injected for 120 s at 20 μl/min over all FCs followed by a 120 s dissociation step. Full regeneration was achieved using a 15–30-s injection of 1 M MgCl at 100 μl/min. FCs were washed with a 30-s injection of running buffer at 20 μl/min. KDs were generated using a 1:1 Langmuir binding model. Kinetics were run in duplicate.

Tumor growth models

Mice were inoculated subcutaneously with 8×10^6 EL4E7 tumor cells in the flank. Tumor volume was determined by measuring the width (w) and length (l) of the tumor and using the following formula: $(w^2*l)*0.5$. Mice bearing tumors with a length of 5 mm on Day 7 and 8 mm on Day 10 were randomized into two groups that received a first injection of 100 μg of either Rat IgG or 2A by intraperitoneal injection on Day 7 or Day 10 and a second injection, 3 days later. Tumor growth was monitored until mice appeared moribund or the diameter of the tumors reached 25 mm. Mice were killed, based on these endpoints, and this was recorded as the date of death for survival studies. Mice with complete tumor resolution were challenged with EL4E7 and B16-F10 tumor cells. Tumor growth was monitored until the combined tumor volume reached approximately 20 % of the mouse's total body weight, and then, the mice were killed.

To evaluate immune cell kinetics, mice bearing tumors with an average length of 5 mm on Day 7 were randomized into two groups, which received 100 μg of either Rat

IgG or 2A, by intraperitoneal injection on Days 7 and 10. Two or three mice per group were euthanized on Day 7, Day 12, Day 16, and Day 21, and spleens were processed. Cell counts from individual spleens were taken manually or with the Countess automated cell counter (Invitrogen) using trypan blue exclusion to count live cells. Total cell numbers were calculated by multiplying the cell count by the lymphocyte gate and then by the appropriate gate of the cell population of interest.

Competition assay for CD137L binding

HEK-293 cells were stably transfected with mouse CD137 using lipofectamine (Invitrogen) transfection with a pcDNA3.3 vector containing CD137. The HEK-mCD137 cells were incubated with Rat IgG or anti-CD137 mAb (clone 2A or 3H3) at 0.1, 1, 10, or 100 μg/ml, followed by incubation with 31 ng/ml of His-tagged recombinant mouse CD137L (R&D systems). Finally, cells were incubated with anti-polyhistidine PE (R&D systems) to determine CD137L binding. Data were acquired on an LSRII instrument (BD Biosciences) and analyzed using FlowJo (TreeStar).
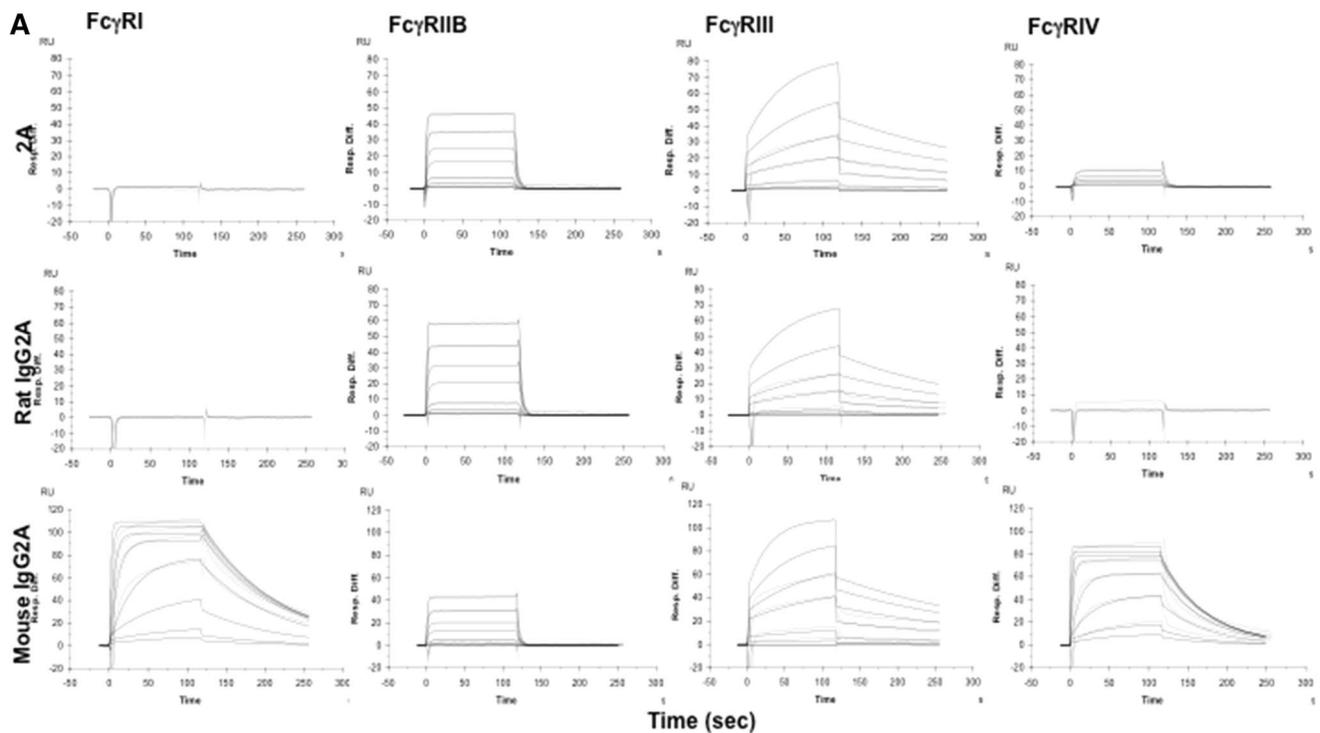
Statistical analysis

All Kaplan–Meier survival curves were compared using the log-rank test. Statistical significance was determined using a one-way ANOVA with Tukey's multiple comparison test. All statistical analyses were performed using GraphPad (Prism). A p value < 0.05 was considered significant.

Results

Rat anti-mouse CD137 binds to mouse FcγRIIB, FcγRIII, and FcγRIV

The majority of anti-CD137 mAbs used to study the impact of CD137 ligation on anti-tumor immunity are of rat origin [2, 12, 13]. As a first step in understanding the potential of the Fc fragments of these rat mAbs to impact the anti-tumor immune response in mice, we characterized the interactions between rat IgG2a and mouse FcγRs by surface plasmon resonance. We found that mouse FcγRIIB, FcγRIII, and FcγRIV, but not FcγRI, bound to immobilized rat anti-CD137 (clone 2A) (Fig. 1). FcγRIIB and FcγRIII bound comparably to both immobilized 2A and control mouse IgG2a (anti-CD137 mAb: FcγRIIB KD = 528 nM, FcγRIII KD = 238 nM; mouse IgG2a: FcγRIIB KD = 501 nM, FcγRIII KD = 138 nM). In contrast, the interactions between FcγRIV and 2A (KD = 3180 nM) were dramatically weaker than correlate interactions between FcγRIV

Fig. 1 2A binds to mouse FcγRIIB, FcγRIII, and FcγRIV but not to FcγRI. **a** Mouse IgG2a, Rat IgG2a, and 2A were bound to a SPR chip at 10 μg/ml. Recombinant mouse FcγRs were serially diluted from 1 μM to less than 1nM with running buffer (HBS-EP), injected for 120 s at 20 μl/min over all FCs, and followed by a 120 s dissociation step. **b** The KD values (nM) and the Chi^2 in parenthesis

and control mouse IgG2a (KD = 8.63 nM). These data indicate that rat IgG2a, the isotype of the anti-CD137 mAbs used in this study, has the potential to interact with select murine FcγRs.

Anti-CD137 has enhanced activity against EL4E7 in mice lacking the common Fcγ-chain

We validated the absolute requirement for host expression of CD137 on the anti-tumor activity of 2A. As anticipated, 2A failed to mediate tumor regression in CD137$^{-/-}$ mice, while retaining its therapeutic efficacy in wild-type (WT) animals (supplementary Fig. 1A, WT 2A vs. WT IgG $p < 0.0001$; WT 2A vs. CD137$^{-/-}$ 2A $p = 0.0013$). In order to rule out the possibility that EL4E7 might express CD137 and/or FcγRs and thus confound our findings, we determined the levels of CD137 and FcγRs on the EL4E7 tumors. The ex vivo EL4E7 tumor samples lacked

expression of CD137 (supplementary Fig. 1B). Furthermore, the EL4E7 tumor cells expressed exceptionally low levels of FcγRIII and/or FcγRIIB (supplementary Fig. 1C). These data confirm that the EL4E7 tumor is an appropriate model for determining the role of Fc–FcγR interactions on the anti-tumor function of anti-CD137, independent of direct antibody–tumor interactions.

As an initial step in defining the potential role of Fc–FcγR interactions on the anti-tumor activity of anti-CD137, we employed Fc common γ-chain (FcRγ) and FcγRIIB-deficient mice. The FcR mice do not express activating FcγRs, yet maintain expression of FcγRIIB, whereas the FcγRIIB$^{-/-}$ mice only express the activating FcγRs [14]. The FcR and FcγRIIB$^{-/-}$ mice were challenged with EL4E7 tumors and treated with 2A on days 7 and 10 post-tumor inoculation. Unexpectedly, the both FcγRIIB$^{-/-}$ and WT mice had a similar anti-tumor response to 2A, with both experiencing progressive disease (Fig. 2a,
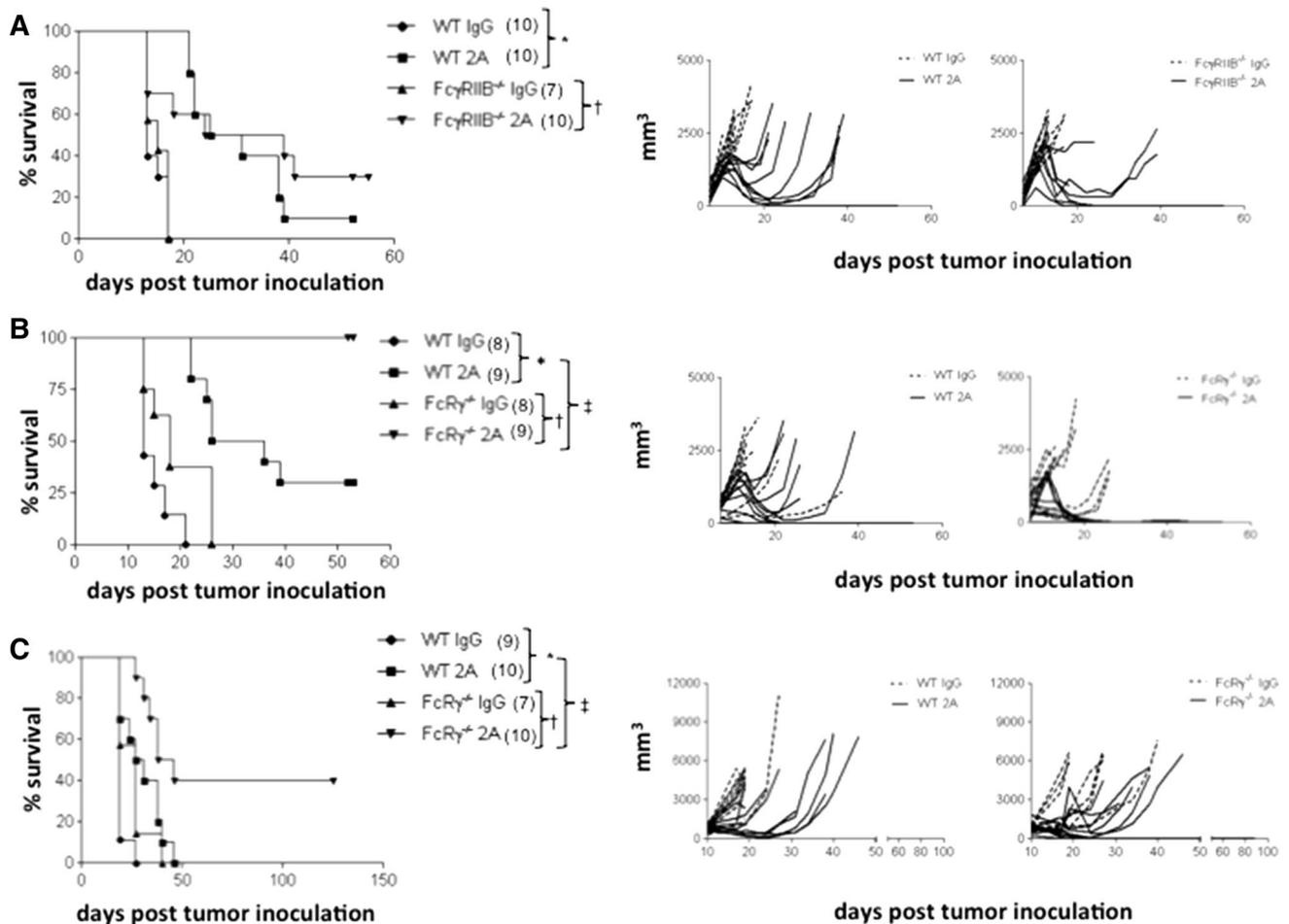
Fig. 2 2A treatment preferentially enhances survival of EL4E7 tumor-bearing FcRγ$^{-/-}$ mice. Mice were inoculated with 8×10^6 EL4E7 tumor cells on Day 0. Survival and growth curves for mice, **a** FcγRIIB$^{-/-}$ *WT 2A versus WT IgG $p < 0.0001$ and †FcγRIIB$^{-/-}$ 2A versus FcγRIIB$^{-/-}$ IgG $p = 0.0128$, and **b** Fcγ$^{-/-}$ *WT 2A versus WT IgG $p < 0.0001$, †FcRγ$^{-/-}$ 2A versus FcRγ$^{-/-}$ IgG $p < 0.0001$, and ‡FcRγ$^{-/-}$ 2A versus WT 2A $p = 0.002$, treated with 100 µg of either

Rat IgG or 2A on Day 7 and Day 10. **c** Survival and growth curves for FcRγ$^{-/-}$ mice treated with 100 µg of either Rat IgG or 2A on Day 10 and Day 13, *WT 2A versus WT IgG $p = 0.0063$, †FcRγ$^{-/-}$ 2A versus FcRγ$^{-/-}$ IgG $p = 0.0027$, and ‡FcRγ$^{-/-}$ 2A versus WT 2A $p = 0.0161$. *Numbers* in parentheses indicate the number of mice. Statistical significance determined by log-rank test

FcγRIIB$^{-/-}$ 2A vs. FcγRIIB$^{-/-}$ IgG $p = 0.0128$; WT 2A vs WT IgG $p < 0.0001$). In contrast, the FcR mice had improved survival compared to both WT animals treated with 2A and control mice treated with rat IgG, (Fig. 2b, FcRγ$^{-/-}$ 2A vs. FcRγ$^{-/-}$ IgG $p < 0.0001$, FcRγ$^{-/-}$ 2A vs. WT 2A $p = 0.002$, WT 2A vs. WT IgG $p < 0.0001$). These data indicate FcγRIIB engagement is not involved in enhancing the 2A-mediated anti-tumor immune response. We further assessed the enhanced anti-tumor response in the 2A-treated FcRγ$^{-/-}$ mice by delaying treatment with 2A to days 10 and 13 post-tumor inoculation. The 2A-treated FcRγ$^{-/-}$ mice had significantly improved anti-tumor responses compared to the 2A treated WT mice and IgG controls (Fig. 2c, FcRγ$^{-/-}$ 2A vs. FcRγ$^{-/-}$ IgG $p = 0.0027$; FcRγ$^{-/-}$ 2A vs. WT 2A $p = 0.0161$, WT 2A vs. WT IgG $p = 0.0063$). The FcRγ$^{-/-}$ mice, which

enjoyed initial 2A-mediated tumor rejection, were immune to distant challenge with EL4E7 but not B16 melanoma (data not shown). Taken in concert, these data indicate the expression of the Fcγ chain impairs the 2A-mediated immune response against EL4E7.

Anti-CD137 has enhanced activity against EL4E7 in FcγRIII$^{-/-}$ mice

Next, we sought to identify the individual activating FcγR contributing to the improved 2A-mediated anti-tumor immunity in FcRγ$^{-/-}$ mice. We studied the contribution of FcγRIII and FcγRIV, excluding FcγRI based on the lack of binding to rat IgG2a (Fig. 1). 2A treatment significantly improved survival of tumor-bearing FcγRIII$^{-/-}$, but not FcγRIV$^{-/-}$ mice versus WT controls (Fig. 3a,
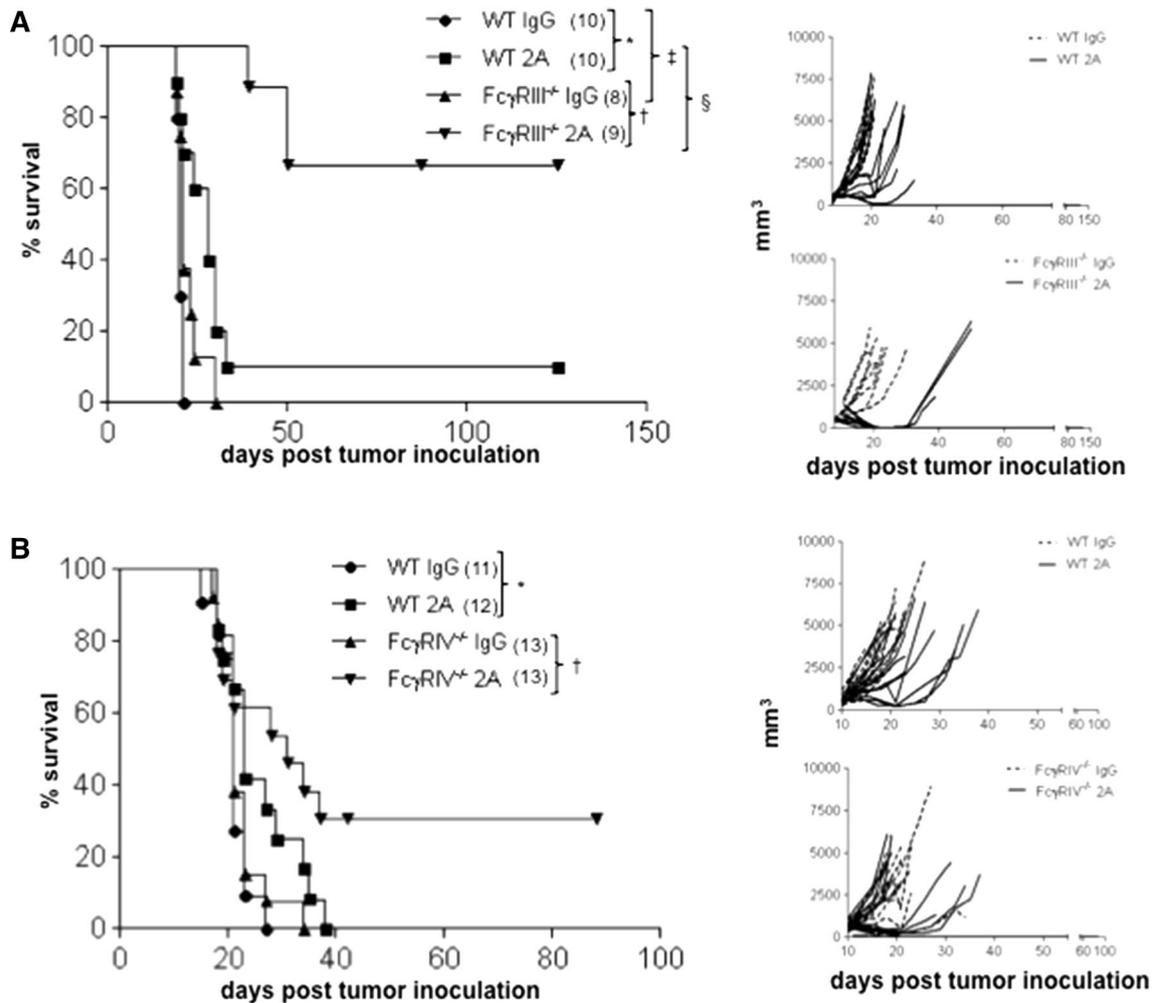
Fig. 3 2A treatment preferentially enhances survival of EL4E7 tumor-bearing FcγRIII$^{-/-}$ mice. Mice were inoculated with 8×10^6 EL4E7 tumor cells on Day 0. On Day 10 and Day 13 mice were treated with either 100 μg of 2A or Rat IgG. **A** FcγRIII$^{-/-}$; *WT IgG versus WT 2A $p = 0.0023$, †FcγRIII$^{-/-}$ IgG versus FcγRIII$^{-/-}$ 2A $p < 0.0001$, ‡WT IgG versus FcγRIII$^{-/-}$ IgG $p = 0.0311$, and §WT 2A versus FcγRIII$^{-/-}$ 2A $p = 0.0006$. **b** FcγRIV$^{-/-}$; *WT IgG versus WT 2A $p = 0.0484$ and †FcγRIV$^{-/-}$ IgG versus FcγRIV$^{-/-}$ 2A $p = 0.0217$. *Number* in parenthesis indicates the number of mice. Both figures are the results of two pooled experiments. Statistical significance was determined by the Log-rank Test

FcγRIII$^{-/-}$ 2A vs. FcγRIII$^{-/-}$ IgG $p < 0.0001$; WT 2A vs. WT IgG $p = 0.0023$; FcγRIII$^{-/-}$ IgG vs. WT IgG $p = 0.0311$, and FcγRIII$^{-/-}$ 2A vs. WT 2A $p = 0.0006$ and, 3B, FcγRIV$^{-/-}$ 2A vs. FcγRIV$^{-/-}$ IgG $p = 0.0217$; WT 2A vs. WT IgG $p = 0.0484$; and FcγRIV$^{-/-}$ 2A vs. WT 2A $p = 0.1062$). FcγRIII$^{-/-}$ mice, which enjoyed initial 2A-mediated tumor rejection, were immune to second challenge with EL4E7 but not B16-F10 melanoma (data not shown). These data identify FcγRIII as the receptor involved in the modulation of the anti-CD137-mediated anti-tumor response against EL4E7.

To exclude the possibility that our observations were 2A specific, we evaluated a second clone of anti-CD137 mAb, 3H3 (rat IgG2a), in FcγRIII$^{-/-}$ mice. 3H3 preferentially prolonged survival of EL4E7 tumor-bearing

FcγRIII$^{-/-}$ mice, but, unlike 2A, did not result in complete tumor resolution (Fig. 4a, FcγRIII$^{-/-}$ 3H3 vs. FcγRIII$^{-/-}$ IgG $p = 0.015$ and FcγRIII$^{-/-}$ 3H3 vs. WT 3H3 $p = 0.0010$). Differences in the absolute efficacy of these individual mAbs may relate to the fact that at higher doses, 3H3, but not 2A, blocks naturally occurring CD137–CD137L interactions (Fig. 4b and c). These data demonstrate that FcγRIII impairs anti-CD137-mediated anti-tumor immunity with antibody-dependent variation in therapeutic benefit.

To determine whether the modulation of the 2A-mediated anti-tumor response in the FcγRIII$^{-/-}$ mice could be recapitulated in another tumor type, we utilized the MC38 colon cancer model, which has a weak therapeutic response to 2A treatment [15]. Both WT and FcγRIII$^{-/-}$ mice
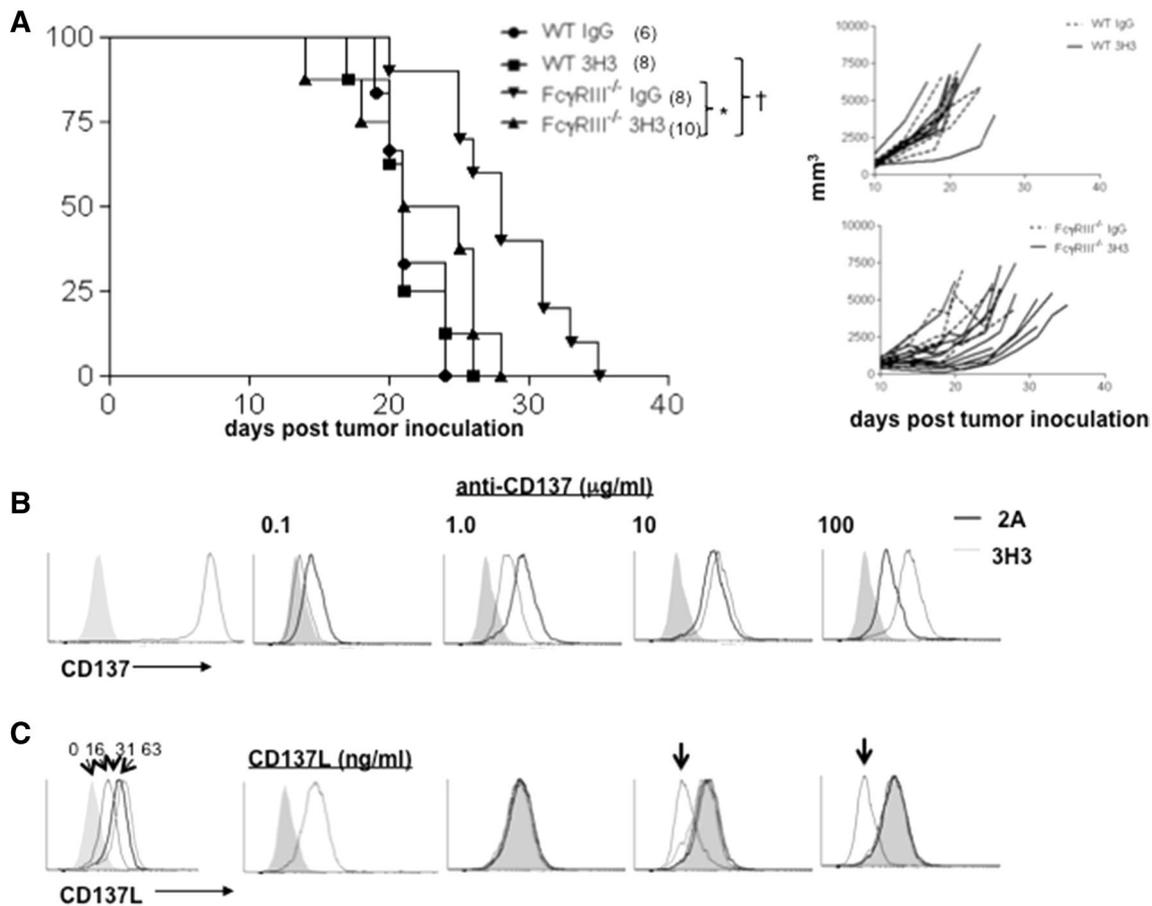
Fig. 4 The 3H3-mediated anti-EL4E7 response is enhanced in FcγRIII$^{-/-}$ mice. **a** Mice were inoculated with 8×10^6 EL4E7 tumor cells on Day 0. On Day 10 and Day 13, mice were treated with either 100 μg of anti-CD137 mAb or Rat IgG. *Numbers* in parentheses indicate the number of mice. Statistical significance was determined by the log-rank test: *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$. Survival curve and growth curves represent the sum of two experiments. **b** HEK-mCD137L expressing cells incubated with anti-CD137 mAb clone 2A or 3H3. **c** HEK-mCD137L expressing cells incubated with recombinant mCD137L at 31 ng/ml (2nd panel). Cells were initially incubated with anti-CD137 mAb at concentrations listed in **a**, followed by secondary incubation with mCD137L at 31 ng/ml

experienced equivalent MC38 tumor growth in response to 2A (data not shown). This result suggests that FcγRIII deficiency is not sufficient for further enhancement of the 2A-mediated anti-tumor response in the MC38 model.

Anti-CD137 therapy preferentially enhances CD8β$^+$ T cell and CD11c$^+$ dendritic cell populations in FcγRIII$^{-/-}$ mice

To understand the mechanisms underlying the improved anti-tumor efficacy of anti-CD137 in FcγRIII$^{-/-}$ mice, we performed a detailed kinetic analysis of CD8β$^+$CD11c$^+$ T cells and DCs, both of which are implicated in 2A-mediated tumor rejection [5, 16]. As anticipated, anti-CD137 treatment resulted in expansion of the CD8β$^+$ T cell population in WT and FcγRIII$^{-/-}$ mice (Fig. 5a). However, the increase of the CD8β$^+$ cell population began at an earlier time point and was dramatically more pronounced in

the spleens of FcγRIII$^{-/-}$ mice (Fig. 5a). Next, based on reports that anti-CD137 treatment mediates CD103 up-regulation on CD8β$^+$CD11c$^+$ cells and that CD103 expression is associated with CD8$^+$ regulatory T cell function, we employed CD103 as a phenotypic marker of suppressor activity [17–19]. Remarkably, in anti-CD137-treated FcγRIII$^{-/-}$ mice, while the ratio of CD103 negative to positive T cells remained stable, there was almost a 1 log order difference in absolute numbers of CD103 negative to positive T cells in the spleen (Fig. 5a). Direct comparison of the number of CD8β$^+$CD11c$^+$CD103$^-$ T cells to tumor volume using Pearson's correlation coefficient suggested that the CD8β$^+$CD11c$^+$CD103$^-$ T cells were involved in tumor resolution (Fig. 5b). Importantly, the expansion of the CD8β$^+$ T cells and tumor resolution were not due to differences in expression of CD137 or CD137L in the WT and FcγRIII$^{-/-}$ mice (Fig. 5c). Finally, there
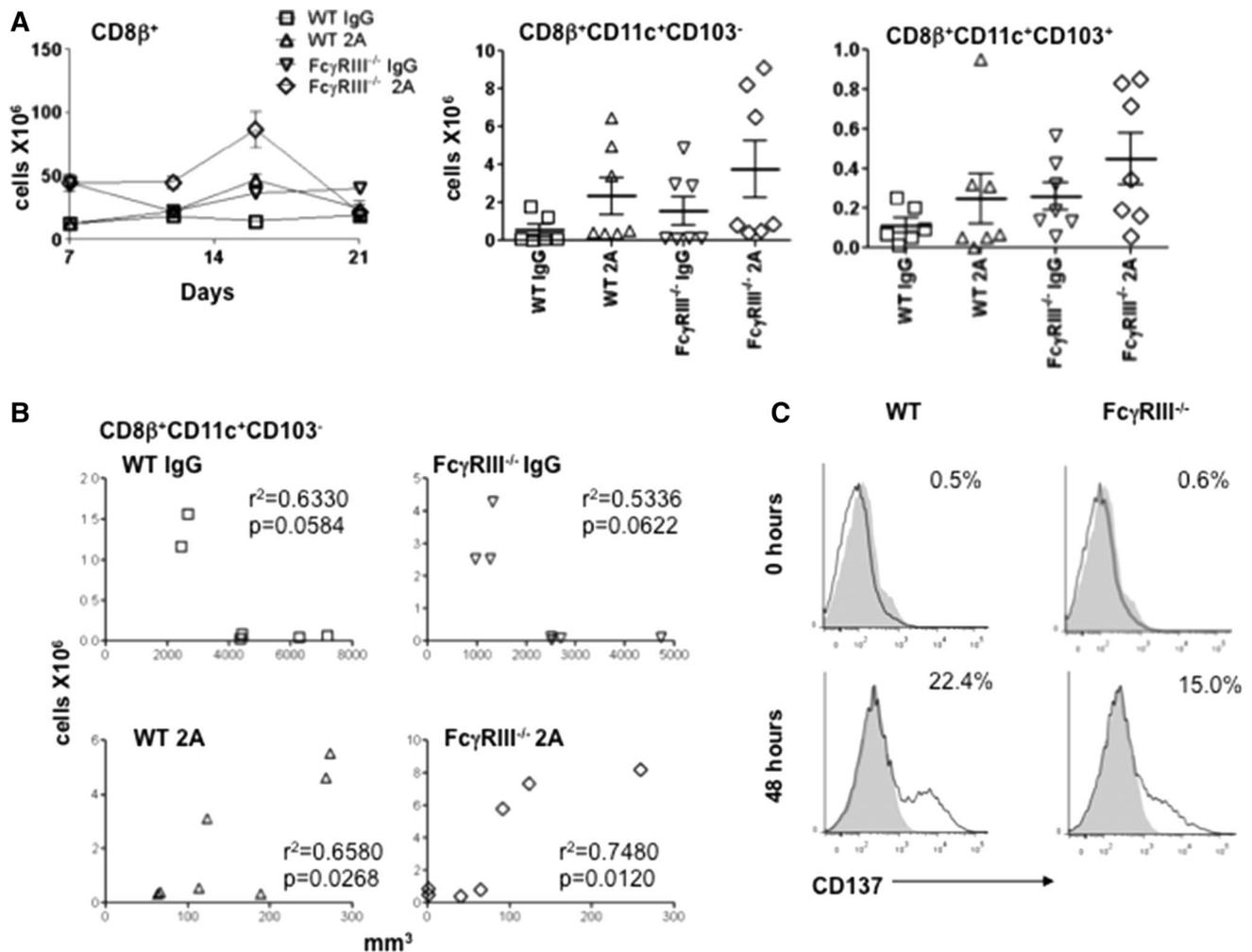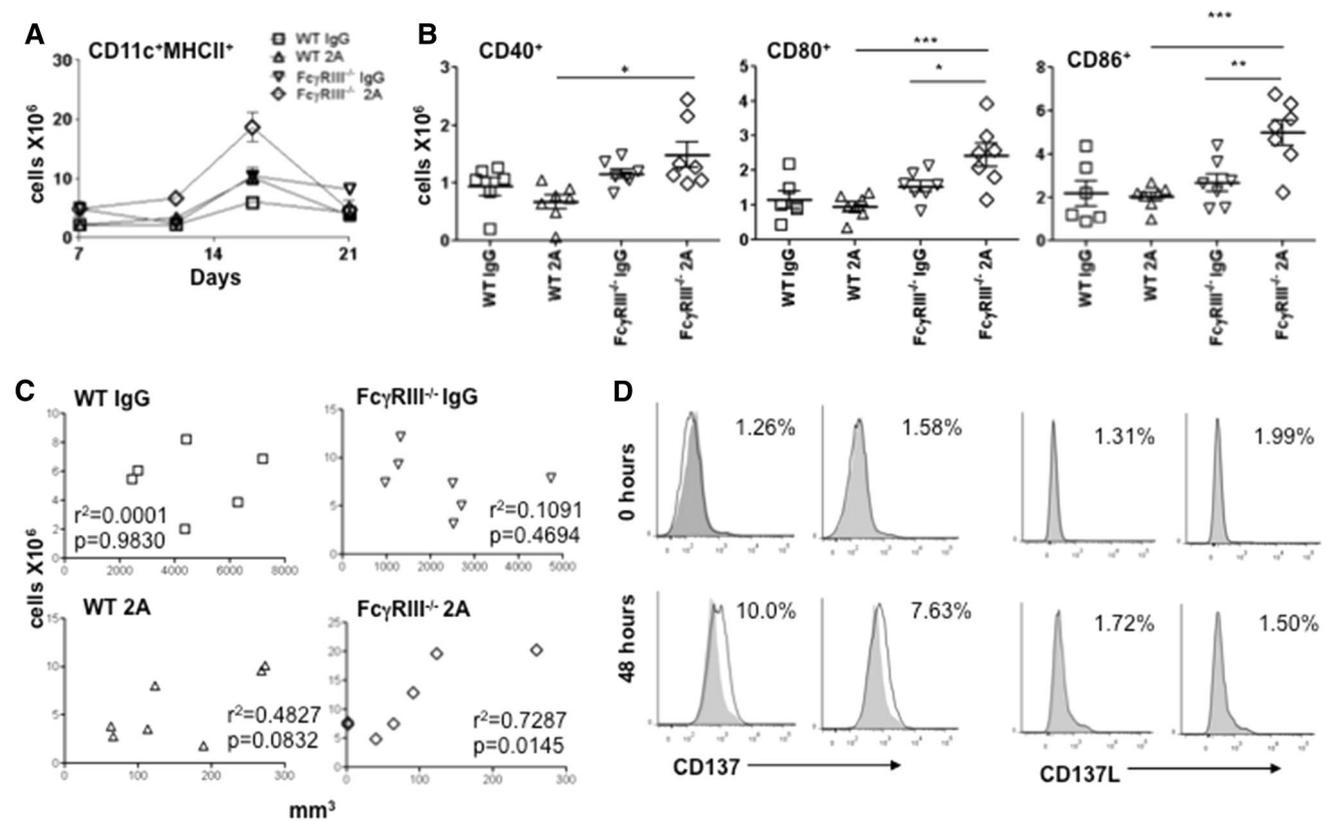
Fig. 5 2A-treated tumor-bearing FcγRIII$^{-/-}$ mice have an increase in CD8β$^+$CD11c$^+$ T cells. **a** Mice were inoculated with 8×10^6 EL4E7 tumor cells on Day 0. On Day 7 and Day 10, mice received either 100 µg of 2A or Rat IgG. Spleens were harvested from 2 to 3 mice per group on Days 7, 12, 16, and 21. Representative data from one experiment repeated three times. **b** Pearson's correlation of CD8β$^+$CD11c$^+$CD103$^-$ absolute cell number versus tumor volume. Data are combined from two experiments for a final $n = 6$ or 7. **c** Cells isolated by positive selection from spleens of naïve WT and FcγRIII$^{-/-}$ mice and stimulated with anti-CD3 and anti-CD28 at 1 µg/ml for 48 h. CD137 expression was determined by flow cytometry. Representative data from one experiment repeated four times

was no antibody-dependent cellular cytotoxicity (ADCC) detected using either WT or FcγRIII$^{-/-}$ NK incubated with 2A-opsonized ConA-stimulated T cells, suggesting the enhanced CD8β$^+$ T cells in the 2A-treated FcγRIII$^{-/-}$ mice was not due to ADCC mediated T cell depletion in the WT mice (Supplementary Fig. 2). These data indicate the FcγRIII$^{-/-}$ mice produce a more rapid and robust CD8β$^+$CD11c$^+$ T cell response after 2A treatment in the EL4E7 tumor model.

DCs have been implicated in the anti-tumor function of anti-CD137 treatment [6]. FcγRIII is not expressed on CD8β$^+$ T cells, suggesting FcγR$^+$ DCs are likely responsible for the regulation of the CD8β$^+$ T cell response. Interestingly, the CD11c$^+$MHCII$^+$ cell population was preferentially expanded in the spleens of 2A-treated

FcγRIII$^{-/-}$ mice (Fig. 6a). Three lines of evidence support the role of these CD11c$^+$MHCII$^+$ cells in mediating the enhanced anti-tumor function of 2A in FcγRIII$^{-/-}$ mice. First, these FcγRIII$^{-/-}$ CD11c$^+$MHCII$^+$ cells evidenced increased expression of CD40, CD80, and CD86, suggesting their potential for enhanced antigen presentation (Fig. 6b). From a functional perspective, the enhanced antigen presentation capacity of these cells was indirectly supported by observed increases in the numbers of OT-I transferred T cells in 2A-treated FcγRIII$^{-/-}$ mice (data not shown). Second, similar to our T cell data, a significant positive Pearson's correlation was found between the CD11c$^+$MHCII$^+$ cell population and the tumor volume in the 2A-treated FcγRIII$^{-/-}$ mice (Fig. 6c). Third, the increased numbers of CD11c$^+$MHCII$^+$ cells in

Fig. 6 2A-treated tumor-bearing FcγRIII$^{-/-}$ mice have an increase in CD11c$^+$ MHCII$^+$ cells with an APC phenotype due to the FcγRIII deficiency. **a** Mice were inoculated with 8×10^6 EL4E7 tumor cells on Day 0. On Day 7 and Day 10, mice received either 100 µg of 2A or Rat IgG. Spleens were harvested from 2 to 3 mice per group on Days 7, 12, 16, and 21. Representative data from one experiment repeated three times. **b** The expression of CD40, CD80, and CD86 on CD11c$^+$ MHCII$^+$ cells from spleen on Day 16. Data combined from two experiments for a final $n = 6$ or 7. A significance differ- ence determined by one way ANOVA and Tukey's multiple compari- son test * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. **c** Pearson's correlation of CD11c$^+$MHCII$^+$ absolute cell number versus tumor volume. Data combined from two experiments for a final $n = 6$ or 7. **d** Cells were isolated by positive selection from spleens of naïve WT and FcγRIII$^{-/-}$ mice. CD11c$^+$ cells were stimulated with 5 µg/ ml of LPS for 48 h. CD137 and CD137L expression were determined by flow cytometry. Representative data from one experiment repeated four times

FcγRIII$^{-/-}$ treated mice corresponded with the presence of disease, suggesting that CD11c$^+$MHCII$^+$ cells are involved in tumor regression. Importantly, the expansion of the CD11c$^+$MHCII$^+$ cell population was not due to differences in the expression of CD137 or CD137L in the WT and FcγRIII$^{-/-}$ mice (Fig. 6d). Taken together with the increase in the CD8β T cell population, our data suggest a paradigm in which 2A treatment preferentially enhances DC numbers and function in FcγRIII$^{-/-}$ mice, with resultant secondary expansion of effector T cells, at least in the EL4E7 tumor model.

Discussion

In this study, we investigated the contributions of Fc–FcγR interactions to the anti-tumor activity of anti-CD137. Our data indicate that anti-CD137-treated FcγRIII$^{-/-}$ mice

have improved responses against EL4E7, which are associ- ated with an increase in CD8β$^+$ T cells and phenotypically activated DCs in the spleen. Our results suggest a paradigm in which anti-CD137 treatment preferentially enhances DC function in tumor-bearing FcγRIII$^{-/-}$ mice, with resultant increases in effector T cell activity against EL4E7.

The anti-tumor function of anti-CD137 was defined in preclinical mouse models using rat–anti-mouse Abs [2, 4, 13]. Despite the recognized cross-reactivity between mouse FcγRs and rat IgG, little is known about the binding affinity and kinetics of rat Fc–mouse FcγR interactions. Our data indicate that mouse FcγRIIB and FcγRIII interact with rat IgG2a in a manner similar to control mouse IgG2a, whereas FcγRI and FcγRIV, the high-affinity receptors, have differ- ent binding affinities and kinetics for rat and mouse IgG2a (Fig. 1). Our binding prolife for rat IgG2a to mouse FcγRs is similar that of mouse IgG1 [7]. This similarity in binding profiles between rat IgG2a and mouse IgG1 is anticipated

based on their structural homology [20]. Collectively, these data and findings from other investigators suggest that rat IgG2a has the potential to interact with mouse FcγRs in a manner similar to mouse IgG1.

The import of Fc–FcγR interactions in mediating the anti-tumor activity of immunomodulatory mAbs has been acknowledged in recent publications. For example, engagement of activating FcγRs is required for the anti-tumor function of anti-GITR and anti-CTLA-4 mAbs [9, 10]. Interestingly, agonistic mAbs against CD40 are functionally dependent on interactions with FcγRIIB [7, 8]. Our data add to this growing body of literature, showing that Fc–FcγRIII interactions dampen the anti-CD137-mediated anti-tumor response against EL4E7.

The exact mechanism by which FcγRIII attenuates anti-CD137 immunity against EL4E7 is uncertain. FcγRIII is associated with both pro- and anti-inflammatory responses [21–25]. For example, FcγRIII is associated with inhibitory ITAM signaling resulting from tonic Fc–FcγR interactions that initiate inhibisome formation [21, 26]. A recent publication demonstrates anti-CD137 mAb is rapidly internalized after Fab-CD137 interactions, which would limit Fc exposure to FcγRIII [27]. Therefore, the combined tonic Fc interaction with FcγRIII may inhibit the NF-κB signaling induced by anti-CD137 mAb interactions resulting in a reduction of CD137 stimulation [27]. Besides the induction of inhibitory signaling, FcγRIII is involved in the clearance of apoptotic cells [25]. The reduction in the clearance of apoptotic cells would allow for enhanced tumor antigen exposure. Taken in concert with our data, these published findings suggest that the improved anti-tumor activity of anti-CD137 in FcγRIII$^{-/-}$ is likely a result of several non-mutually exclusive and potentially overlapping mechanisms.

Our data do not rule out the potential role of FcγRIV in modulating anti-CD137 mAb tumor responses. FcγRIV expression is limited to Ly6C^{lo} monocytes, macrophages, and neutrophils, which are common tumor-infiltrating leukocytes [29, 30]. CD137 expression is highest on tumor-infiltrating lymphocytes, potentially enabling enhanced localization of Fc–FcγRIV interactions at the tumor site [28]. These expression patterns of CD137 and FcγRIV in vivo may surmount the weaker interaction of FcγRIV to rat IgG2a by increasing the concentration of 2A at the tumor and may explain the trend toward improvement of the anti-tumor response in 2A-treated FcγRIV$^{-/-}$ mice.

To understand the mechanisms underlying the improved anti-tumor efficacy of anti-CD137 mAb in FcγRIII$^{-/-}$ mice, we performed detailed kinetic analysis of CD8$^+$ T cells and DCs, which are implicated in 2A-mediated tumor rejection [4, 5, 31–36]. Following anti-CD137 therapy, we observed a profound increase in the CD8β$^+$ T cell and DC populations, which was dramatically enhanced in the FcγRIII$^{-/-}$ mice. CD8β$^+$ T cells do not express FcγRs suggesting a model in which FcγRIII deficiency on DCs is modulating the anti-tumor immune response elicited by anti-CD137 in the EL4E7 tumor model [6].

DCs are considered professional antigen-presenting cells, and improved antigen presentation would result in an enhanced CD8β$^+$ T cell response [37]. The 2A-treated FcγRIII$^{-/-}$ mice had increases in numbers of DCs expressing costimulatory molecules associated with antigen presentation. CD137 stimulation likely contributed to this phenotype as: (1) FcγRIII$^{-/-}$ DCs have comparable antigen presentation capabilities as WT DCs and (2) anti-CD137 stimulation enhances CD40 stimulation of DCs corresponding to an increase in tumor-infiltrating T cells [38, 39]. The lack of potential Fc–FcγRIII interactions is also functionally relevant as the loss of ITAM signaling on DCs is recognized to improve toll-like receptor stimulation by increasing costimulatory molecule expression and the production of pro-inflammatory cytokines [40]. When taken in combination with our findings, these studies imply that antibody binding to CD137, in the absence of FcγRIII interactions, results in enhanced antigen presentation capabilities that promote the secondary expansion of CD8β$^+$ T cells against EL4E7.

Our data suggest that anti-CD137 mAb interactions with FcγRIII inhibit anti-tumor immune responses to EL4E7 tumors. When taken in combination with the rapidly evolving literature in this field, our studies lend credence to the idea that the functional role of Fc–FcγR interactions on antibody-mediated immune modulatory therapy is defined by both patterns of receptor and relevant FcR expression. These data suggest that Fc–FcγR interactions play a critical role in the biology of antibodies targeting co-signaling molecules and that manipulation of these interactions may allow for improved therapeutic efficacy.

References

1. Wang C, Lin GHY, McPherson AJ, Watts TH (2009) Immune regulation by 4-1BB and 4-1BBL: complexities and challenges. Immunol Rev 229:192–215. doi:10.1111/j.1600-065X.2009.00765

2. Wilcox RA, Flies DB, Zhu G, Johnson AJ, Tamada K, Chapoval AI, Strome SE, Pease LR, Chen L (2002) Provision of antigen and CD137 signaling breaks immunological ignorance, promoting regression of poorly immunogenic tumors. J Clin Invest 109:651–659. doi:10.1172/JCI14184

3. Choi BK, Kim YH, Kang WJ, Lee SK, Kim KH, Shin SM, Yokoyama WM, Kim TY, Kwon BS (2007) Mechanisms involved in synergistic anticancer immunity of anti-4-1BB and anti-CD4 therapy. Cancer Res 67:8891–8899. doi:10.1158/0008-5472.CAN-07-1056

4. Melero I, Shuford WW, Newby SA, Aruffo A, Ledbetter JA, Hellstrom KE, Mittler RS, Chen L (1997) Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. Nat Med 3:682–685. doi:10.1038/nm0697-682

5. Murillo O, Dubrot J, Palazón A, Arina A, Azpilikueta A, Alfaro C, Solano S, Ochoa MC, Berasain C, Gabari I, Pérez-Gracia JL, Berraondo P, Hervás-Stubbs S, Melero I (2009) In vivo depletion of DC impairs the anti-tumor effect of agonistic anti-CD137 mAb. Eur J Immunol 39:2424–2436. doi:10.1002/eji.200838958

6. Bruhns P (2012) Properties of mouse and human IgG receptors and their contribution to disease models. Blood 119:5640–5649. doi:10.1182/blood-2012-01-380121

7. White AL, Chan HT, Roghanian A, French RR, Mockridge CI, Tutt AL, Dixon SV, Ajona D, Verbeek JS, Al-Shamkhani A, Cragg MS, Beers SA, Glennie MJ (2011) Interaction with FcγRIIB Is Critical for the agonistic activity of anti-CD40 monoclonal antibody. J Immunol 187:1754–1763. doi:10.4049/jimmunol.1101135

8. Li F, Ravetch JV (2011) Inhibitory Fc gamma receptor engagement drives adjuvant and anti-tumor activities of agonistic CD40 antibodies. Science 333:1030–1034. doi:10.1126/science.1206954

9. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, Roddie C, Henry JY, Yagita H, Wolchok JD, Peggs KS, Ravetch JV, Allison JP, Quezada SA (2013) Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti–CTLA-4 therapy against melanoma. J Exp Med 210:1695–1710. doi:10.1084/jem.20130579

10. Bulliard Y, Jolicoeur R, Windman M, Rue SM, Ettenberg S, Knee DA, Wilson NS, Dranoff G, Brogdon JL (2013) Activating Fc γ receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies. J Exp Med 210:1685–1693. doi:10.1084/jem.20130573

11. Lin W, Voskens CJ, Zhang X, Schindler DG, Wood A, Burch E, Wei Y, Chen L, Tian G, Tamada K, Wang LX, Schulze DH, Mann D, Strome SE (2008) Fc-dependent expression of CD137 on human NK cells: insights into "agonistic" effects of anti-CD137 monoclonal antibodies. Blood 112:699–707. doi:10.1182/blood-2007-11-122465

12. Kim JA, Averbook BJ, Chambers K, Rothchild K, Kjaergaard J, Papay R, Shu S (2001) Divergent effects of 4-1BB antibodies on antitumor immunity and on tumor-reactive T-cell generation. Cancer Res 61:2031–2037

13. Shuford WW, Klussman K, Tritchler DD, Loo DT, Chalupny J, Siadak AW, Brown TJ, Emswiler J, Raecho H, Larsen CP, Pearson TC, Ledbetter JA, Aruffo A, Mittler RS (1997) 4-1BB costimulatory signals preferentially induce CD8 + T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. J Exp Med 186:47–55. doi:10.1084/jem.186.1.47

14. Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV (1994) FcR gamma chain deletion results in pleiotrophic effector cell defects. Cell 76:519–529. doi:10.1016/0092-8674(94)90115-5

15. Dubrot J, Milheiro F, Alfaro C, Palazón A, Martinez-Forero I, Pérez-Gracia J, Morales-Kastresana A, Romero-Trevejo J, Ochoa M, Hervás-Stubbs S, Prieto J, Jure-Kunkel M, Chen L, Melero I (2010) Treatment with anti-CD137 mAbs causes intense accumulations of liver T cells without selective antitumor immunotherapeutic effects in this organ. Cancer Immunol Immunother 59:1223–1233. doi:10.1007/s00262-010-0846-9

16. Ju SA, Park SM, Lee SC, Kwon BS, Kim BS (2007) Marked expansion of CD11c + CD8 + T-cells in melanoma-bearing mice induced by anti-4-1BB monoclonal antibody. Mol Cells 24:132–138

17. Uss E, Rowshani AT, Hooibrink B, Lardy NM, van Lier RAW, ten Berge IJM (2006) CD103 is a marker for alloantigen-induced regulatory CD8 + T cells. J Immunol 177:2775–2783. doi:10.4049/jimmunol.177.5.2775

18. Myers L, Croft M, Kwon BS, Mittler RS, Vella AT (2005) Peptide-specific CD8 T regulatory cells use IFN-γ to elaborate TGF-β based suppression. J Immunol 174:7625–7632. doi:10.4049/jimmunol.174.12.7625

19. Vinay DS, Kim CH, Choi BK, Kwon BS (2009) Origins and functional basis of regulatory CD11c + CD8 + T cells. Eur J Immunol 39:1552–1563. doi:10.1002/eji.200839057

20. Bruggemann M, Free J, Diamond A, Howard J, Cobbold S, Waldmann H (1986) Immunoglobulin heavy chain locus of the rat: striking homology to mouse antibody genes. Proc Natl Acad Sci USA 83:6075–6079

21. Aloulou M, Ben Mkaddem S, Biarnes-Pelicot M, Boussetta T, Souchet H, Rossato E, Benhamou M, Crestani B, Zhu Z, Blank U, Launay P, Monteiro RC (2012) IgG1 and IVIg induce inhibitory ITAM signaling through FcγRIII controlling inflammatory responses. Blood 119:3084–3096. doi:10.1182/blood-2011-08-376046

22. Ivashkiv LB (2009) Cross-regulation of signaling by ITAM-associated receptors. Nat Immunol 10:340–347. doi:10.1038/ni.1706

23. Siragam V, Crow AR, Brinc D, Song S, Freedman J, Lazarus AH (2006) Intravenous immunoglobulin ameliorates ITP via activating Fc gamma receptors on dendritic cells. Nat Med 12:688–692. doi:10.1038/nm1416

24. Sutterwala FS, Noel GJ, Salgame P, Mosser DM (1998) Reversal of proinflammatory responses by ligating the macrophage Fcγ receptor type I. J Exp Med 188:217–222. doi:10.1084/jem.188.1.217

25. Erdinc Sunay MM, Fox-Talbot K, Velidedeoglu E, Baldwin WM III, Wasowska BA (2013) Absence of FcγRIII results in increased proinflammatory response in FcγRIII-KO cardiac recipients. Transplantation 96:601–608. doi:10.1097/TP.0b013e31829c2455

26. Pfirsch-Maisonnas S, Aloulou M, Xu T, Claver J, Kanamaru Y, Tiwari M, Launay P, Monteiro RC, Blank U (2011) Inhibitory ITAM signaling traps activating receptors with the phosphatase SHP-1 to form polarized "inhibisome" clusters. Sci Signal 4:ra24. doi:10.1126/scisignal.2001309

27. Martinez-Forero I, Azpilikueta A, Bolanos-Mateo E, Nistal-Villan E, Palazon A, Teijeira A, Perez-Chacon G, Morales-Kastresana A, Murillo O, Jure-Kunkel M, Zapata JM, Melero I (2013) T cell costimulation with anti-CD137 monoclonal antibodies is mediated by K63-polyubiquitin-dependent signals from endosomes. J Immunol 190:6694–6706. doi:10.4049/jimmunol.1203010

28. Palazon A, Martinez-Forero I, Teijeira A, Morales-Kastresana A, Alfaro C, Sanmamed MF, Perez-Gracia JL, Penuelas I, Hervas-Stubbs S, Rouzaut A, de Landazuri MO, Jure-Kunkel M, Aragones J, Melero I (2012) The HIF-1alpha hypoxia response in tumor-infiltrating T lymphocytes induces functional CD137 (4-1BB) for immunotherapy. Cancer Discov 2:608–623. doi:10.1158/2159-8290.CD-11-0314

29. Mancardi DA, Iannascoli B, Hoos S, England P, Daeron M, Bruhns P (2008) FcgammaRIV is a mouse IgE receptor that resembles macrophage FcepsilonRI in humans and promotes IgE-induced lung inflammation. J Clin Invest 118:3738–3750. doi:10.1172/JCI36452

30. Biburger M, Aschermann S, Schwab I, Lux A, Albert H, Danzer H, Woigk M, Dudziak D, Nimmerjahn F (2011) Monocyte subsets responsible for immunoglobulin G-dependent effector functions in vivo. Immunity 35:932–944. doi:10.1016/j.immuni.2011.11.009

31. John LB, Howland LJ, Flynn JK, West AC, Devaud C, Duong CP, Stewart TJ, Westwood JA, Guo ZS, Bartlett DL, Smyth MJ,

Kershaw MH, Darcy PK (2012) Oncolytic virus and Anti–4-1BB combination therapy elicits strong antitumor immunity against established cancer. Cancer Res 72:1651–1660. doi:10.1158/0008-5472.CAN-11-2788

32. Ito F, Li Q, Shreiner AB, Okuyama R, Jure-Kunkel MN, Teitz-Tennenbaum S, Chang AE (2004) Anti-CD137 monoclonal antibody administration augments the antitumor efficacy of dendritic cell-based vaccines. Cancer Res 64:8411–8419. doi:10.1158/0008-5472.CAN-04-0590

33. Cuadros C, Dominguez AL, Lollini P, Croft M, Mittler RS, Borgström P, Lustgarten J (2005) Vaccination with dendritic cells pulsed with apoptotic tumors in combination with anti-OX40 and anti-4-1BB monoclonal antibodies induces T cell–mediated protective immunity in Her-2/neu transgenic mice. Int J Cancer 116:934–943. doi:10.1002/ijc.21098

34. Houot R, Goldstein MJ, Kohrt HE, Myklebust JH, Alizadeh AA, Lin JT, Irish JM, Torchia JA, Kolstad A, Chen L, Levy R (2009) Therapeutic effect of CD137 immunomodulation in lymphoma and its enhancement by Treg depletion. Blood 114:3431–3438. doi:10.1182/blood-2009-05-223958

35. Murillo O, Arina A, Hervas-Stubbs S, Gupta A, McCluskey B, Dubrot J, Palazon A, Azpilikueta A, Ochoa MC, Alfaro C, Solano S, Perez-Gracia JL, Oyajobi BO, Melero I (2008) Therapeutic antitumor efficacy of anti-CD137 agonistic monoclonal antibody in mouse models of Myeloma. Clin Cancer Res 14:6895–6906. doi:10.1158/1078-0432.ccr-08-0285

36. Kim YH, Choi BK, Oh HS, Kang WJ, Mittler RS, Kwon BS (2009) Mechanisms involved in synergistic anticancer effects of anti-4-1BB and cyclophosphamide therapy. Mol Cancer Ther 8:469–478. doi:10.1158/1535-7163.mct-08-0993

37. Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392:245–252. doi:10.1038/32588

38. French RR, Taraban VY, Crowther GR, Rowley TF, Gray JC, Johnson PW, Tutt AL, Al-Shamkhani A, Glennie MJ (2007) Eradication of lymphoma by CD8 T cells following anti-CD40 monoclonal antibody therapy is critically dependent on CD27 costimulation. Blood 109:4810–4815. doi:10.1182/blood-2006-11-057216

39. Miller RE, Jones J, Le T, Whitmore J, Boiani N, Gliniak B, Lynch DH (2002) 4-1BB-specific monoclonal antibody promotes the generation of tumor-specific immune responses by direct activation of CD8 T cells in a CD40-dependent manner. J Immunol 169:1792–1800. doi:10.4049/jimmunol.169.4.1792

40. Chu C, Yu Y, Shen K, Lowell C, Lanier L, Hamerman J (2008) Increased TLR responses in dendritic cells lacking the ITAM-containing adapters DAP12 and FcRγ. Eur J Immunol 38:166–173. doi:10.1002/eji.200737600