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# **In vivo effector functions of high-affinity mouse IgG receptor FcγRI in disease and therapy models**

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## ABSTRACT

Two activating mouse IgG receptors (FcγRs) have the ability to bind monomeric IgG, the high-affinity mouse FcγRI and FcγRIV. Despite high circulating levels of IgG, reports using FcγRI<sup>-/-</sup> or FcγRIV<sup>-/-</sup> mice or FcγRIV-blocking antibodies implicate these receptors in IgG-induced disease severity or therapeutic Ab efficacy. From these studies, however, one cannot conclude on the effector capabilities of a given receptor, because different activating FcγRs possess redundant properties *in vivo*, and cooperation between FcγRs may occur, or priming phenomena. To help resolve these uncertainties, we used mice expressing only FcγRI to determine its intrinsic properties *in vivo*. FcγRI<sup>only</sup> mice were sensitive to IgG-induced autoimmune thrombocytopenia and anti-CD20 and anti-tumour immunotherapy, but resistant to IgG-induced autoimmune arthritis, anaphylaxis and airway inflammation. Our results show that the *in vivo* roles of FcγRI are more restricted than initially reported using FcγRI<sup>-/-</sup> mice, but confirm effector capabilities for this high-affinity IgG receptor *in vivo*.

## 1. INTRODUCTION

IgG receptors (FcγR) in both humans and mice are divided into high-affinity IgG receptors that are able to retain monomeric IgG, and low-affinity IgG receptors that do not. Both high- and low-affinity FcγRs are, however, able to bind to IgG-immune complexes or IgG-opsonised cells and surfaces. In humans only hFcγRI is a high-affinity IgG receptor, for human IgG1, IgG3 and IgG4; and in mice both mFcγRI (for mouse IgG2a) and mFcγRII (for mouse IgG2a and IgG2b) are high-affinity receptors [1]. Although it was proposed that high-affinity FcγRs are occupied by circulating IgG *in vivo* (discussed in [2]), multiple effector roles for hFcγRI, mFcγRI and mFcγRII have been reported using mouse models of disease and therapy [3-6].

hFcγRI has been studied by exogenous expression in hFcγRI<sup>tg</sup> mice, demonstrating its role on dendritic cells in the enhancement of antigen presentation and cross-presentation [7], and on neutrophils and monocyte/macrophages in inflammation, autoimmunity and systemic anaphylaxis [8]. These studies indicate that hFcγRI can, by itself, induce clinical signs of autoimmune diseases, by triggering local inflammation (*e.g.* autoimmune rheumatoid arthritis) or phagocytosis (*e.g.* autoimmune thrombocytopenia and anaemia [9]). Additionally, hFcγRI was reported to induce allergic shock (anaphylaxis) triggered by IgG-immune complexes [8]. Finally, hFcγRI may also be a therapeutic target as it can mediate antibody-based therapies such as anti-malaria [10], anti-metastatic melanoma [8] and angiogenesis prevention [6]. The mouse counterpart of hFcγRI, mFcγRI, has been so far studied only by the effect of its absence. Compared to wild-type, mFcγRI<sup>-/-</sup> mice demonstrate reduced reaction severity in models of autoimmune diseases such as experimental haemolytic anaemia and arthritis [11, 12]. In addition

mFcγRI<sup>-/-</sup> mice are less susceptible to IgG-mediated systemic anaphylaxis, Arthus reactions [13, 14], and show reduced efficacy of anti-melanoma [15-17], anti-lymphoma [18] and anti-angiogenic therapies [6]. mFcγRIV was also initially studied by its absence in mFcγRIV<sup>-/-</sup> mice or by using blocking anti-mFcγRIV mAbs. These studies reported reduced IgG-mediated autoimmune anaemia, thrombocytopenia, rheumatoid arthritis and experimental nephrotoxic nephritis, but also reduced anaphylaxis and less efficient subcutaneous melanoma therapy in the absence or after blockade of mFcγRIV [19, 20]; some of the latter results should be taken with caution since mFcγRIV-blocking antibody 9E9 may also block mFcγRIII *in vivo* [21]. Effector functions could nevertheless be definitively attributed to mFcγRIV through the generation of mice expressing mFcγRIV without other FcγR. Indeed, using mFcγRIV<sup>only</sup> mice we could demonstrate that mFcγRIV can individually induce autoimmune thrombocytopenia and rheumatoid arthritis, as well as IgG-mediated airway inflammation and anaphylaxis, but not anti-metastatic melanoma therapy [8, 22-24]. Altogether these data propose multiple effector functions for high-affinity receptors hFcγRI, mFcγRI and mFcγRIV in autoimmune and inflammatory disease models, and therapy, with direct evidence provided by studies using hFcγRI<sup>tg</sup> mice and mFcγRIV<sup>only</sup> mice, but only indirect evidence provided by mFcγRI<sup>-/-</sup> mice.

The *in vivo* effector functions proposed for mFcγRI in IgG-mediated autoimmune disease and therapy models are surprising, considering its expression is restricted to monocytes, monocyte-derived dendritic cells and some tissue-resident macrophages, and is absent on neutrophils. As several reports suggest redundant functions among mFcγRs (reviewed in [1, 2]), it is uncertain if mFcγRI can induce IgG-mediated autoimmune diseases and therapeutic efficacy by itself, or if this receptor is indirectly involved: either for optimal activation via other mFcγRs or priming of effector cells. Therefore we analysed the *in vivo* effector functions of mFcγRI in

47 mFcγRI<sup>only</sup> mice, *i.e.* in the absence of mFcγRIIB, mFcγRIII and mFcγRIV, in comparison with  
48 mFcγR<sup>null</sup> mice that express no mFcγR. Our results identify the effector functions of mFcγRI as  
49 more restricted than initially reported, but confirm that mFcγRI does function independently *in*  
50 *vivo*, in particular for depletion of IgG-opsonised cells.

51

## 2. MATERIALS & METHODS

### 2.1 Mice

C57BL/6J mice (WT) were purchased from Charles River. VG1505 (FcγRI<sup>only</sup>) mice were reported previously [17] and generated by Regeneron Pharmaceuticals, Inc. FcγR<sup>null</sup> mice were generated by crossing FcγRI<sup>only</sup> mice to FcγRI<sup>-/-</sup> mice. FcγRI<sup>only</sup> and FcγR<sup>null</sup> mice were bred at Institut Pasteur, used for experiments at 8-11 weeks of age and all protocols were approved by the Animal Ethics committee CETEA (Institut Pasteur, Paris, France) registered under #C2EA-89.

### 2.2 K/BxN serum-induced passive arthritis

K/BxN serum was generated from a pooled collection of >40 animals. Arthritis was induced by i.v. transfer of indicated volumes of K/BxN serum, and scored as described [25]. In some experiments mice were sacrificed on day 8 for blinded histological assessment.

### 2.3 Airway Inflammation

As previously described [26], mice were injected with 50 μL of rabbit anti-OVA serum i.n. and 500 μg of OVA i.v. 16-18h later 4 bronchoalveolar lavages (BALs) were performed with cold PBS (1x 0.5ml, then 3x 1mL) under lethal anaesthetic. Cells were pooled and stained for flow cytometry after RBC lysis; and haemorrhage was determined by OD570nm in the supernatant. We confirmed that mFcγRI, like all mouse FcγR, can indeed bind rabbit IgG immune complexes (Supplementary Fig.2).

## **2.4 Passive Systemic Anaphylaxis (PSA)**

Mice were sensitised by i.v. injection of 500µg anti-DNP IgG2a (clone Hy1.2) and challenged 16h later with 200µg TNP(21-31)-BSA i.v. Alternatively, mice were injected with 1mg of heat-aggregated (1 hour at 63°C in BBS pH8) human IVIG; considering that mouse FcγRI cross-binds human IgG subclasses 1, 3 and 4 [27]. Central temperature was monitored using a digital thermometer with rectal probe (YSI).

## **2.5 Experimental Thrombocytopenia**

Blood samples were taken in EDTA before and at indicated time points after i.v. injection of 3 or 10µg anti-platelet mAb 6A6. Some mice were treated 32h before 6A6 injection with 300µL PBS- or clodronate- liposomes i.v. Platelet counts were determined using an ABC Vet automatic blood analyser (Horiba ABX).

## **2.6 Tumour Immunotherapy**

Mice were depilated and received  $5 \times 10^5$  B16-Luc2+ cells s.c. on d0. Where indicated, mice were injected i.v. with 200µg mAb TA99 on d1, d2 and d3 (Figure 4A, closed symbols), and control groups were untreated (Figure 4A, open symbols). Bioluminescence was acquired from anaesthetised mice on d1, d7 and d13, 10 min after injection of 75µg luciferin s.c. (IVIS Spectrum CT, Caliper Life Sciences), and images were analysed with Living Image software.

## **2.7 Anti-mouse CD20 treatment**

Mice received a single i.v. injection of 50µg anti-mouse CD20 (clone 5D2, IgG2a, Genentech) to deplete endogenous B cells, or saline control, and CD19<sup>+</sup>B220<sup>+</sup> B cells in the blood, spleen and

inguinal lymph nodes were assessed 16 hours later by flow cytometry. Remaining B cells were calculated as a percentage of the average of vehicle-treated controls (Fig. 4B).

## **2.7 Statistics**

Data was analysed using one-way ANOVA with Bonferroni post-test (Fig.2C) or a Tukey's multiple comparisons test (Fig.1 B) to compare individual timepoints (Fig.2 B, D & E, and Fig.3 A, B & E), or a Kruskal-Wallis test with Dunn's multiple comparisons (Fig. 2A & 2C, bottom panel); a Student's t-test (Fig.3 C&D, Fig.4B) or a Mann-Whitney test (Fig.4A). Statistical significance is indicated (ns:  $p>0.05$ ; \* $p<0.05$ ; \*\* $p<0.01$ , \*\*\*  $p<0.001$ , \*\*\*\*  $p<0.0001$ ).

**Please refer to supplemental Methods** for information on reagents, flow cytometry, histology, surgical procedures and Active Systemic Anaphylaxis (ASA).

### 3. RESULTS & DISCUSSION

To evaluate the *in vivo* effector functions of mouse FcγRI, we investigated mice expressing this receptor in the absence of other endogenous classical FcγR (FcγRIIB, FcγRIII and FcγRIV-deficient), termed FcγRI<sup>only</sup> mice [17] or VG1505 mice [28], in comparison with mice deficient for all four endogenous classical FcγRs (FcγRI, FcγRIIB, FcγRIII and FcγRIV-deficient), termed FcγR<sup>null</sup> mice (Fig.1A). Both strains retain FcRn and non-classical IgG receptor expression. FcγRI<sup>only</sup> and FcγR<sup>null</sup> mice display normal breeding patterns and development, and no overt pathological signs up until 1 year of age. We assessed the circulating immune cell populations in these novel strains, compared to WT mice, using an automated blood cell analyser (Fig.1B). Across a large sample size, WT, FcγRI<sup>only</sup> and FcγR<sup>null</sup> mice display comparable total leukocyte counts in the blood, and similar frequencies of leukocytes, granulocytes and eosinophils (Fig.1B). Notably, FcγRI<sup>only</sup> and FcγR<sup>null</sup> mice have a slightly higher frequency of circulating monocytes. The pattern of expression of FcγRI in FcγRI<sup>only</sup> mice was comparable to wt mice: FcγRI was detected on circulating Ly6C<sup>hi</sup> and Ly6C<sup>low</sup> monocytes (Fig.1D), with greater expression on the latter, and on CD11b<sup>+</sup>CD11c<sup>+</sup> spleen cells, liver kupffer cells and liver macrophages, alveolar macrophages and bone marrow monocytes (not shown) and prominently on CD11b<sup>+</sup> skin resident cells (Fig.1E), but barely detected on F4/80<sup>+</sup> peritoneal macrophages, in agreement with previous reports [24, 29, 30].

An understanding of the participation of FcγRI in autoimmune and inflammatory pathologies remains elusive [4]. FcγRI<sup>-/-</sup> mice seem to experience reduced inflammation associated with immune complex tissue deposition [13] and severity of antigen-induced arthritis [11, 12]. Yet redundancy between FcγRI and FcγRIII certainly exists [12] and many of these



earlier studies did not consider the potential contribution of FcγRIV [14, 24]. To address whether FcγRI can play an effector role in inflammatory autoimmunity, we subjected FcγRI<sup>only</sup> and FcγR<sup>null</sup> mice to the K/BxN passive serum transfer model of autoimmune rheumatoid arthritis (K/BxN PA) [31]. Transfer of K/BxN serum into wt mice induced inflammatory signs of arthritis, and an arthritic score which peaked at day 4-6 and remained elevated until day 10 following serum transfer (Fig.2A&B), yet the same volume transferred into FcγRI<sup>only</sup> or FcγR<sup>null</sup> mice did not induce arthritis. Histological assessment of ankle joints confirmed marked to severe arthritis in wt mice, and no microscopic signs of arthritis observed in either FcγRI<sup>only</sup> or FcγR<sup>null</sup> mice (Supplementary Fig.1A-F). Moreover, K/BxN serum transfer at a dose higher than which was sufficient to induce arthritis in FcγRIV<sup>only</sup> mice [24] did not result in arthritis induction in FcγRI<sup>only</sup> mice (Supplementary Fig.1G), indicating that FcγRI alone cannot induce significant cellular infiltration and inflammation associated with this K/BxN model of autoimmune rheumatoid arthritis.

During experimental antigen-induced arthritis (EAIA), FcγRI<sup>-/-</sup> mice were reported to experience comparable joint swelling but a reduction in severe cartilage destruction compared to wt mice [11, 12]. Importantly, EAIA relies first on antigen uptake and presentation, a known functionality of FcγRI [13] on monocyte-derived dendritic cells, leading to the activation of antigen-specific T and B cells and to pathogenic antibody production, and thereafter to FcγR-mediated effector functions. Antigen uptake and presentation is not required in the K/BxN passive arthritis model, as pathogenic antibodies are directly injected into the recipient mouse. Because the severity of K/BxN passive arthritis is not affected in FcγRI<sup>-/-</sup> mice [25], and because FcγRI<sup>only</sup> mice are resistant to this arthritis model (this report), FcγRI evidently does not induce the required effector functions to engender arthritic symptoms after K/BxN serum transfer. If this inability extends also to the EAIA model, one may propose that the reduced severity of EAIA

observed in FcγRI<sup>-/-</sup> mice is attributable rather to reduced antigen uptake and presentation. Furthermore, the cardinal marker of cartilage destruction used in EAIA studies relies on the release of matrix metalloproteinases (MMPs) and the creation of neo-epitopes [11, 32]. Therefore although FcγRI does not mediate cell recruitment and joint inflammation in either of these arthritis models (Fig.2A and [11, 12]), it may contribute to reaction severity via local events in the tissue, *i.e.* MMP release and cartilage destruction.

The K/BxN model of autoimmune rheumatoid arthritis relies on local generation of IgG-immune complexes on the cartilage surface, that trigger the activation of FcγR-expressing cells. To determine if FcγRI can induce local inflammation in another context, we induced IgG-immune complexes at another anatomical site, the airways, and examined the resulting alveolitis. The inflammatory response in wt mice is characterised in the bronchoalveolar lavage (BAL) by massive neutrophil infiltration, significant infiltration of Ly-6C<sup>+</sup> monocytes, and damage to the airways with haemorrhage into the BAL 16h after challenge (Fig.2C) [26]. Neither FcγR<sup>null</sup> mice nor FcγRI<sup>only</sup> mice experienced significant local inflammation: only a mild neutrophil infiltration was observed in the absence of inflammatory monocytes and haemorrhage. Together, our data support the notion that FcγRI does not mediate significant immune cell recruitment and inflammation associated with IgG-immune complex deposition, but may contribute to reaction severity via local events in the tissue, as suggested by studies using FcγRI<sup>-/-</sup> mice [13, 14].

Local inflammatory reactions, such as that which drive autoimmune arthritis, require the activation of several cell populations including monocytes/macrophages and neutrophils. Systemic inflammatory reactions like severe hypersensitivity reactions, or anaphylaxis, can also proceed through pathways dependent on IgG and IgG receptors, yet symptoms may arise

following the activation of only one cell population, among monocyte/macrophages, neutrophils and basophils (reviewed in [5, 33]). FcγRI may participate in such reactions by its expression on monocyte/macrophages. Although FcγRI<sup>-/-</sup> mice were reported to experience mouse IgG2a-induced passive systemic anaphylaxis (PSA) with reduced severity [11], we could not reproduce these findings [34]. Here we demonstrate that FcγRI<sup>only</sup>, like FcγR<sup>null</sup> mice, were resistant to mouse IgG2a-mediated anaphylaxis (Fig.2D), heat-aggregated human IVIG-mediated anaphylaxis (Fig.2E) and even to an active model of anaphylaxis induced by BSA immunization and challenge (Supplementary Fig.3). Two possibilities emerge from these findings: firstly, that FcγRI alone is incapable of triggering sufficient inflammatory mediator release to cause systemic symptoms; secondly, that FcγRI expression is insufficient when expressed only on monocyte/macrophages to mediate anaphylaxis induction.

Since we found that FcγRI is not sufficient to mediate IgG-induced local or systemic inflammation that require cell recruitment and activation, and release of mediators, we wondered if FcγRI was able to induce IgG-mediated cell depletion through phagocytosis and/or ADCC mechanisms that contribute to several autoimmune diseases (*e.g.* autoimmune thrombocytopenia and anaemia) and to immunotherapies. Earlier studies using FcγRI<sup>-/-</sup> mice indicated that FcγRI contributes to experimental autoimmune haemolytic anaemia induced by RBC-targeting mIgG2a antibodies [11], particularly to more severe manifestations at high Ab doses [35]. To determine if FcγRI has autoimmune destructive properties in FcγRI<sup>only</sup> mice, we examined another model of autoimmunity characterised by circulating immune complexes, immune thrombocytopenic purpura (ITP) induced by injecting anti-platelet antibodies intravenously. ITP could be induced in wt and FcγRI<sup>only</sup> mice, but not FcγR<sup>null</sup> mice (Fig.3A). Within 4 hours of mAb injection, circulating platelet levels were reduced to <20% of their initial concentration in both wt and

206 FcγRI<sup>only</sup> mice (Fig.3A&B), and platelet counts remained low even 24 hours later. Administration  
207 of platelet-targeting mAb at a threefold-reduced dose was also sufficient to induce platelet  
208 clearance in FcγRI<sup>only</sup> mice, comparable to that of WT mice (Supplementary Fig.4A&B). High-  
209 dose toxic clodronate-containing liposomes, administered i.v. to deplete monocyte/macrophages  
210 mainly in the blood, spleen, and liver, protected against ITP induction in both WT and FcγRI<sup>only</sup>  
211 mice (Fig.3C), indicating that monocytes and macrophages are responsible for platelet clearance.

212

213 To investigate the organ-specific macrophage population responsible for FcγRI-dependent  
214 autoimmune platelet clearance, we subjected FcγRI<sup>only</sup> mice to either removal of the spleen  
215 (splenectomy), or partial removal of the liver (hemi-hepatectomy), prior to ITP induction (Figure  
216 3D&E). ITP induction in FcγRI<sup>only</sup> mice was mildly inhibited by splenectomy (Figure 3D):  
217 comprising an average reduction in platelet clearance from 88% (±4.2%) to 77% (±9%) 3.5 hours  
218 after mAb injection. Splenectomised FcγRI<sup>only</sup> and WT mice had somewhat elevated platelet  
219 counts at baseline, whether sampled 1 week or 3 weeks post-surgery, yet notably spleen removal  
220 did not inhibit ITP induction in WT mice (Supplementary Fig 4C, and data not shown). These  
221 findings suggest that splenic macrophages have a minor contribution to FcγRI-dependent ITP.  
222 Conversely, partial removal of the liver, which amounted to about 50% reduction in liver mass  
223 [36], did not affect ITP induction in FcγRI<sup>only</sup> mice (Figure 3E and Supplementary Fig 4F),  
224 suggesting that liver macrophages may not be mandatory for FcγRI-dependent ITP. We  
225 performed ITP experiments starting 4-5 days after hepatectomies or sham surgeries, due to the  
226 rapid regenerative potential of the liver. Importantly, despite the inflammatory effect of the  
227 surgical procedures, we did not see differences in FcγRI expression on circulating cells between  
228 sham operated and hemi-hepatectomised mice (data not shown). It is difficult to completely  
229 exclude a role for the liver in this model, as the part of the organ remaining after hemi-

hepatectomy may be sufficient to efficiently mediate ITP. Moreover, considerable platelet clearance was still observed in splenectomised mice, which implies the involvement of another physiological site; potentially blood monocytes [37]. Collectively, our data indicate a minor contribution of splenic macrophages to FcγRI-dependent ITP, but do not provide evidence of a role for liver macrophages.

Antibody-mediated therapies are now a frontline treatment for many malignancies, including a number of autoimmune diseases. Reports using FcγRI<sup>-/-</sup> mice suggest that FcγRI contributes to IgG-induced tumour cell depletion in the lung [15] and liver [16] but not the skin [20]. As the latter finding may be due to redundant functions among mouse FcγRs in the skin, we followed TYRP-1<sup>+</sup> Luc2<sup>+</sup> B16 melanoma cells tumour growth by bioluminescent imaging *in vivo* [17] in mice treated or not with anti-TYRP-1 mouse IgG2a TA99 mAb. Identical growth kinetics were detected in FcγRI<sup>only</sup> and FcγR<sup>null</sup> mice (Fig.2F; WT mice in Supplementary Fig.5A), and repeated TA99 injections dramatically reduced tumour load in FcγRI<sup>only</sup> mice, but not in FcγR<sup>null</sup> mice, to that of background levels (Fig.4A and Supplementary Fig.5). Thus FcγRI<sup>only</sup> mice reveal FcγRI-mediated functions that can remain masked in FcγRI<sup>-/-</sup> mice. Furthermore, these data reinforce the previously reported anti-tumour effector function of FcγRI in lung and liver tissues [15-18] and extends it to the skin tissue.

Anti-CD20 therapy to deplete B cells has been highly successful in the treatment of B cell malignancies and autoimmune disorders. B cell depletion is known to depend on FcR-dependent mechanisms [38], primarily phagocytosis by Kupffer cells in the liver [36]. A role for FcγRI in the clearance of both malignant and endogenous B cells, in cooperation with FcγRIII and FcγRIV, has been suggested by several studies [18, 39], but has not been formally demonstrated.

Since FcγRI was sufficient to mediate destructive platelet clearance (Fig.3), and is expressed on liver Kupffer cells, we tested the capacity of FcγRI to deplete endogenous B cells in a model of anti-CD20 therapy. Administration of mouse CD20-targeting mAb 5D2 (IgG2a) induced B cell depletion in the blood, spleen and lymph nodes of FcγRI<sup>only</sup>, but not FcγR<sup>null</sup> mice. 16 hours after treatment 85% of B cells were cleared from the blood and 25-30% of B cells from the secondary lymphoid organs of anti-CD20 treated FcγRI<sup>only</sup> mice. These data demonstrate that FcγRI, in the absence of FcγRIII and FcγRIV, is sufficient to mediate endogenous B cell clearance, and support a contribution for FcγRI to the efficacy of anti-CD20 therapy in models of lymphoma and autoimmunity.

In conclusion, genetically modified FcγRI<sup>only</sup> mice enabled us to demonstrate that the mouse high-affinity IgG receptor FcγRI is sufficient to mediate IgG-induced autoimmune thrombocytopenia and IgG-based immunotherapy targeting either B cells (anti-CD20) or subcutaneous melanoma, in the absence of FcγRIIB, FcγRIII and FcγRIV. FcγRI alone is, however, insufficient to induce IgG-induced autoimmune rheumatoid arthritis, airway inflammation and systemic anaphylaxis, probably due to its inability to efficiently mediate Ab-induced cell recruitment or release of inflammatory mediators. Rather we identify that FcγRI mediates Ab-induced cell depletion/destruction, in both pathogenic autoimmune and therapeutic anti-tumour contexts; which agrees with the important contributions of this receptor to pathogen elimination [11, 40, 41] and antigen uptake and presentation [13]. Furthermore, our data attribute FcγRI-dependent phagocytic function to macrophages in the skin and the spleen. Finally, the effector capabilities of mouse FcγRI appear aligned with its restricted expression profile: low to moderate expression on monocytes, tissue macrophages and monocyte-derived DCs, who are

277 indeed responsible for clearance of foreign bodies and antigen uptake, whereas that of its human  
278 homolog hFcγRI extend to pro-inflammatory and pro-anaphylactic functions attributable to more  
279 promiscuous expression, particularly high on circulating monocytes and neutrophils. In  
280 conclusion, mice models expressing only one particular IgG receptor, *e.g.* FcγRI<sup>only</sup> or FcγRIV<sup>only</sup>  
281 mice [17, 22-24], are particularly useful to ascribe independent functions to FcγRs, as distinct  
282 from potential cooperative roles with other FcγR, the latter of which may be implied from studies  
283 using specific FcγR<sup>-/-</sup> mice.

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285

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#### 5. CONFLICT OF INTEREST STATEMENT

L.E.M and A.M. are employees of Regeneron Pharmaceuticals, Inc. and hold stock in the company. C.G., H.B., F.J., L.F., S.C., D.A.M., P.P.Z., P.Bousso and P.Bruhns declare no competing financial interests.



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## FIGURE LEGENDS

**Figure 1: FcγRI only mice have normal blood leukocyte composition and show comparable FcγRI expression to that of WT mice.** (A) Schematic representation of WT, FcγRI<sup>only</sup> and FcγR<sup>null</sup> mice. (B) Leukocyte counts and relative percentages of immune cell populations in the blood of WT (n=20), FcγRI<sup>only</sup> (n=35) and FcγR<sup>null</sup> (n=24) mice were enumerated using an automatic blood cell analyser. (C) Representative flow cytometry profiles of FcγRI expression on indicated cell populations from the blood and organs of WT, FcγRI<sup>only</sup> and FcγR<sup>null</sup> mice (MΦ; macrophages). Shaded histograms indicate background staining of a mIgG1 isotype control. Staining is representative of at least 2 independent experiments, n≥2.

**Figure 2: FcγRI alone is insufficient to mediate IgG-induced arthritis, airway inflammation or systemic anaphylaxis,** (A-B) Arthritis was evaluated by clinical score (A) and ankle thickness (B) measured following transfer of K/BxN serum (5μL/g body weight) into WT (triangles), FcγRI<sup>only</sup> (circles) or FcγR<sup>null</sup> (squares) mice. (A) \*p<0.05 on d3-6, WT compared to FcγR<sup>null</sup> mice; (B) \*p<0.05 on d2, \*\*p<0.01 on d3, \*\*\*p<0.001 on d4-6, WT compared to FcγR<sup>null</sup> and FcγRI<sup>only</sup> mice. Data is representative of >2 independent experiments, n≥3 per group. (C) Bronchoalveolar lavage (BAL) was performed on naive (open symbols, n=3 per group) WT (triangles), FcγRI<sup>only</sup> (circles) and FcγR<sup>null</sup> (squares) mice, or mice 16-18h after challenge with antiserum i.n. and OVA antigen i.v. (closed symbols). Neutrophils and Ly6C<sup>+</sup> macrophages (MΦ) in the BAL were determined by flow cytometry. Haemorrhage was determined by measuring haemoglobin concentration in the BAL supernatant. \*\*p<0.01, \*\*\* p<0.001, \*\*\*\* p<0.0001; challenge data is pooled from 2 independent experiments, n=8-10 mice per group. (D-E) Temperature monitoring during passive systemic anaphylaxis (PSA) in WT, FcγR<sup>null</sup> or

457 FcγRI<sup>only</sup> mice, induced by (D) mIgG2a anti-TNP sensitization and TNP-BSA i.v. challenge or  
458 (E) i.v. injection of aggregated human IVIG. *n*=3-5 per group; data is representative of 2  
459 independent experiments. \**p*<0.05, \*\**p*<0.01, \*\*\*\* *p*<0.0001 at all time points from 30min; WT  
460 compared to FcγR<sup>null</sup> and FcγRI<sup>only</sup> mice.

461

462 **Figure 3: FcγRI-mediated thrombocytopenia is dependent on monocyte/macrophages, and**  
463 **partially inhibited by splenectomy.** (A-B) Circulating platelets in the blood of WT (*n*=3),  
464 FcγRI<sup>only</sup> (*n*=7) and FcγR<sup>null</sup> (*n*=7) mice were quantified at baseline and following i.v. injection of  
465 10μg anti-platelet mAb (clone 6A6) and are represented as (A) percentage over time and (B)  
466 number of platelets 4 hours after mAb injection; \*\*\*\* *p*<0.0001 at 4h, 9h and 24h, WT vs  
467 FcγR<sup>null</sup> and FcγRI<sup>only</sup> vs FcγR<sup>null</sup>. (C) Platelet counts at baseline and 4 hours after mAb injection  
468 in mice pre-treated with PBS- (open symbols) or toxic- (closed symbols) liposomes; *n*=4-5 per  
469 group; \* *p*<0.05, \*\*\*\* *p*<0.0001, significance values indicated for each group at 4 hours  
470 compared to baseline. (D-E) ITP induction and percentage of circulating platelets in (D) FcγRI<sup>only</sup>  
471 mice following splenectomy (closed symbols, *n*=11) compared to controls (open symbols, *n*=10);  
472 and (E) FcγR<sup>null</sup> mice (*n*=4) or FcγRI<sup>only</sup> mice following hemi-hepatectomy (*n*=5), compared to  
473 sham operated (*n*=4), or controls (*n*=3); \*\* *p*<0.01, splenectomised mice compared to controls;  
474 \*\*\*\* *p*<0.0001 at 3.5h, 8h and 24h, all groups compared to FcγR<sup>null</sup>; ns not significant. Data in  
475 (D&E) is pooled from two independent experiments.

476

477 **Figure 4: FcγRI is sufficient for anti-melanoma and B cell depletion therapies.**  
478 (A) FcγRI<sup>only</sup> (circles) or FcγR<sup>null</sup> (squares) mice were injected with B16-Luc2+ cells s.c. on d0  
479 and received no treatment (open symbols) or mAb TA99 i.v. on day 1, 2 and 3 (closed symbols);  
480 tumour growth was monitored by bioluminescent signal after s.c. injection of luciferin.

481 **(B)** FcγRI<sup>only</sup> (circles) or FcγR<sup>null</sup> (squares) mice were injected with anti-CD20 mAb 5D2 (closed  
482 symbols) or vehicle (open symbols). The percentage of remaining CD19<sup>+</sup>B220<sup>+</sup> B cells  
483 (compared to the average of vehicle-treated controls) was determined in the blood, spleen and  
484 inguinal lymph nodes after 16h later. Data in **(A)** is representative of 2 independent experiments,  
485  $n=4-5$  per group. Data in **(B)** is pooled from 2 independent experiments,  $n=3-5$  per group.  
486 \* $p<0.05$  on day 7 and day 13, \*\*\* $p<0.001$ , \*\*\*\* $p<0.0001$ , FcγRI<sup>only</sup> controls compared to mAb-  
487 treated; ns not significant, FcγR<sup>null</sup> controls compared to mAb-treated.  
488

## **Supplemental Materials and Methods**

### **Reagents**

Human IVIG (Gamunex®) was from Grifols, containing 63% hIgG1, 29% hIgG2, 5% hIgG3 and 3% hIgG4. B16-Luc2+ cells were from Caliper-Life Sciences. IgG were purified by Protein G-affinity purification from supernatants of hybridomas producing anti-gp75 mAb (TA99) from American Type Culture Collection, mIgG2a anti-platelet mAb (clone 6A6) provided by Dr R. Good (USFCM, Tampa, FL, USA), and mIgG2a anti-TNP mAb (Hy1.2) provided by Shozo Izui (University of Geneva, Geneva, Switzerland). Luciferin was from Invitrogen, rabbit anti-OVA antiserum, OVA, BSA, and Freund's adjuvant (CFA/IFA) were from Sigma-Aldrich, TNP<sub>(21-31)</sub>-BSA was from Santa Cruz and PBS- and clodronate-liposomes were prepared as previously described [1]. Vivotag-680 was from Perkin Elmer and OVA-vivotag-680 was prepared as recommended by the manufacturer.

### **Tissue processing and flow cytometry**

Spleens were dissociated through a 70µm cell strainer into MACS buffer (PBS /0.5%BSA /2mM EDTA) and RBC lysis was performed using an ammonium chloride-based buffer. For isolation of skin cells, ears were split into dorsal and ventral halves and roughly chopped before digestion with 0.25mg/mL Liberase TL ResearchGrade (Roche) + 0.1mg/mL DNase (Sigma) for 1h at 37°C (800rpm; Eppendorf Thermomixer), washed with 10x volume of PBS/ 10%FBS /2mM EDTA and processed through a 100µm cell strainer. Livers were perfused with cold PBS before dissection, and liver leukocytes were isolated using the Liver Dissociation Kit and gentleMACS Octo Dissociator from Miltenyi, according to the manufacturer's

instructions. Cells were isolated from the peritoneum by lavage with 6mL cold PBS; BALs were performed 3x with 1mL PBS. For blood leukocyte analysis, heparinised blood was subjected to RBC lysis with either Red Blood Cell Lysis Solution (Miltenyi) or BD Pharm Lyse Lysing Buffer (BD Biosciences) and washed with MACS buffer. Single cell suspensions were washed with MACS buffer, incubated with 2.4G2 (Fab')<sub>2</sub> fragments (anti-CD16/32, 40µg/mL; 15min on ice) and stained with fluorochrome-conjugated antibodies in MACS buffer for 30min on ice. Data was collected on a MACSQuant flow cytometer (Miltenyi), and analysed using FlowJo Software (TreeStar, Inc.).

Cell populations were defined by FSC/SSC properties and surface markers as indicated (Fig.1B), or in the BAL (Fig.2B): alveolar macrophages (CD11c<sup>+</sup>/ SiglecF<sup>+</sup>), eosinophils (CD11c<sup>neg</sup>/ SiglecF<sup>+</sup>), neutrophils (CD11c<sup>neg</sup>/ SiglecF<sup>neg</sup>/ CD11b<sup>+</sup>/ Ly-6G<sup>+</sup>), Ly-6C<sup>+</sup> macrophages (CD11c<sup>neg</sup>/ SiglecF<sup>neg</sup>/ CD11b<sup>+</sup>/ Ly-6G<sup>neg</sup>/ Ly-6C<sup>+</sup>).

### **Active Systemic Anaphylaxis (ASA)**

Mice were immunised i.p. on d0 with 200µg BSA in Complete Freund's Adjuvant, and boosted on d14 with 200µg BSA in Incomplete Freund's Adjuvant. BSA-specific IgG1, IgG2a/b/c and IgE serum antibodies were titrated by ELISA on d21 as described [2]. Mice with comparable antibody titers were challenged 13-14 days after the last immunisation i.v. with 500µg BSA. Central temperature and mortality was monitored.

### **Partial hepatectomy and splenectomy**

Partial hepatectomy was performed as described[3]. Mice were anaesthetised and a transverse abdominal incision was made. The superior lobes of the liver were laid on the diaphragm and the ligaments of the caudate lobe dissected. The caudate lobe was



then pulled in front of the stomach and resected after in-bloc ligation of its hilum (6/0 silk). The lateral left lobe was resected using the same technique. The abdomen was closed using 4/0 silk running sutures. The procedure removed approximately half of the initial liver mass. Mice were rested for 2 days before experimental procedure. For splenectomy, a small vertical incision was made on the left flank, the spleen was gently pulled outside the abdomen, and the splenic ligaments and vessels were cut. The abdomen was closed with a 4/0 silk suture, and the skin with a surgical staple. Mice were rested for 1-3 weeks before experimental procedure.

## **Histology**

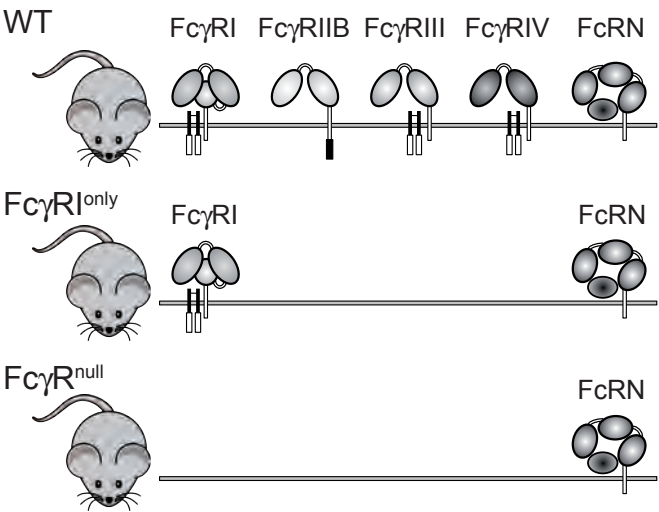
Ankle joints and surrounding tissues (from the extremity of the femur/tibia to the digits) were sampled on day 8 after K/BxN serum transfer (batch #1, 5mL/kg), then fixed and simultaneously decalcified using Formical-4<sup>®</sup> (StatLab Medical Products) for 2 weeks. Samples were routinely embedded in paraffin, and 4 mm sections were stained with haematoxylin and eosin (H&E). Sections were evaluated microscopically and histological changes (*i.e.* inflammation, pannus formation, bone erosion and cartilage damage) were scored from 0 (no change) to 5 (severe).

## **Supplemental references**

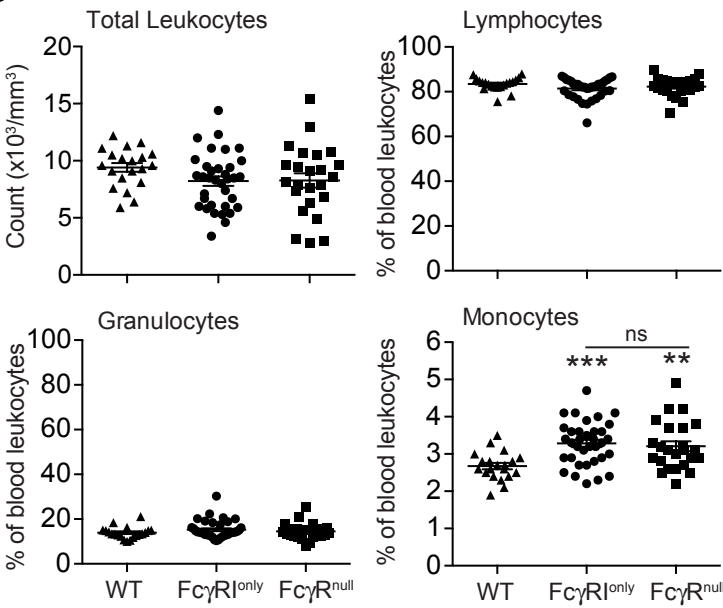
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Figure 1

A



B



C

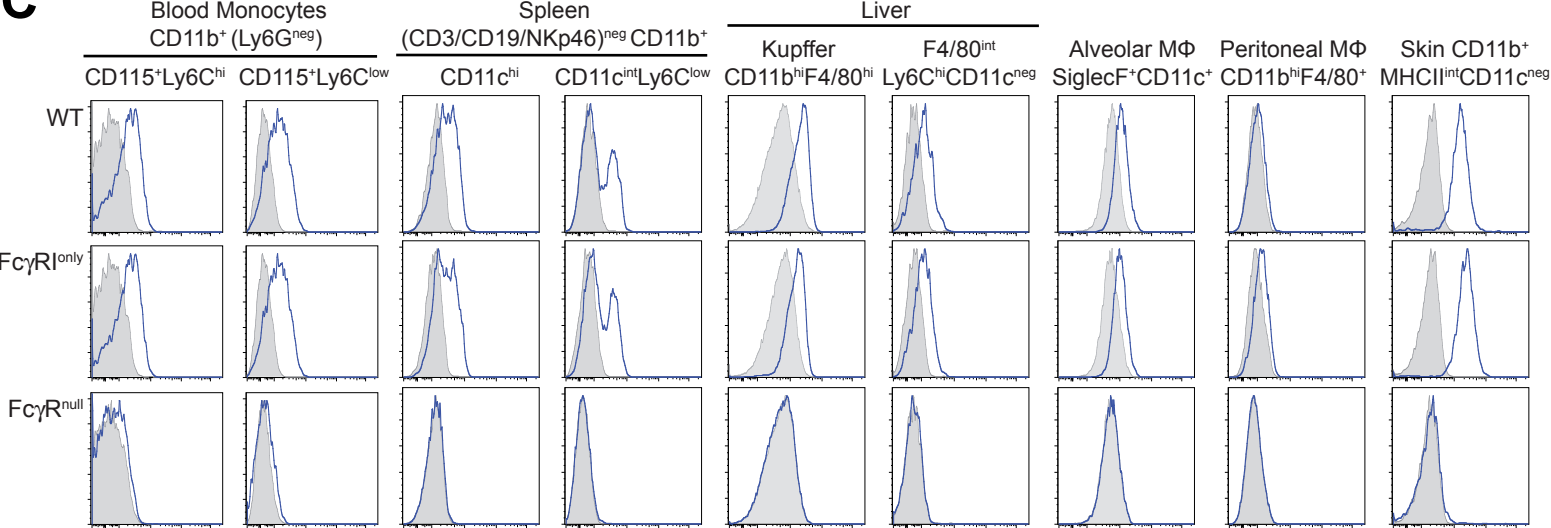


Figure 2

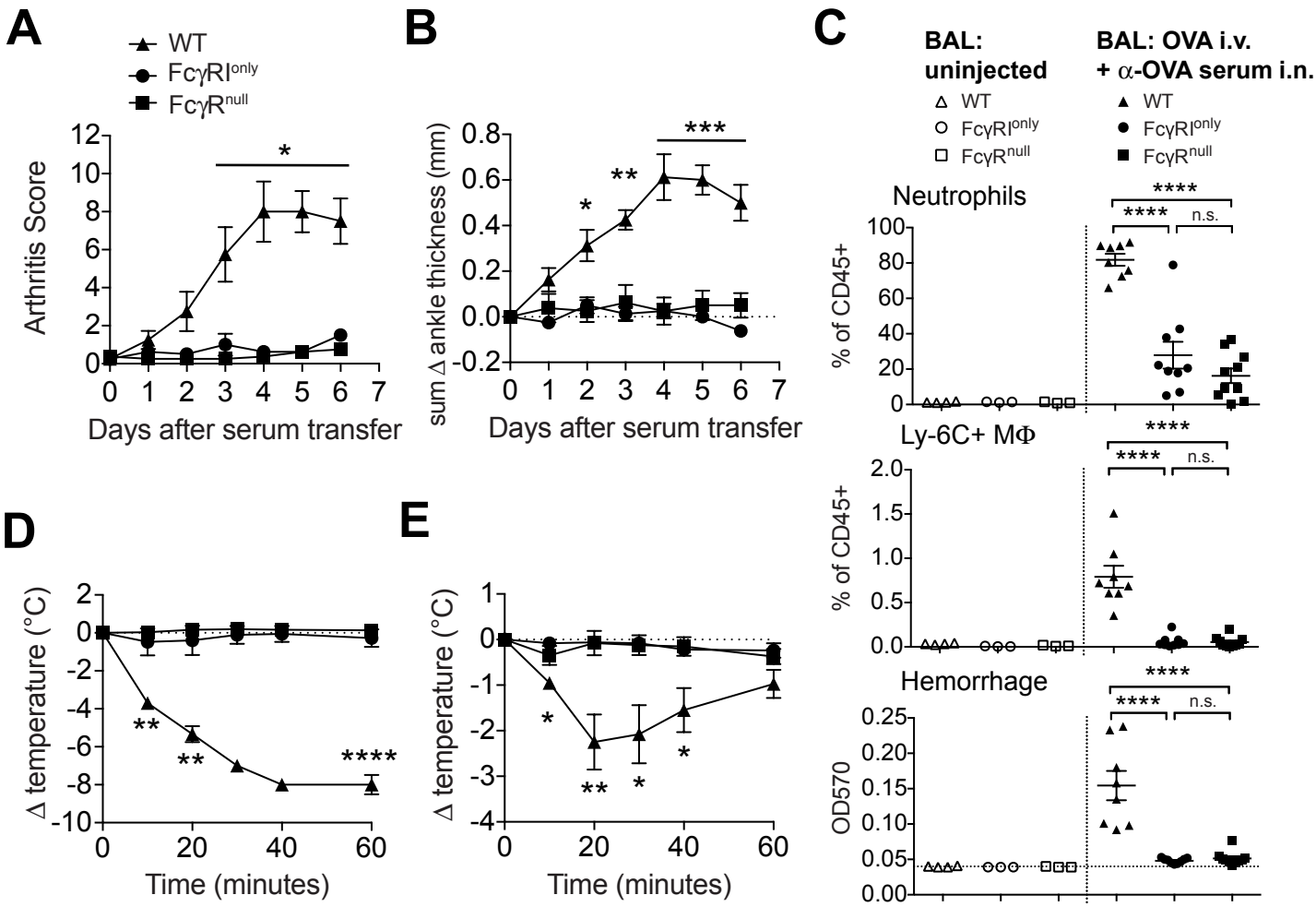


Figure 3

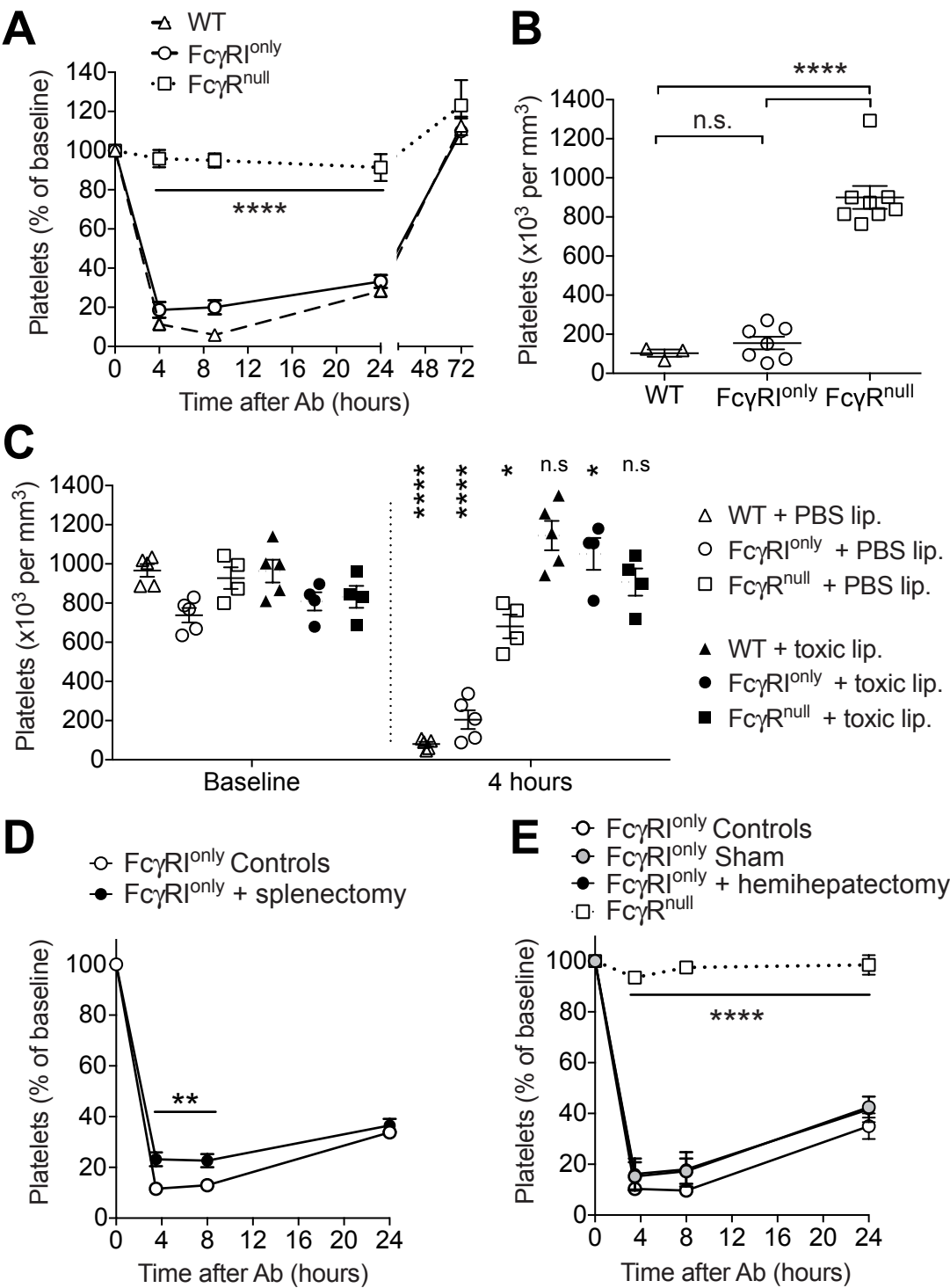
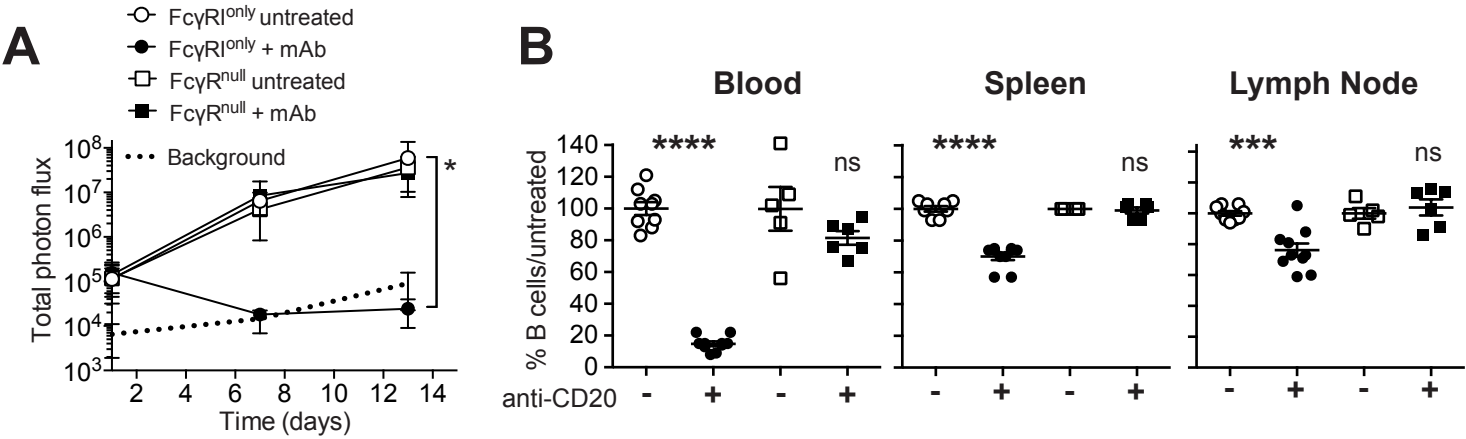
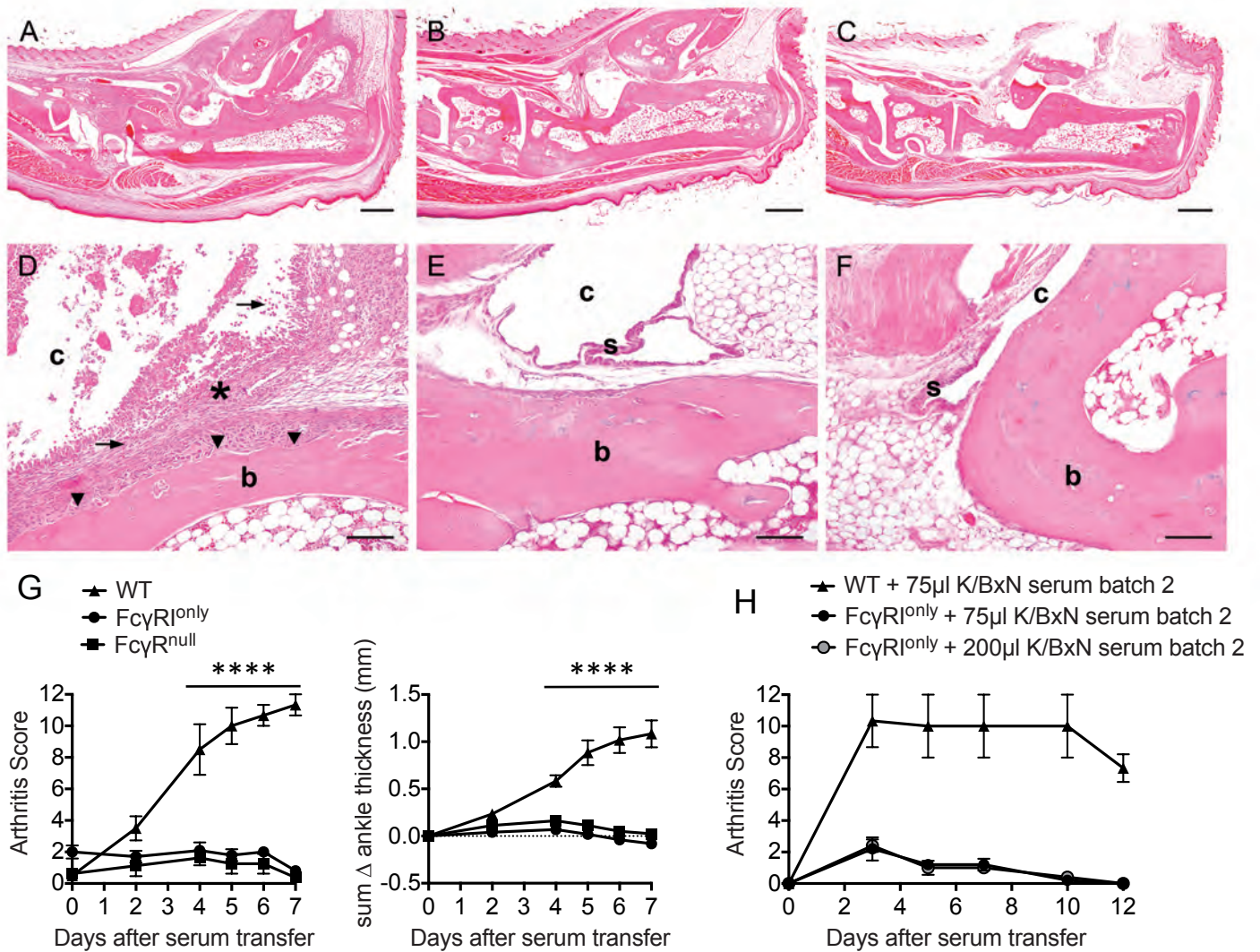


Figure 4

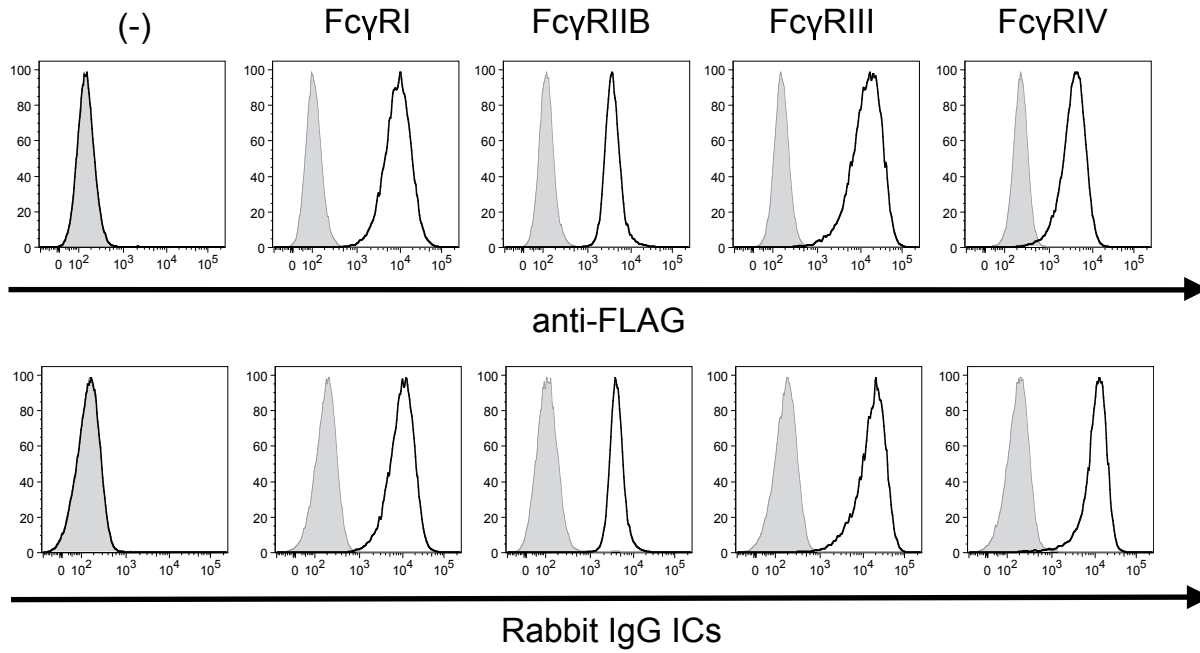


## Supplementary Figure 1



**Supplementary Figure 1: mFcγRI alone is insufficient to induce arthritis, even with increased dose of administration, or a different batch of K/BxN serum.** (A-F) H&E stained paraffin-embedded sections of decalcified ankle joints on day 8 after K/BxN serum transfer (batch #1, 5mL/kg). Histological signs of marked to severe arthritis were noted in WT mice (A, D), and were characterised by neutrophil and mononuclear cell infiltrates (arrow), pannus formation (asterisk), cartilage damage and bone resorption (arrowhead). No microscopic signs of arthritis were observed in *FcγR<sup>null</sup>* (B, E) or *FcγR<sup>I</sup><sup>only</sup>* (C, F) mice. (b: bone; c: synovial cavity, s: synovial membrane). Scale bar: (A, B, C) 500 μm, (D, E, F) 100 μm (G) Arthritic score and ankle thickness following transfer of high-dose K/BxN serum (batch #1, 10mL/kg) into WT (triangles), *FcγR<sup>null</sup>* (squares) or *FcγR<sup>I</sup><sup>only</sup>* (circles) mice, or (H) arthritic score following transfer of K/BxN serum (batch #2, indicated volumes) into WT or *FcγR<sup>I</sup><sup>only</sup>* mice.

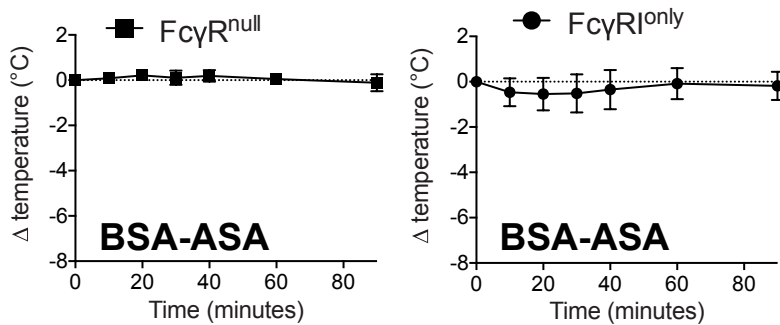
## Supplementary Figure 2



**Supplemental Figure 2: FcγRI binds OVA immune complexes.** CHO cells stably transfected to express FLAG-tagged mFcγRI, mFcγRIIB, mFcγRIII, or mFcγRIV, as indicated, or control cells (-) were stained with an anti-FLAG antibody (upper panel) to confirm receptor expression; shaded histograms represent isotype control staining. CHO transfectants were incubated with immune complexes (ICs) formed by rabbit anti-OVA serum and fluorescently (vivotag680)-tagged OVA (lower panel). Shaded histograms represent background fluorescence (OVA-vivotag680 alone); open histograms IC binding.

## Supplementary Figure 3

	<b>FcγR<sup>null</sup></b>		<b>FcγRI<sup>only</sup></b>	
Maximum temp. loss	IVIG PSA	BSA ASA	IVIG PSA	BSA ASA
<1°C	56 (100%)	14 (100%)	67 (82,7%)	36 (83,7%)
1-2°C	0	0	6 (7,4%)	5 (11,6%)
>2°C	0	0	8 (9,9%)	2 (4,7%)

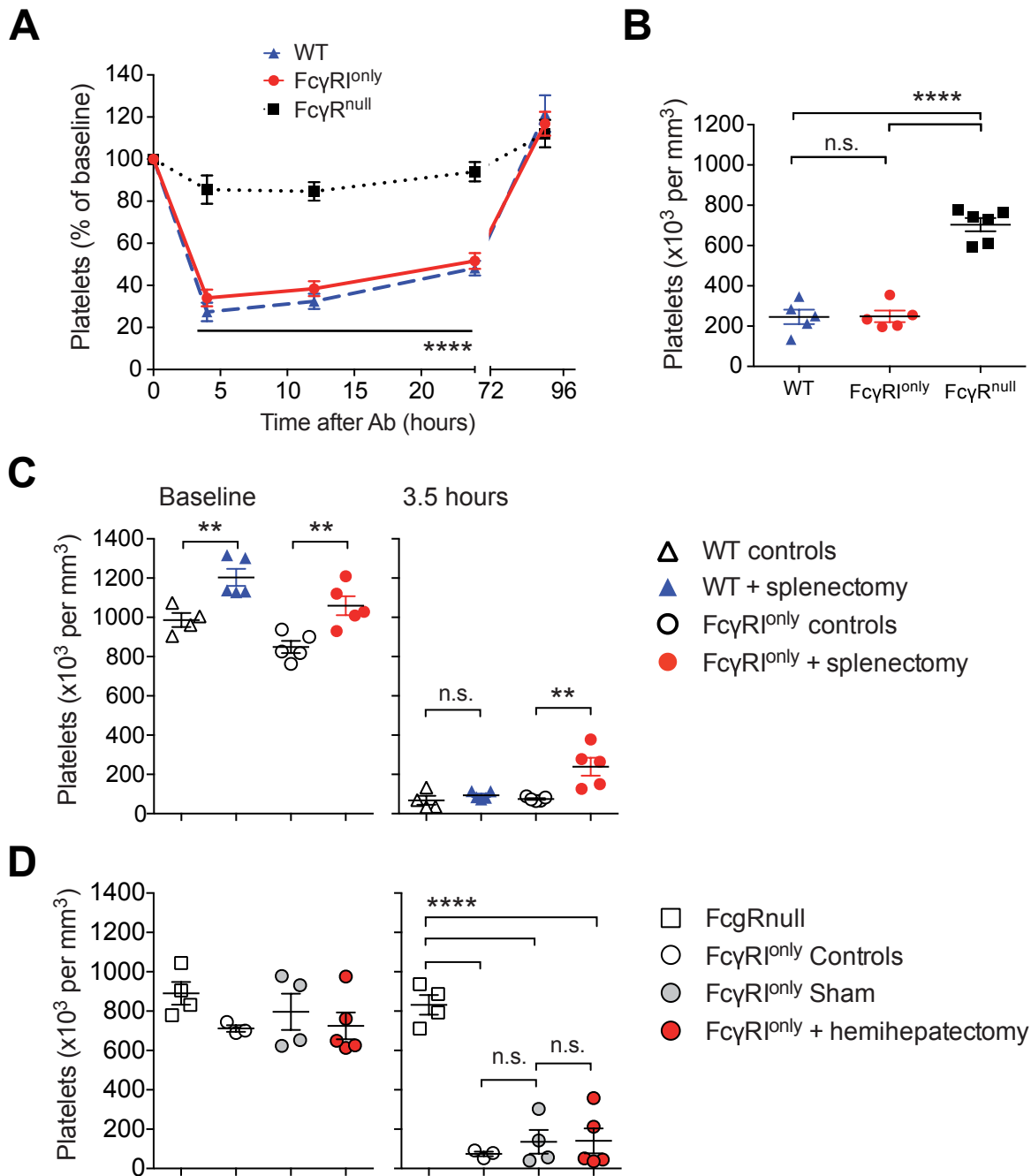


### Supplementary Figure 3: FcγRI<sup>only</sup> mice are resistant to BSA-ASA and IVIG-PSA.

(A) FcγR<sup>null</sup> (left) and FcγRI<sup>only</sup> (right) mice were injected with 1mg heat-aggregated IVIG (IVIG-PSA) or immunized and challenged with BSA antigen (BSA-ASA) and central temperatures were monitored. Only a small percentage of FcγRI<sup>only</sup> animals across all experiments demonstrated a mild hypothermia. Table summarises individual values and graphs represent mean  $\pm$  SEM. Data is pooled from >4 individual experiments.

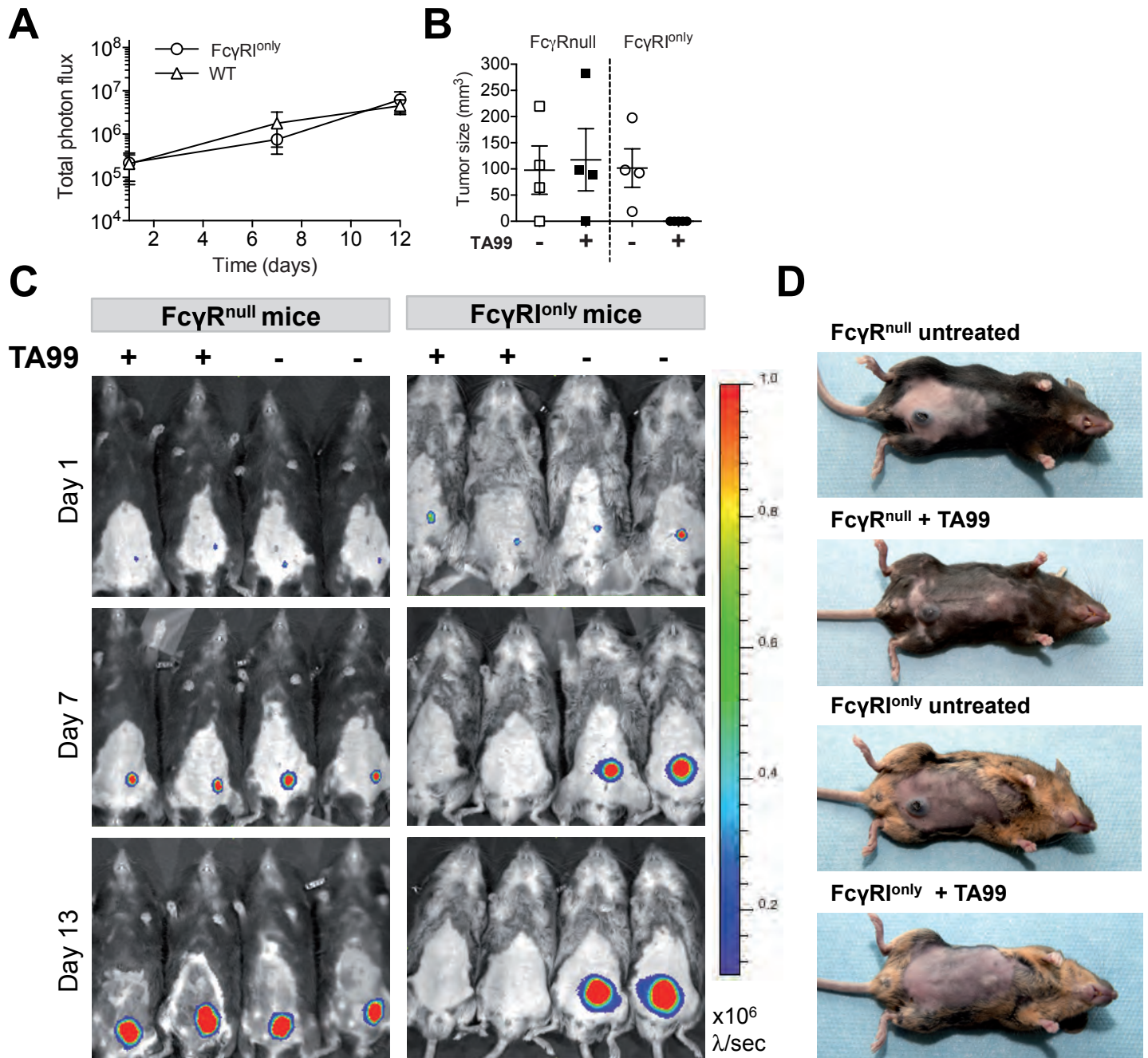


# Supplementary Figure 4



**Supplementary Figure 4: FcγRI<sup>only</sup> and WT mice are susceptible to thrombocytopenia at a low-dose of platelet-targeting Ab; platelet clearance is partially inhibited by splenectomy but not affected by hemihepatectomy.** Circulating platelets were quantified in the blood of (A-B) WT, FcγRI<sup>only</sup> and FcγR<sup>null</sup> mice at baseline and after injection of 3μg anti-platelet mAb 6A6, and are represented as (A) percentage over time and (B) number of platelets 4 hours after mAb injection.  $n=5-6$  per group; \*\*\*\*  $p<0.0001$  at 4, 12 and 24 hours. (C-D) Number of platelets in the blood of WT or FcγRI<sup>only</sup> controls, sham operated, or mice with splenectomy (C) or hemihepatectomy (D), at baseline (1 week after splenectomy or 2 days after hemihepatectomy or sham) and 3,5 hours after 10μg mAb 6A6. (C)  $n=4-5$  per group, data shown from one of two experiments; \*\*  $p<0.01$ . (D)  $n=3-5$  per group, data pooled from two independent experiments; \*\*\*\*  $p<0.0001$ .

## Supplementary Figure 5



**Supplemental Figure 5: mFcγRI alone is sufficient to mediate mAb therapy of subcutaneous melanoma.** Mice were injected subcutaneously with B16-Luc2+ melanomas cells with or without therapeutic mAb TA99 treatment and (A,C) tumour growth was monitored by bioluminescent signal 10min after s.c. luciferin injection at indicated timepoints or (B) tumour size measured on day 12. (A) Tumour growth is comparable between untreated WT (triangles, n=5) and  $Fc\gamma RI^{only}$  (circles, n=5 until d7, n=3 at d12) mice. (B-D)  $Fc\gamma RI^{only}$  mice eliminate tumours with TA99 treatment, and  $Fc\gamma R^{null}$  mice do not. (D) Representative photographs taken at day 16. (B-D) correspond to the experiment shown in Figure 2F and are representative of 2 individual experiments.