The Inhibitory Fcγ Receptor is Unnecessary for IVIG Efficacy

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Intravenous gamma globulin (IVIG) is used as an effective therapy for many different autoimmune and inflammatory diseases[1-5]. The current paradigm for the mechanism underpinning anti-inflammatory effects links IVIG administration to increased expression and activity of the inhibitory Fcγ receptor (FcγRIIB) on splenic macrophages[6]. This hypothesis has been disputed in the literature[7]. Here we show that IVIG administered in the context of passive antibody-mediated thrombocytopenia to be therapeutically efficacious in both splenectomized and unmanipulated Balb/c mice despite no upregulation of FcγRIIB mRNA in the spleen. Moreover, IVIG effectively ameliorated immune thrombocytopenia (ITP) in FcγRIIB-deficient Balb/c mice, but not in FcγRIIB$^{-/-}$ or FcγRIIB$^{+/+}$ control mice with a C57BL6/129S background. Our results demonstrate that FcγRIIB is not relevant to the beneficial effects of IVIG. We anticipate that investigators will now shift their research focus away from FcγRIIB and seek out other possible mechanism(s) to explain the plethora of diseases and conditions treatable with IVIG.

IVIG was first used to treat ITP in 1981 and its use to treat this and other autoimmune inflammatory diseases has continued to increase, particularly in relation to acute ITP in

children[1-5]. The therapeutic benefit of IVIG has been ascribed to Fc receptor blockade, antibody-mediated autoantibody neutralization, inhibition of complement-mediated damage, modulation of cytokine production, down-regulation of B or T cell responses, and modulation of dendritic cells[8-13].

In 2001[6], it was reported that the inhibitory Fc receptor, FcγRIIB, is upregulated in the splenic macrophages of immune thrombocytopenic mice following administration of IVIG and that FcγRIIB knockout mice fail to respond to IVIG therapy. These data raised the possibility that the therapeutic effect of IVIG reflects modulation of FcγRIIB inhibitory signaling, and this possibility is now generally regarded as the explanation for IVIG-mediated suppression of autoimmune cytopenia[6, 13-15]. However, not all data from studies addressing this issue are consistent with this mechanistic paradigm[7, 16]. Accordingly, we have capitalized on a new mouse model of ITP[17] to re-examine the role of the FcγRIIB in IVIG therapy.

Our model involves the IP injection of wild-type mice with escalating doses of a rat monoclonal anti-platelet antibody (MWReg30) that recognizes the platelet-specific integrin αIIβ (CD41) glycoprotein and induces in the animals a rapid decrease in platelet count[17]. In this setting, thrombocytopenia reaches a nadir 24 hours after injection and is maintained over time by daily escalation of anti-platelet antibody doses. This protocol creates a clinical model that more closely mimics the thrombocytopenic state of human ITP than do most other mouse models. Also, as seen in human ITP, administration of IVIG in MWReg30-treated mice when the platelet count is at its nadir induces a rapid increase in platelet number[17]. Importantly, in this system, neither ITP induction nor its amelioration by IVIG depends on the spleen, both being comparable in splenectomized and nonsplenectomized

wild-type mice (Fig. 1). Also importantly, FcγRIIB transcript levels, as evaluated using reverse transcriptase-polymerase chain reaction (RT-PCR), do not increase in spleen, liver or bone marrow of the mice after IVIG administration (Fig. 2), although FcγRIIB mRNA levels are mildly increased in lymph nodes[18].

In contrast to the system described above, the more-commonly studied mouse model of ITP involves administration of monoclonal anti-platelet antibody, 6A6 or MWReg30, with the consequent induction of a ~50% drop in platelet number. Importantly, in the original study[6], this drop occurs primarily during the initial 2-8 hours, platelet counts recovering to normal or near normal numbers by 24 hours later. Also, in this and other mouse models of ITP, IVIG is usually administered prior to induction of thrombocytopenia. By contrast, in human ITP, platelet counts usually fall to very low levels and their levels remain low until therapeutic intervention. Thus our mouse model of passive antibody-induced ITP[17] more closely resembles the human condition, the monoclonal antibody evoking a significant (up to 80%-90%) reduction in platelet number by 24 hours that is sustained until IVIG is administered.

Importantly, the major evidence supporting the importance of FcγRIIB to IVIG effects was derived using FcγRIIB knockout mice to show that the effect is lost in the context of FcγRIIB deficiency. However, using our mouse model of ITP, we have found the effects of IVIG on thrombocytopenia to be no different in FcγRIIB-deficient compared to wild-type mice on either the C57BL6/129S or Balb/c backgrounds (Fig. 3).

A lack of FcγRIIB contribution to IVIG therapeutic effect is also supported by our finding that FcγRIIB expression is unaltered in the spleen of wild-type Balb/c mice following IVIG treatment and the lack of any detectable difference in IVIG therapeutic

effect in splenectomized compared to unmanipulated FcγRIIB$^{-/-}$ mice on the Balb/c background. These findings are consistent with previous data showing that FcγRIIB is not upregulated in monocyte-macrophages obtained from Kawaski's disease patients treated with IVIG[16] and also with data showing no differences in IVIG effect on ITP manifested by mice deficient for SHP-1 or SHIP-1, signaling effectors that are key to inhibitory activity of Fc receptors[18-21].

Our data contradict prior suggestions of a role for the FcγRIIB inhibitory receptor in the therapeutic effect of IVIG in ITP. We find the effects of IVIG on thrombocytopenia to be no different in FcγRIIB-deficient Balb/c compared to wild-type Balb/c mice or in FcγRIIB-deficient mice produced on a mixed C57BL/6/129S background compared to wild-type C57BL/6/129S F2 mice, the recommended control animals for these studies[22] (Fig. 3). Importantly, in at least some reports in which FcγRIIB-deficient mice were used to explore IVIG therapeutic effects, it appears that mutant and wild-type mice were not fully congenic, FcγRIIB$^{-/-}$ mice having C57BL/6/129S mixed backgrounds, but the wild-type mice being C57BL/6[7, 19]. In other studies, the congeneity of the mice is not certain[6, 23]. By contrast, the controls and FcγRIIB$^{-/-}$ mice used in our studies were completely matched for genetic background. Thus we believe that the use of an inappropriate control strain may have led to incorrect conclusions as to the effect of FcγRIIB deficiency on effectiveness of IVIG therapy. Importantly, the C57BL/6/129S F2 mice that were confirmed FcγRIIB$^{+/+}$ failed to respond to IVIG. Further studies are thus required to pinpoint the key mechanisms accounting for IVIG beneficial effects in ITP and other autoimmune and inflammatory clinical conditions.

Fig.1. Splenectomy does not affect ability of IVIG to ameliorate experimental ITP. **(A)** Nonsplenectomized Balb/c mice respond to IVIG (1.0 g/kg) treatment. **(B)** Splenectomized Balb/c mice respond to IVIG (1.0 g/kg) treatment. *=significant compared to untreated group ($P \leq 0.01$).

Fig. 2. FcγRIIB expression following IVIG treatment. **(A)** RT-PCR for FcγRIIB mRNA 6 hours after administration of IVIG (1.0 g/kg) to Balb/c mice made thrombocytopenic using MWReg30: Lanes 1 and 5, lymph nodes; lanes 2 and 6, bone marrow; lanes 3 and 7, liver; lanes 4 and 8, spleen. HPRT =hypoxanthine-guanine phosphoribosyl transferase **(B)**. FcγRIIB expression level densitometry compared to HPRT.

Fig. 3. FcγRIIB receptor is not involved in the mechanism of action of IVIG. **(A)** FcγRIIB$^{-/-}$ mice congenic on a Balb/c background respond to IVIG treatment (1.0 g/kg), means +/- SEM (n=9) *P=0.0001, **P=0.00003, ***P=0.001 by Student's *t* test; all mice were confirmed for genotype. **(B)** IVIG ameliorates ITP in wild-type C57BL/6 mice, means +/- SEM (n=3) *P=0.0263; **P=0.0022. **(C)** IVIG treatment fails to work using non-fully congenic B6;129S FcγRIIB$^{-/-}$ knockout mice (n=6). **(D)** IVIG treatment fails to work using recommended control wild-type B6129SF2/J FcγRIIB$^{+/+}$ mice (n=7). **(E)** FcγRIIB genotyping results; -/- = 232bp fragment; +/+=161bp fragment.

METHODS SUMMARY

We used wild-type Balb/c (normal or splenectomized) and FcγRIIB knockout mice on a Balb/c background as well as wild-type C57BL6, B6129SF2/J and FcγRIIB knockout mice on a B6 background. All experiments were performed following animal-use protocols that were approved by the University Health Network Animal Research Committee in Toronto. The rat monoclonal anti-mouse glycoprotein IIb (CD41; clone MWReg30, rat IgG1) antibody was used to induce ITP using a dose-escalation protocol whereby mice received daily intraperitoneal (IP) injections of 68 μg/kg of MWReg30 on days 0 and 1, followed by a daily increase in the dose by 34 μg/kg day until the end of the experiment. Platelets in whole blood samples were quantified on a daily basis using a calibrated flow cytometer as

previously published. For genotyping, DNA extraction was done using a Mouse Tail Direct Lysis Buffer using the protocol and primers recommended by the Jackson Laboratory. For gene expression analysis, total RNA was extracted from mouse tissues (lymph nodes, bone marrow, liver and spleen) using the TRIzol Reagent according to the manufacturer's protocol. Reverse transcription was performed with a First-Strand cDNA Synthesis Kit with appropriate forward and reverse primers using a Perkin-Elmer GeneAmp PCR 9700 thermocycler. Amplicons were visualized on a 2% agarose gel containing 0.2 μg/μl ethidium bromide, and quantified using a Bio-Rad GS 800 Laser Densitometer and a Bio-Rad Quantity One software. Group means and standard deviations (SDs) of total platelet counts were determined and plotted. Student's two-tailed t test for equal variance was used to determine the significance ($p < 0.05$) of total platelet counts by comparing the counts of test mice to control mice.

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Figure 1

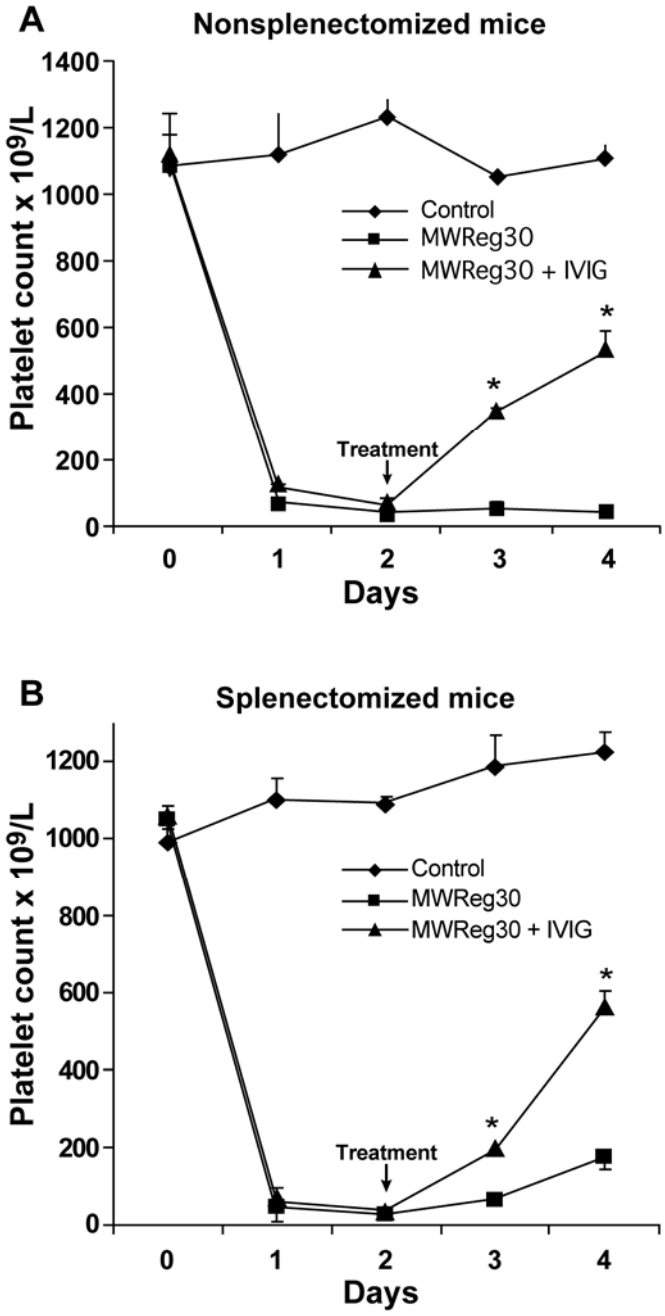


Figure 2

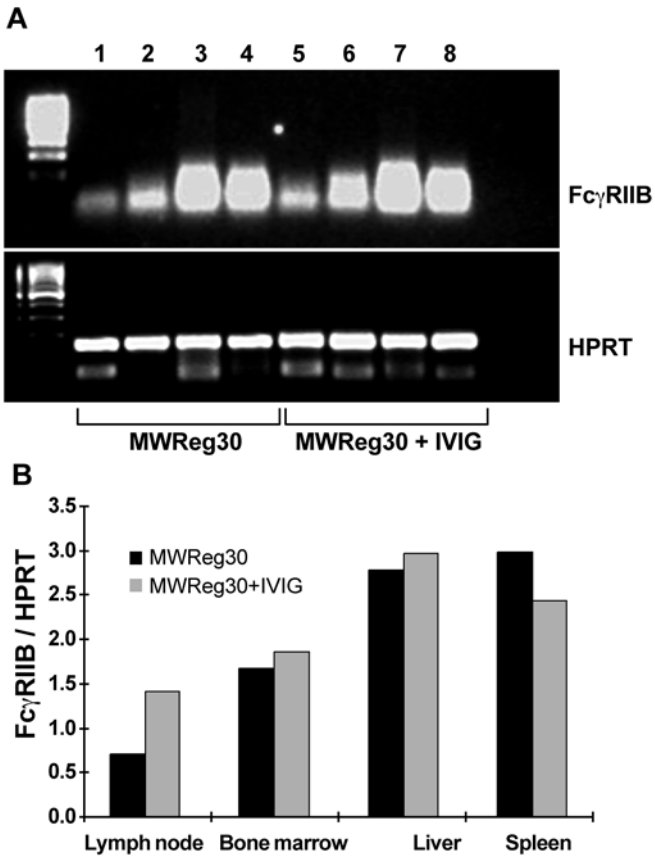
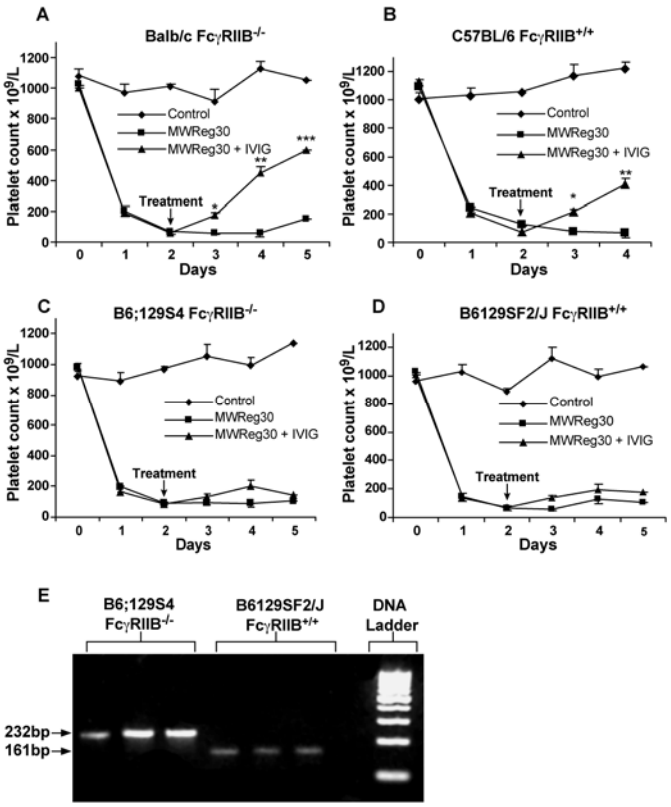


Figure 3



METHODS

Mice. Wild-type Balb/c (normal or splenectomized) and FcγRIIB knockout mice on a Balb/c background (Model Number 579) were purchased from Taconic (Germantown, NY, USA) at 6-8 weeks of age. Also, wild-type C57BL6 (stock #000664), B6129SF2/J (stock #101045) and FcγRIIB knockout mice on a B6 background (stock #002848) at 6-8 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were kept under a natural light/dark cycle, maintained at 22 $\pm$ 4°C, and fed with standard diet and water ad libitum. All experiments were performed following animal-use protocols that were approved by the University Health Network Animal Research Committee in Toronto.

Reagents. The rat monoclonal anti-mouse glycoprotein IIb (CD41; clone MWReg30, rat IgG1,κ) antibody, used to induce ITP was purchased from BD PharMingen (Mississauga, Ontario, Canada). The IVIG (Gammagard S/D), administered IP 2 hours post anti-platelet antibody administration day 2 of the experiment at 1g/kg, was from Baxter Healthcare Corp. (Glendale, CA).

Mouse model of ITP. ITP was induced and sustained using a dose escalation protocol as previously described (12). Mice received daily intraperitoneal (IP) injections of 68 μg/kg of monoclonal PLT antibody (anti-CD41 in 200 μL phosphate-buffered saline [PBS], pH 7.2) on days 0 and 1, followed by a daily increase in the dose by 34 μg/kg day until the end of the experiment. Platelets in whole blood samples were quantified on a daily basis using a calibrated flow cytometer (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ) exactly as previously described (Katsman et al., Transfusion 2010).

PCR-analysis. For genotyping, DNA extraction was done using a Mouse Tail Direct Lysis Buffer (Allele Biotechnology, San Diego, CA, USA). PCR was perforemed using the protocol and primers recommended by the Jackson Laboratory (Bar Harbor, ME, USA) for $Fcgr2b^{tm1Ttk}$ genotyping. For gene expression analysis, total RNA was extracted from mouse tissues (lymph nodes, bone marrow, liver and spleen) using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription was performed with a First-Strand cDNA Synthesis Kit (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's protocol. Briefly, 1μg of total RNA and random hexamer oligonucleotides were used in a 15μl total reaction volume. Samples were heated to 65°C for 10 min, chilled on ice for 5 min, incubated at 37°C for 1 hr,

followed by a 10-min incubation at 80°C. Primers used to amplify the mouse Fcgr2b receptor are: 5'CTCTCCCAGGATACCCTGAGT3' (forward) and 5'GGTGCATGAGAAGTGAATAGGTG3' (reverse), and primers used to amplify the mouse hypoxanthine guanine phosphoribosyl transferase (HPRT, house-keeping gene) are: 5'TCAGTCAACGGGGGACATAAA3' (forward) and 5'GGGGCTGTACTGCTTAACCAG3' (reverse). PCR reactions were carried out in a Perkin-Elmer GeneAmp PCR 9700 thermocycler. Briefly, 25µl total reaction volumes were composed of 1x REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO, USA), 25µM forward and reverse primers and 2µl of template cDNA or a no-template control. Following initial denaturation at 95°C for 2 min, 35 cycles of amplification were carried out at 94°C for 30 seconds, 60^{o}C for 30 seconds, and 72°C for 60 seconds, followed by a final extension step at 72°C for 7 min. Amplicons were visualized on a 2% agarose gel containing 0.2 µg/µl ethidium bromide, and quantified using a Bio-Rad GS 800 Laser Densitometer (Hercules, CA, USA) and a Bio-Rad Quantity One software.

Data and statistical analysis. Group means and standard deviations (SDs) of total PLT counts were determined and plotted. Student's two-tailed t test for equal variance was used to determine the significance ($p < 0.05$) of total PLT counts by comparing the counts of test mice to control mice.