

# Pathology and protection in nephrotoxic nephritis is determined by selective engagement of specific Fc receptors

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**Introduction of heterologous anti-glomerular basement membrane antiserum (nephrotoxic serum, NTS) into presensitized mice triggers the production of IgG anti-NTS antibodies that are predominantly IgG2b and the glomerular deposition of pathogenic immune complexes, leading to accelerated renal disease. The pathology observed in this model is determined by the effector cell activation threshold that is established by the coexpression on infiltrating macrophages of the IgG2a/2b restricted activation receptor FcγRIV and its inhibitory receptor counterpart, FcγRIIB. Blocking FcγRIV with a specific monoclonal antibody thereby preventing IgG2b engagement or treatment with high dose intravenous γ-globulin (IVIg) to down-regulate FcγRIV while up-regulating FcγRIIB, protects mice from fatal disease. In the absence of FcγRIIB, IVIg is not protective; this indicates that reduced FcγRIV expression alone is insufficient to protect animals from pathogenic IgG2b immune complexes. These results establish the significance of specific IgG subclasses and their cognate FcγRs in renal disease.**

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Abbreviations used: BUN, blood urea nitrogen; GBM, glomerular basement membrane; ITP, idiopathic thrombocytopenic purpura; IVIG, intravenous γ-globulin; NTS, anti-GBM antiserum; PAS, periodic acid-Schiff.

It is now well-established that IgG antibodies mediate their effector activities *in vivo* through their engagement of FcγRs (1). Mice that are deficient in activation FcγRs, by virtue of the targeted disruption of the common γ chain, are protected from the pathogenic effects of cytotoxic antibodies and immune complexes in both passive and active models of inflammation (2–6). In contrast, disruption of the inhibitory Fc receptor, FcγRIIB, enhances the pathogenic activity of IgG antibodies in many of these models (7–11). Although individual subclasses of IgG display different *in vivo* activities (12), it has been difficult, until recently, to account for the hierarchy of these activities based on their interaction with the activation FcγRs I and III. With the identification of a third, γ chain-dependent, IgG activation FcγR, FcγRIV (13), which selectively binds to IgG2a and 2b with intermediate affinity ( $K_d = 10^{-7}$ ), a direct association between IgG subclass activity and FcγR binding could be established (13, 14). This association is based on the respective binding affinities for each subclass to

its cognate activation and inhibitory FcγR, thus resulting in a ratio of activation to inhibition that is predictive of the *in vivo* activity of an antibody (13, 14). Thus, IgG2a has an A/I ratio of 70 and IgG2b has an A/I ratio of 7, whereas IgG1 has an A/I ratio of 0.1, which fits with the hierarchy of activities of these subclasses *in vivo* such that IgG2a > IgG2b >> IgG1. However, these activities can be modified by the relative expression levels of the activation and inhibitory receptor on a specific effector cell, thus changing the avidity of an antibody-antigen complex for a specific activation/inhibitory pair. Inflammatory cytokines, such as IFNγ and C5a, up-regulate activation receptors, while down-regulating the inhibitory receptor (15–17). In contrast, high dose intravenous γ-globulin (IVIg) has been shown to up-regulate the inhibitory receptor (18, 19). The net result of these responses is to change the thresholds required for effector cell triggering and initiation of an inflammatory cascade at a given concentration of cytotoxic antibodies or immune complexes. IgG antibodies that interact with their restricted, cognate FcRs resulting in low A/I ratios are more sensitive to

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these avidity considerations than antibodies which interact with FcR with high A/I ratios (13, 14).

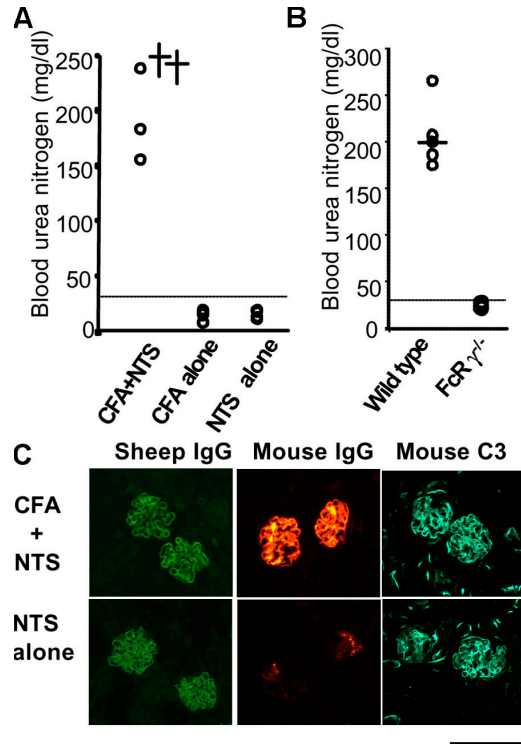
To investigate the consequences of subclass affinity for specific FcRs and the impact of Fc receptor modulation on IgG-mediated inflammation in an active model of inflammation, we selected the murine nephrotoxic nephritis model. This model resembles human Goodpasture's Disease (20–22) by virtue of its dependence on the targeting effect of anti-glomerular basement membrane (GBM) antibodies to direct immune complex deposition to the GBM and initiate an inflammatory response. The introduction of heterologous rabbit or sheep anti-GBM antiserum (NTS) results in an acute, complement-dependent, transient phase of renal injury (day 0–4) that is characterized by mildly elevated blood urea nitrogen (BUN) and proteinuria (21–24). The development of mouse anti-rabbit or -sheep antibodies in the later, autologous stage of this model (day 4–14) results in immune complex deposition and frank glomerulonephritis with renal failure and death (21, 22, 25). This autologous phase has been shown to be Fc $\gamma$ R dependent (25, 26); mice lacking the common  $\gamma$  chain are protected from renal failure and death. An accelerated model of nephrotoxic nephritis has been developed in which mice are presensitized to the heterologous anti-GBM serum by preimmunization with normal, heterologous serum at day -4, followed by introduction of the heterologous anti-GBM serum at day 0, thus initiating the autologous phase of disease concomitant with the introduction of the anti-GBM serum (25, 26). Renal disease is evident by day 4, with significant mortality by day 8 through an Fc $\gamma$ R-dependent pathway.

We now report the contribution of specific IgG subclasses and their cognate Fc $\gamma$ Rs to this accelerated autologous model of nephrotoxic nephritis and demonstrate the role of FcR modulation by IVIG in this active model of inflammation. The intrinsic Fc $\gamma$ R affinities of IgG2b for the specific activating (Fc $\gamma$ RIV) and inhibitory (Fc $\gamma$ RIIB) receptor pair determines the pathology observed, whereas the selective receptor modulation by IVIG of both of these molecules alters their ratio on the infiltrating effector cells and contributes to the protection observed.

## RESULTS

### The nephrotoxic nephritis model

Presensitization of C57BL/6 mice with normal sheep IgG followed 4 d later by the introduction of sheep anti-mouse GBM serum induces acute glomerulonephritis with renal injury, as seen in Fig. 1. Elevated BUN was observed by day 4 in wild-type mice presensitized with normal sheep IgG and exposed to sheep NTS (CFA + NTS). Presensitization with sheep IgG alone, or exposure to anti-GBM serum alone did not result in elevated BUN (Fig. 1 A), despite the localization of the heterologous anti-GBM antibodies to the glomeruli (Fig. 1 C). Renal pathology was dependent, therefore, on the presence of mouse anti-sheep antibodies which colocalized to the glomeruli in the CFA + NTS-treated animals (Fig. 1 C)

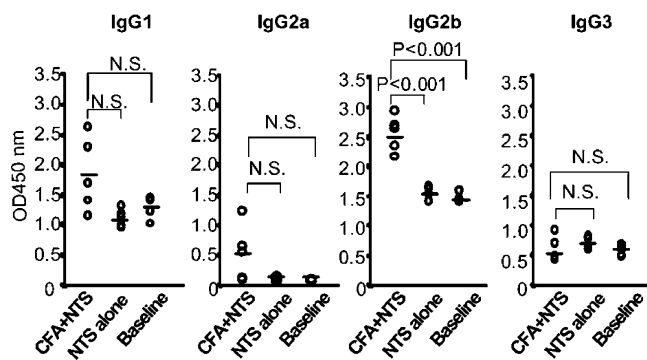


**Figure 1. Renal disease in presensitized, NTS-treated mice is FcR dependent.** (A) Serum samples were collected from five C57BL/6 mice in each group on day 8 after anti-GBM antiserum (NTS) administration alone (NTS alone) or with sheep IgG and CFA preimmunization 4 d before (CFA + NTS), or preimmunization alone (CFA alone). CFA + NTS treatment was necessary for the induction of nephritis. The dagger indicates mice that died before day 8. (B) Serum samples were collected on day 8 after NTS administration from 5 C57BL/6 mice or FcR $\gamma$ <sup>-/-</sup> mice preimmunized with sheep IgG and CFA. Renal pathology was dependent on FcR. (C) Deposition of sheep anti-GBM antibody, autologous mouse anti-sheep IgG antibody, and complement C3 along the GBM. C57BL/6 mice were injected with NTS alone or with sheep IgG and CFA preimmunization (CFA + NTS), and kidneys were removed on day 4 (Bar, 200  $\mu$ m). Autologous mouse IgG deposition was essential for the induction of nephritis in CFA + NTS treated mice. Data shown are representative of three independent experiments.

in a “ribbon” pattern, characteristic of Goodpasture's Disease, and was completely dependent on FcR expression. Mice deficient in the common  $\gamma$  chain, hence deficient in surface expression of the activation Fc receptors Fc $\gamma$ RI, III and IV, as well as Fc $\epsilon$ RI, were protected from the pathogenic consequences of CFA + NTS (Fig. 1 B).

### IgG2b anti-sheep Ig is dominantly induced in the CFA + NTS model of nephrotoxic nephritis

Individual,  $\gamma$  chain-dependent FcRs interact with specific isotypes and subclasses of immunoglobulins (13, 14). To further define the pathology observed in the CFA + NTS model, wild-type mice were characterized for the specific subclasses of IgG anti-sheep GBM antibodies they produced at day 4 after CFA + NTS treatment. As shown in Fig. 2,

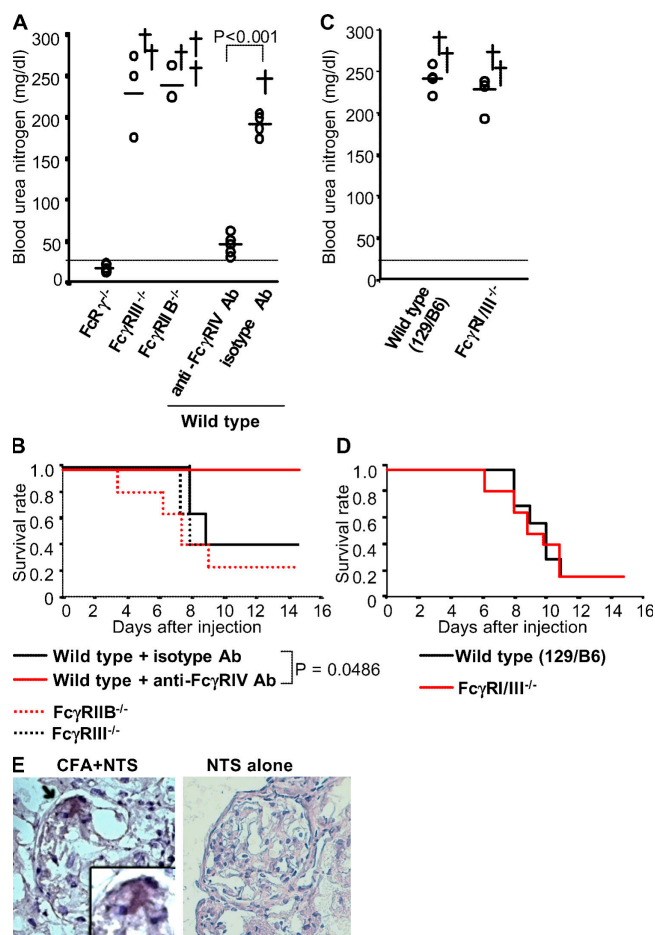


**Figure 2. IgG subclass-restricted production of mouse anti-sheep IgG antibody in presensitized, NTS-treated mice.** Serum titer of each isotype of anti-sheep IgG-specific IgG in C57BL/6 mice was measured by ELISA on day 4 after NTS injection alone or with sheep IgG and CFA pre-immunization (CFA + NTS). Only mouse IgG2b anti-sheep IgG in mice treated with CFA + NTS showed statistically significant elevation compared with baseline level or with sera from mice treated with NTS alone. Significance was determined by Student's *t* test. Data shown are representative of three independent experiments.

only mouse IgG2b anti-sheep Ig showed a statistically significant elevation ( $P < 0.001$ ) after CFA + NTS treatment. NTS alone did not induce measurable levels of mouse anti-sheep Ig at day 4 after exposure to anti-GBM serum. IgG1 anti-sheep Ig was elevated, although the level was not statistically significant over baseline or NTS alone (Fig. 2).

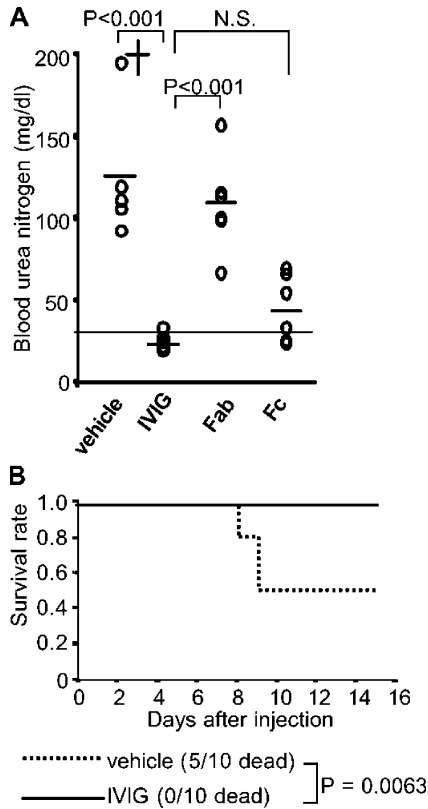
### The renal pathology induced by mouse IgG2b is Fc $\gamma$ RIV dependent

We recently described a novel IgG Fc receptor in the mouse with selective specificity for IgG2a and 2b and with a binding affinity  $\sim 10$ -fold higher than that observed for these subclasses binding to Fc $\gamma$ RIII (13). To determine the relative contributions of these Fc receptors to the renal pathology observed in the nephrotoxic nephritis model described here, we induced disease in either common  $\gamma$  chain-deficient (FcR $\gamma^{-/-}$ ), Fc $\gamma$ RIII-deficient (Fc $\gamma$ RIII $^{-/-}$ ) or wild-type mice treated with a monoclonal anti-Fc $\gamma$ RIV antibody shown to specifically block this activation Fc $\gamma$ R (13, 14; Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20051900/DC1>). Common  $\gamma$  chain-deficient mice were protected, as expected, as were mice treated with the anti-Fc $\gamma$ RIV blocking mAb (Fig. 3, A and B, and Fig. S2). Fc $\gamma$ RIII-deficient mice developed disease comparable with wild-type mice, whereas Fc $\gamma$ RIB-deficient mice displayed enhanced disease, as revealed by the accelerated mortality of this strain when treated with CFA + NTS (Fig. 3 B). We also induced nephrotoxic nephritis in Fc $\gamma$ RI and RIII double-deficient (Fc $\gamma$ RI/III $^{-/-}$ ) mice, and these animals developed disease comparable with wild-type controls (Fig. 3, C and D and Fig. S2). Because no differences in the titer of mouse IgG2b anti-sheep Ig was observed for any of the genotypes studied (Fig. S3), the data indicate that the critical activation Fc $\gamma$ R in this model is Fc $\gamma$ RIV, which is consistent with studies



**Figure 3. Nephrotoxic nephritis is exclusively Fc $\gamma$ RIV dependent.** (A) Serum samples were collected on day 8 after NTS administration from five individual FcR $\gamma^{-/-}$ , Fc $\gamma$ RIII $^{-/-}$ , Fc $\gamma$ RIB $^{-/-}$ , or wild-type mice treated with anti-Fc $\gamma$ RIV antibody or its isotype control antibody. The dagger indicates mice that died before day 8. NTS nephritis was Fc $\gamma$ RIII independent, whereas it was Fc $\gamma$ RIV dependent. Significance of the results was determined by Student's *t* test. (B) Kaplan-Meier analysis of survival rate of Fc $\gamma$ RIII $^{-/-}$ , Fc $\gamma$ RIB $^{-/-}$ , or wild-type mice treated with anti-Fc $\gamma$ RIV antibody or its isotype control antibody ( $n = 5$ ). Treatment with anti-Fc $\gamma$ RIV antibody showed protection in wild-type NTS nephritis mice. The log rank test was used to determine significance between mouse groups treated with anti-Fc $\gamma$ RIV antibody or treated with its isotype control antibody. (C) Serum samples were collected on day 8 after NTS administration from six individual Fc $\gamma$ RI/III $^{-/-}$  or control mice. The dagger indicates mice that died before day 8. NTS nephritis was both Fc $\gamma$ RI and Fc $\gamma$ RIII independent. (D) Kaplan-Meier analysis of the survival rate of Fc $\gamma$ RI/III $^{-/-}$  or control mice ( $n = 6$ ). There was no significant difference in survival rate. (E) Immunohistochemical staining for Fc $\gamma$ RIV on kidney section of wild-type mice on day 4 after NTS administration alone or with sheep IgG and CFA preimmunization (CFA + NTS). The arrow highlights an Fc $\gamma$ RIV-positive cell infiltrating in the diseased glomerulus (brown). Data shown are representative of three independent experiments.

in passive models of idiopathic thrombocytopenic purpura (ITP) and B cell and tumor cell clearance (13, 14; unpublished data). Staining of infiltrating macrophages with anti-Fc $\gamma$ RIV antibody revealed accumulation of these cells in the

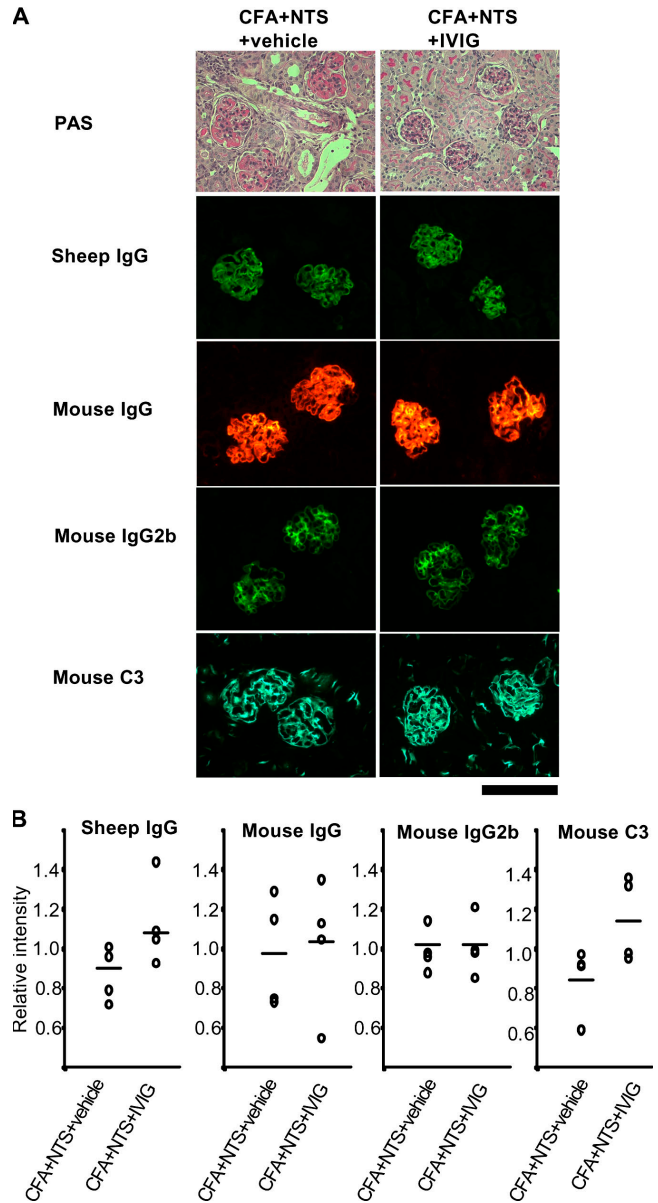


**Figure 4. IVIG protects mice from nephrotoxic nephritis.** (A) Six C57BL/6 mice in each group were injected with IVIG (1 g/kg), IVIG-Fc portion (0.33 g/kg), IVIG-Fab portion (0.67 g/kg), or 10% maltose (vehicle of IVIG) 1 h before NTS administration. Serum samples were collected on day 8 after NTS administration. 1 g/kg of mouse weight of IVIG and equimolar concentrations of IVIG-Fc portion showed the same protective effect in NTS nephritis mice. The dagger indicates mice that died before day 8. (B) Kaplan-Meier analysis of survival rate in NTS nephritis mice treated with IVIG or its vehicle alone ( $n = 10$ ). IVIG treatment improved survival rate of NTS mice. Significance of differences was verified by log rank test. Data shown are representative of three independent experiments.

glomeruli of CFA + NTS-treated animals, thus providing a mechanism for their engagement of IgG2b immune complex deposited on the GBM and the inflammation observed in this model (Fig. 3 E).

**Modulation of FcRs by IVIG attenuates disease in nephrotoxic nephritis**

We have previously demonstrated in passive models of ITP and arthritis that IVIG is able to protect from the pathogenic consequences of cytotoxic antibodies or immune complex deposition by induction of the inhibitory receptor, FcγRIIB (18, 19). Similarly, IVIG treatment attenuates the renal pathology observed in CFA + NTS nephrotoxic nephritis and is dependent on the Fc fragment for its activity (Fig. 4 A). This protection results in extended survival for IVIG-treated animals (Fig. 4 B). IVIG treatment did not modify the development of mouse IgG2b anti-sheep Ig antibodies



**Figure 5. Histopathology and deposition along the GBM of kidney section in NTS nephritis mice with or without IVIG treatment.**

(A) PAS staining of kidney section on day 8 from NTS nephritis mice treated with either 10% maltose (vehicle) alone or IVIG. Vehicle alone shows severe tissue damage with intracapillary cellular proliferation, glomerular thrombosis, and mesangiolysis. In contrast, renal pathology of NTS mice treated with IVIG was markedly attenuated. Kidney sections from mouse groups on day 4 were equally stained with FITC-conjugated anti-sheep IgG antibody, Cy3-conjugated anti-mouse IgG antibody, FITC-conjugated anti-mouse IgG2b antibody, and FITC-conjugated anti-mouse complement 3 antibody (Bar, 200 μm). (B) Quantitative immunofluorescence of glomerular deposits of sheep IgG, mouse IgG, mouse IgG2b, and mouse C3 on day 4. There was no significant difference between the groups. Data shown are representative of three independent experiments.

(Fig. S3) or the deposition of either sheep anti-GBM antibodies, total mouse IgG or IgG2b antibodies, or complement C3 in the glomeruli (Fig. 5). To determine the mechanism

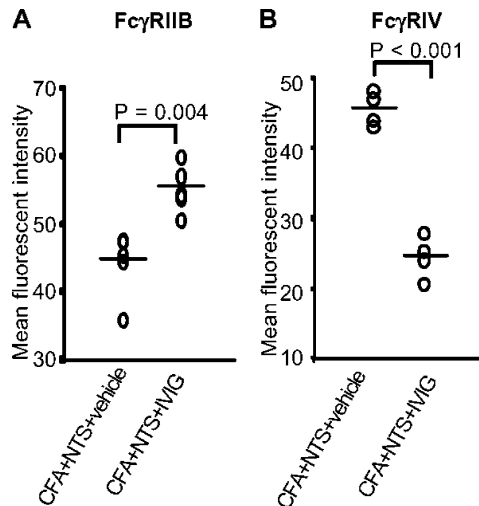
by which IVIG protects CFA + NTS-treated mice from developing renal pathology, we examined the expression of Fc $\gamma$ RIIIB and Fc $\gamma$ RIV on macrophages infiltrating the kidney in this disease. As shown in Fig. 6 A, IVIG significantly induces the surface expression of Fc $\gamma$ RIIIB on infiltrating macrophages ( $P = 0.004$ ), as we have previously described for splenic (18) and infiltrating synovial macrophages (19). However, and in contrast to these previous studies, IVIG treatment also resulted in reduced surface expression of Fc $\gamma$ RIV (Fig. 6 B and Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20051900/DC1>). Fc $\gamma$ RIII expression was unchanged (unpublished data) as was Fc $\gamma$ RIIIB and Fc $\gamma$ RIV expression on granulocytes (unpublished data). The combined effect of reduced Fc $\gamma$ RIV and elevated Fc $\gamma$ RIIIB alters the activation threshold for IgG2b engagement of Fc $\gamma$ RIV and protects mice from the pathological consequences of IgG2b immune complex deposition in the glomeruli.

#### Fc $\gamma$ RIIIB up-regulation by IVIG is required to protect mice from NTS + CFA induced nephrotoxic nephritis

Because IgG2b can bind to both Fc $\gamma$ RIII and Fc $\gamma$ RIV, albeit with significantly different affinities, the relative contributions of Fc $\gamma$ RIIIB, Fc $\gamma$ RIII and Fc $\gamma$ RIV modulation to the protective effect of IVIG was examined. As we have observed previously (18, 19), IVIG protection was ablated in Fc $\gamma$ RIIIB-deficient mice (Fig. 7 and Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20051900/DC1>), which is consistent with Fc $\gamma$ RIIIB expression contributing to the protective effect of IVIG. Thus, the down-regulation of Fc $\gamma$ RIV by IVIG treatment was not sufficient to provide protection, and thus required additional modulation of Fc $\gamma$ RIIIB. CFA + NTS-treated Fc $\gamma$ RIIIB-deficient mice were protected by anti-Fc $\gamma$ RIV, indicating that Fc $\gamma$ RIV is the sole activation FcR involved in the pathology of this disease, despite the ability of IgG2b to engage Fc $\gamma$ RIII. Consistent with this observation, IVIG protection was not affected by Fc $\gamma$ RIII deficiency (Fig. 7).

#### DISCUSSION

The selective engagement of IgG subclasses with specific Fc $\gamma$ Rs is a determining factor in the pathophysiology of a variety of antibody-mediated inflammatory disorders and antibody based immunotherapeutics. Simultaneous engagement of both activating (Fc $\gamma$ RIII and Fc $\gamma$ RIV in the mouse model; Fc $\gamma$ RIIA and Fc $\gamma$ RIIIA in the human model) and inhibitory receptors (Fc $\gamma$ RIIIB in both mouse and human models) determines the threshold for triggering activation of effector cells such as macrophages, mast cells, and neutrophils. Detailed studies performed in the murine system have revealed the specifics of these interactions. Thus, IgG1 antibodies mediate their *in vivo* activity through simultaneous engagement of the low-affinity receptors Fc $\gamma$ RIIIB and Fc $\gamma$ RIII, whereas IgG2a and IgG2b are dependent on the Fc $\gamma$ RIIIB and Fc $\gamma$ RIV pair. Several studies have confirmed the role of Fc $\gamma$ RIIIB and Fc $\gamma$ RIII in IgG1-mediated inflam-

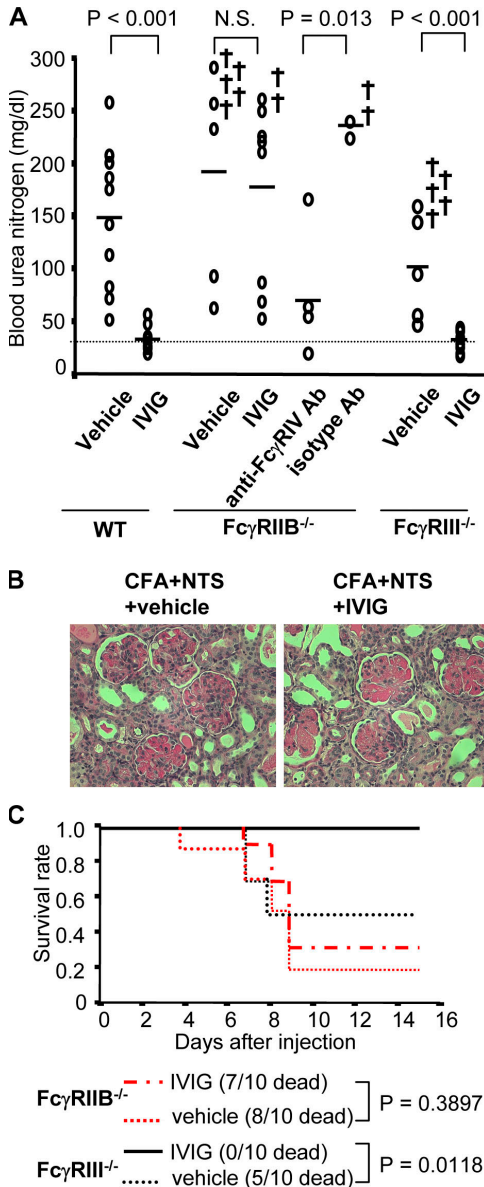


**Figure 6. Up-regulation of Fc $\gamma$ RIIIB and down-regulation of Fc $\gamma$ RIV by IVIG on F4/80-positive cells infiltrating in the kidney.**

Leukocytes infiltrating in the kidney of NTS nephritis mice treated with IVIG or its vehicle (10% maltose) were isolated on day 2 after NTS administration, and mean fluorescent intensities of Fc $\gamma$ RIIIB (A) and Fc $\gamma$ RIV (B) on F4/80-positive cells were evaluated by flow cytometry using the Fc $\gamma$ R-specific antibody Ly 17.2 (reference 17) and 9G8.1 (reference 13). F4/80-positive cells in the kidney of NTS mice treated with IVIG showed significant up-regulation of Fc $\gamma$ RIIIB and down-regulation of Fc $\gamma$ RIV expression. Significance was verified by Student's *t* test. Data shown are representative of two independent experiments.

mation *in vivo*, based on studies performed in experimental models of cytotoxic antibody-triggered clearance including ITP (13), hemolytic anemia (27, 28) B cell clearance (29) and tumor metastases (9); and immune complex-mediated diseases, such as the cutaneous and alveolar Arthus reactions (30) and KRNxNOD serum-induced arthritis (19). Passive models of IgG2a- and 2b-mediated clearance, although sensitive to deletion of the common  $\gamma$  chain and hence deficient in Fc $\gamma$ RI, III and RIV, were not sensitive to either Fc $\gamma$ RI or RIII deletion (13, 14). Instead, blockade of Fc $\gamma$ RIV abrogates IgG2a- or 2b-mediated effector responses *in vivo* in passive models of ITP, B cell clearance; and tumor metastasis (13, 14; unpublished data). Thus, despite the ability of IgG2a to bind with high affinity to the activating receptor Fc $\gamma$ RI (31), this receptor has not been shown to be central to the mechanism by which IgG2a mediates its biological activity *in vivo* (28, 32). This general lack of Fc $\gamma$ RI involvement is likely to result from the occupancy of this receptor by circulating immunoglobulin during the steady state, thereby rendering it unavailable for specific IgG2a-mediated cross-linking.

Several active models of antibody-mediated inflammation have suggested that skewing of the IgG subclass response occurs in response to a variety of inflammatory stimuli. Thus, in spontaneous murine lupus models, anti-DNA antibodies of the IgG2a or 2b class appear to dominate (unpublished data). Similarly, active antiviral protection models have observed



**Figure 7. IVIG protection in nephrotoxic nephritis requires Fc $\gamma$ RIIB.** (A) Serum samples were collected on day 8 after administration of NTS from wild-type (WT), Fc $\gamma$ RIIB<sup>-/-</sup>, or Fc $\gamma$ RIII<sup>-/-</sup> presensitized mice treated with IVIG or its vehicle (10% maltose) alone ( $n = 10$ ). Additionally, four Fc $\gamma$ RIIB<sup>-/-</sup> mice in each group were treated with anti-Fc $\gamma$ RIV antibody or its isotype control antibody. The dagger indicates mice that died before day 8. IVIG was protective in wild-type and Fc $\gamma$ RIII<sup>-/-</sup> NTS nephritis mice, but not in Fc $\gamma$ RIIB<sup>-/-</sup> mice. Anti-Fc $\gamma$ RIV antibody treatment in Fc $\gamma$ RIIB<sup>-/-</sup> mice showed the same effect as IVIG on wild-type or Fc $\gamma$ RIII<sup>-/-</sup> mice. Significance was verified by Student's  $t$  test. (B) PAS staining of kidney section on day 8 from NTS nephritis. Fc $\gamma$ RIIB<sup>-/-</sup> mice treated with IVIG or its vehicle alone showed equally severe tissue damage with intracapillary cellular proliferation, glomerular thrombosis, and mesangiolysis. (C) Kaplan-Meier analysis of survival rate of Fc $\gamma$ RIII<sup>-/-</sup> and Fc $\gamma$ RIIB<sup>-/-</sup> mice treated with IVIG or its vehicle alone ( $n = 10$ ). Survival rate was significantly improved by IVIG treatment in Fc $\gamma$ RIII<sup>-/-</sup> mice, but not in Fc $\gamma$ RIIB<sup>-/-</sup> mice. Significance was determined by log rank test.

a dominant role for IgG2a antiviral antibodies (33–35). Our present study reveals that skewing to IgG2b is prominent in accelerated anti-GBM nephritis, perhaps as a result of the contribution of TGF $\beta$  to the glomerular injury seen in this model (36). TGF $\beta$  up-regulation has been shown to occur in a variety of conditions of renal injury (37, 38) and has also been shown to induce class switching to IgG2b in vitro (39, 40). It is likely that skewing of IgG subclasses will be a common feature of inflammatory conditions, in response to the specific cytokine milieu present in these states. Although previous studies of accelerated nephrotoxic nephritis all demonstrated a dependence on Fc $\gamma$ R by virtue of the protection observed in the common  $\gamma$  chain-deficient strain, these studies differed on the role of Fc $\gamma$ RIII in this model. Fujii et al. (41) demonstrated that renal disease was substantially attenuated in a rabbit anti-GBM model in Fc $\gamma$ RIII-deficient mice, although to a lesser degree than Fc $\gamma$  chain deficiency for some parameters of renal injury. These investigators did not define the IgG subclasses involved in their model, which could account for the differences with our study. It is tempting to speculate that the mouse anti-rabbit GBM antibody response is dominated by IgG1 antibodies, hence Fc $\gamma$ RIII dependent, in contrast to the mouse anti-sheep GBM antibody response that is IgG2b and Fc $\gamma$ RIV dependent. Tarzi et al. (42), using a sheep anti-GBM model of nephrotoxic nephritis, similar to that used in this study, observed protection in Fc $\gamma$ R-deficient mice, but not Fc $\gamma$ RIII-deficient animals, similar to our observations. They did not investigate Fc $\gamma$ RI-deficient mice, but did report attenuation in the double Fc $\gamma$ RI/III-deficient strain. They, however, used a fivefold lower dose of sheep GBM antiserum in their experiments and mainly histological changes as readout. Moreover, the specific IgG subclasses were not described. In contrast, we observe severe BUN elevation and almost 50% mortality under our experimental conditions.

Consistent with our previous studies (18, 19), we demonstrate that high dose intravenous IgG protects mice from nephrotoxic nephritis and this protection is dependent on Fc $\gamma$ RIIB. Fc $\gamma$ RIIB-deficient mice are not protected by IVIG, because one activity of IVIG is to up-regulate Fc $\gamma$ RIIB on infiltrating macrophages. This mechanism has also been observed in the serum transfer arthritis model, KRNxNOD (19), which is an IgG1-mediated immune complex-driven model of arthritis and results in an elevated threshold for Fc $\gamma$ RIII-driven inflammation. However, in contrast to that Fc $\gamma$ RIII-driven model, the effect of IVIG in this study is to both up-regulate Fc $\gamma$ RIIB and down-regulate Fc $\gamma$ RIV. Because IgG2b has an activation/inhibitory affinity ratio 70 times that of IgG1, resulting from the higher affinity of IgG2b for Fc $\gamma$ RIV (13), up-regulation of Fc $\gamma$ RIIB alone would not be sufficient to raise the threshold required to prevent macrophage triggering by IgG2b immune complexes. Thus, the mechanism of protection by IVIG differs for IgG subclasses and suggests that the normal physiological mechanism underlying this activity reflects an in vivo feedback inhibition pathway that is subclass specific and has evolved to prevent

sustained and inappropriate effector cell activation after resolution of an inflammatory state.

Our present study highlights the importance of specific IgG subclasses and their cognate Fc receptors in defining the pathophysiology of disease states. This knowledge is of importance in identifying the molecular pathways involved and thereby selecting the appropriate targets for intervention.

## MATERIALS AND METHODS

**Mice.** C57BL/6 mice were purchased from the Jackson Laboratory. FcR $\gamma$ <sup>-/-</sup> (2), Fc $\gamma$ RIIB<sup>-/-</sup> (8), and Fc $\gamma$ RIII<sup>-/-</sup> mice were generated in our laboratory and backcrossed for 12 generations to the C57BL/6 background. Fc $\gamma$ RI/III<sup>-/-</sup> mice on a hybrid C57BL/6/129F2 background were compared with a genetically matched control line. Female mice at 6–10 wk of age were used for all experiments and maintained at the Rockefeller University animal facility. All experiments were done in compliance with federal laws and institutional guidelines and have been approved by the Rockefeller University.

**Antibodies and transfectants.** Human IVIG (5% in 10% maltose, chromatography purified) was purchased from Octapharma. Digestion of human IVIG was performed as described previously (19). In brief, IVIG was digested by 0.5 mg/ml papain for 1 h at 37°C, and stopped by the addition of 2.5 mg/ml iodoacetamide. The resulting Fab and Fc fragments were separated from nondigested IVIG on a HiPrep 26/60 S-200HR column (GE Healthcare), and then Fc fragments were separated from Fab fragments using a protein G column (GE Healthcare) and a protein L column (Pierce Chemical Co.). Fragment purity was checked by immunoblotting using anti-human IgG Fab- or Fc-specific antibodies (Jackson ImmunoResearch Laboratories). Purity was judged to be >99%. Antibodies 2.4G2, Mac-1, Gr-1 were purchased from BD Biosciences. The anti-FLAG antibody was purchased from Sigma-Aldrich. The F4/80 and CD68 antibody was obtained from Serotec. The Ly 17.2 antibody was purchased from Caltag. Hamster monoclonal antibodies against mouse Fc $\gamma$ RIV, 9E9, and 9G8.1 were generated in our laboratory (13). 200  $\mu$ g of the 9E9 antibody, which interferes with immune complex binding to Fc $\gamma$ RIV, was injected intravenously every other day from day 0 to 14. Hamster IgG was used as an isotype control. The 9G8.1 antibody was conjugated with Alexa 647 (Invitrogen) and used for flow cytometric analysis. Chinese hamster ovary cells stably transfected with FLAG-tagged murine versions of Fc $\gamma$ RI, Fc $\gamma$ RIIB, Fc $\gamma$ RIII, and Fc $\gamma$ RIV were generated in our laboratory (13).

**Preparation of sheep NTS.** Sheep anti-GBM antigen was prepared as described previously (43). In brief, glomeruli were isolated from normal C57BL/6 mouse kidneys by differential sieving, washed extensively, and used to hyperimmunize sheep as described previously (43). The hyperimmune serum was heat inactivated, and then absorbed with excess amount of murine red blood cells.

**Induction of anti-GBM glomerulonephritis.** Mice were preimmunized intraperitoneally with 200  $\mu$ g of sheep IgG (Serotec) in CFA, followed by intravenous injection of sheep NTS (2.5  $\mu$ l of serum per gram of mouse) 4 d later. IVIG (1 g/kg) or its vehicle alone was injected 1 h before anti-GBM antiserum injection.

**Histological analysis.** Kidneys were removed, fixed in 10% buffered formalin, and embedded in paraffin. 4  $\mu$ m paraffin sections were stained with periodic acid-Schiff (PAS) and evaluated by light microscopy in a blind manner as previously described (44). In brief, the clinical scores of glomerular injury were graded into five grades: 0 (normal), 1 (mild increase in cellularity), 2 (definite glomerular enlargement, focal hypercellularity and mild increase of matrix), 3 (focal hypercellularity and proliferation in >50% of glomeruli), and 4 (diffuse proliferative change with crescents and sclerosis in >50% of

glomeruli). Tubulointerstitial lesions were also graded from 0 to 4 according to the severity of inflammatory cell infiltration. Crescent formation was evaluated as the number of the crescents per 10 glomeruli. The mean number of macrophages infiltrating in the glomeruli were counted in 50 glomeruli in each sample.

**Immunofluorescence and immunohistochemical staining.** 4- $\mu$ m frozen sections were fixed in acetone and stained with FITC-conjugated anti-sheep IgG, Cy3-conjugated anti-mouse IgG (Jackson ImmunoResearch), FITC-conjugated anti-mouse C3 (Cedarlane), and FITC-conjugated anti-mouse IgG2b (BD Biosciences) antibody, respectively. For quantitative immunofluorescence, blinded sections were examined at 100 magnification using MetaMorph version 6.1 (Molecular Devices). The mean intensity of 20 glomeruli for each sample was measured for evaluation. For immunohistochemical analysis, frozen sections were incubated with 5  $\mu$ g/ml of biotinylated anti-Fc $\gamma$ RIV monoclonal antibody (clone 9E9) after blocking with 500  $\mu$ g/ml of hamster IgG in 5% goat serum and biotin blocking (Dako-Cytomation). After blocking of endogenous peroxidase was performed, HRP-conjugated rabbit anti-biotin antibody (Dako-Cytomation) was used as the secondary antibody and 3–3'-diaminobenzidine was used for visualization. Biotinylated anti-CD68 antibody (clone FA11) was used to detect macrophages in the kidney.

**Measurement of sheep IgG-specific circulating IgG levels.** 96-well ELISA plates coated with 5  $\mu$ g/ml of sheep IgG were incubated with 1:500 diluted test sera after blocking with 5% bovine serum albumin. After washing with PBS containing 0.05% Tween 20, the plates were incubated with HRP-conjugated anti-mouse IgG1, IgG2c (Igh 1b allele of IgG2a), IgG2b, or IgG3 antibody (Bethyl Laboratories). For the color development, 3,3',5,5'-tetramethylbenzidine was used.

**Measurement of blood urea nitrogen.** BUN in sera was measured by the urease-indophenol method with an Enzymatic Urea Nitrogen kit (Stambio Laboratory).

**Preparation of kidney-infiltrating cells and flow cytometric analysis.** Kidneys were pressed through a mesh, and the cells were resuspended in PBS. After washing, the cells were suspended in 33% Percoll solution and centrifuged 2,000 rpm for 20 min at room temperature. After red blood cell lysis, the cells were stained with the indicated monoclonal antibodies, and were subjected to flow cytometric analysis. 2,000 F4/80-positive cells infiltrating in the kidneys of each mouse were evaluated for mean fluorescence intensity of FITC-conjugated Ly 17.2 or Alexa 647-conjugated 9G8.1 antibody.

**Statistical analysis.** Statistical differences in each group for the BUN, circulating IgG levels, and mean fluorescent intensity, were calculated with Student's *t* test. Mann-Whitney U test was used for histological analysis. Survival rate was analyzed with Kaplan-Meier estimates, and groups were compared with the log rank test. *P* < 0.05 was considered significant. SPSS version 11.0 for Windows (SPSS Inc.) was used for statistical analysis.

**Online supplemental material.** Fig. S1 shows specificity of anti-Fc $\gamma$ RIV monoclonal antibody 9E9. Fig. S2 demonstrates histological evaluation of kidney sections from wild-type C57BL/6 mice, anti-Fc $\gamma$ RIV antibody-treated or isotype-matched antibody-treated wild-type C57BL/6 mice, Fc $\gamma$ RI/III<sup>-/-</sup> mice or its genetically matched control (129/B6) mice on day 8 from NTS injection. Fig. S3 contains serum titer of each isotype of autologous mouse anti-sheep IgG-specific IgG. Fig. S4 shows histograms of Fc $\gamma$ RIIB and Fc $\gamma$ RIV expression on F4/80-positive cells infiltrating the kidney on day 2. Fig. S5 demonstrates histological evaluation of kidney sections from wild-type C57BL/6 mice and Fc $\gamma$ RIIB<sup>-/-</sup> mice treated with IVIG or its vehicle on day 8 from NTS injection. Supplemental figures are available at <http://www.jem.org/cgi/content/full/jem.20051900/DC1>.

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