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*J Immunol* published online 23 April 2014
http://www.jimmunol.org/content/early/2014/04/23/jimmunol.1301611

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/04/23/jimmunol.130161
1.DCSupplemental

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Therapeutic Effect of IVIG on Inflammatory Arthritis in Mice Is Dependent on the Fc Portion and Independent of Sialylation or Basophils

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High-dose i.v. Ig (IVIG) is used to treat various autoimmune and inflammatory diseases; however, the mechanism of action remains unclear. Based on the K/BxN serum transfer arthritis model in mice, IVIG suppression of inflammation has been attributed to a mechanism involving basophils and the binding of highly sialylated IgG Fc to DC-SIGN—expressing myeloid cells. The requirement for sialylation was examined in the collagen Ab-induced arthritis (CAbIA) and K/BxN serum transfer arthritis models in mice. High-dose IVIG (1–2 g/kg body weight) suppressed inflammatory arthritis when given prophylactically. The same doses were also effective in the CAbIA model when given subsequent to disease induction. In this therapeutic CAbIA model, the anti-inflammatory effect of IVIG was dependent on IgG Fc but not F(ab’)2 fragments. Removal of sialic acid residues by neuraminidase had no impact on the anti-inflammatory activity of IVIG or Fc fragments. Treatment of mice with basophil-depleting mAbs did not abrogate the suppression of either CAbIA or K/BxN arthritis by IVIG. Our data confirm the therapeutic benefit of IVIG and IgG Fc in Ab-induced arthritis but fail to support the significance of sialylation and basophil involvement in the mechanism of action of IVIG therapy. The Journal of Immunology, 2014, 192: 000–000.

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease of unknown cause that targets the synovial joints. It is characterized by synovial inflammation and hyperplasia, autoantibody production to various Ags (e.g., IgG Fc or rheumatoid factor, citrullinated proteins), cartilage and bone erosion, as well as systemic manifestations (e.g., cardiovascular disorders). Cytokines and autoantibodies feature prominently in the disease pathogenesis and current biotherapies that have proven efficacious in the treatment of disease (comprehensively reviewed in Ref. 1) include those targeting TNF (e.g., adalimumab), T cell costimulation (CTLA-4 fusion protein; abatacept), and B cells (anti-CD20; rituximab). However, RA is a heterogeneous disease and not all patients respond to these specifically targeted treatments. Additionally, a variety of complications arise from the immunosuppressive effects of these agents (2).

Intravenous Ig (IVIG) and s.c. Ig are purified IgG preparations made from the pooled plasma of thousands of healthy donors. IVIG was originally prescribed for the treatment of primary immunodeficiency and secondary immunodeficiency syndromes where it replenishes levels of serum Ig and provides life-saving protection from infection (reviewed in Refs. 3–5). Although primary immunodeficiency represents a significant proportion of IVIG usage, the larger market share for IVIG is used for the treatment of patients with various chronic and acute autoimmune and inflammatory diseases (6). High-dose (1–2 g/kg body weight) IVIG is commonly used for the treatment of immune cytopenia, Guillain–Barré syndrome, Kawasaki disease, chronic inflammatory demyelinating polyneuropathy, myasthenia gravis, and several other rare diseases (7–11). In addition to these accepted uses, several other indications are currently under exploration. There are multiple reports of investigational use of IVIG for the treatment of refractory autoimmune and inflammatory disorders, such as RA (12).

Several mechanisms of action have been proposed for the anti-inflammatory efficacy of high-dose IVIG therapy (6). These include FcR blockade (13, 14), anti-idiotypic Abs in IVIG (15, 16), inhibition of complement deposition (17), increased regulatory T cell involvement (18, 19), enhancement of FcyRIIB on regulatory macrophages (20), saturation of neonatal FcR to enhance autoantibody clearance (21, 22), and direct (23–25) and indirect (26, 27) modulation of molecular (cytokines, growth factors, chemokines, adhesion molecules, apoptotic molecules, microbial toxins) and cellular (T cells, B cells, dendritic cells) immune mediators. It is likely that more than one of these potential modes of action accounts for the anti-inflammatory efficacy of IVIG in a particular disease. It is also probable that important modes of
action differ from disease to disease. Several of these proposed mechanisms are not without controversy (28–34); however, it is interesting that many investigational studies have shown that the Fc portion is often the active component of IVIG, suggesting that FcR mechanisms are frequently involved in the anti-inflammatory effects of IVIG.

IVIG contains a broad Ab repertoire representing the plasma donor population. It is used in investigational studies for the treatment of RA and juvenile chronic arthritis; however, despite this, there have been few animal model studies examining its efficacy. One laboratory (35) recently described a possible mechanism of action for IVIG based on studies in an animal model of arthritis, where α2,6-linked terminal sialic acid residues in the Fc region of IgG engage the C-type lectin receptor SIGN-R1 (DC-SIGN in humans) on myeloid regulatory cells stimulating the secretion of IL-33. IL-33 then enhances the expansion of IL-4–producing basophils resulting in the increased expression of the inhibitory Fc receptor FcγRIIB on regulatory macrophages and subsequent suppression of the inflammatory response.

The role of IVIG sialylation in a mouse model of immune thrombocytopenia (ITP) was recently explored. No role for sialylation in the inhibitory effects of IVIG was found in that therapeutic model (28). In another study, contradictory results were reported in a prophylactic model for ITP (36). We therefore wanted to explore the role of sialylation in other animal models of Ab-driven inflammation, such as the K/BxN serum transfer (K/BxN) (37) and collagen Ab-induced arthritis (CAbIA) (38) models of RA. We examined the effect of IVIG in these two models and evaluated the proposed mechanisms of action. We found that IVIG was protective in a dose-dependent manner in both mouse models of Ab-induced arthritis and that the protective activity resided within the Fc fragment. Similar to our findings in mouse ITP (28), sialylation of IVIG was not required for efficacy. We also found that, in contrast to previous reports in Ab-driven arthritis (35), basophils were not required for the protective effect of IVIG.

Materials and Methods

Mice

Male C57BL/6 (B6) mice aged >7 wk were used for all experiments. KRN TCR transgenic mice on the B6 background (a gift from D. Mathis and C. Benoist, Harvard Medical School, Boston, MA) were bred to NOD mice to generate the K/BxN mice expressing both the TCR transgene KRN and the MHC class II molecule I-Ag7. The CSL/Pfizer, St. Michael’s Hospital, and University Health Network (Animal Use Protocol 2915.4) Animal Ethics Committees approved all procedures and protocols.

Production of IgG fragments

Fab′), fragments were produced by papain digestion of IVIG (Privigen: CSL Behring, Bern, Switzerland) as described earlier (29). Briefly, IVIG was digested with papain (0.5 mg/ml IgG; Sigma-Aldrich, Buchs, Switzerland) in acetate buffer (pH 4.0) for 2 h at 37˚C and the reaction was stopped by adding 2 M Tris base until a pH of 8 was reached.

Fc fragments were prepared from IVIG (Privigen) by papain digestion and purification by ion exchange and size exclusion chromatography and polished using Fab′-specific affinity chromatography, as described earlier (29). The endotoxin level was monitored and, when needed, Fc was polished by EndoTrap HD (Hyglos, Regensburg, Germany) resulting in endotoxin levels <0.1 endotoxin unit/mg. Concentration and purification from small digestion products and buffer exchange to PBS was performed by diafiltration. Sialic acid–enriched Fc fragments were prepared by incubation with Sambucus nigra agglutinin (SNA) lectin affinity chromatography as described earlier (29). The sialic acid content was enriched from 1.3 μg per mg Fc in the starting material to 6.6 μg per mg Fc in the resulting enriched fraction (SNAFc). The integrity of the lectin-purified Fc molecule (SNAFc) was controlled by testing binding to protein A using ELISA and biolayer interferometry (measured on an Octet QK® platform; Pall forteBio Portsmouth, UK). With both methods we could show that SNAFc binds in a similar manner to protein A as untreated Fc (data not shown).

Enzymatic desialylation with neuraminidase

IgG and fragments were desialylated by enzymatic digestion with recombinant neuraminidase (NAsne) from Clostridium perfringens expressed in Escherichia coli (New England BioLabs, Ipswich, MA). IgG or Fc was incubated with 7 U enzyme (New England BioLabs units) per mg protein for 48 h at 37˚C followed by concentration and buffer exchange to PBS by diafiltration. The digestion was monitored by sialic acid detection using SNA lectin blot, HPLC, or liquid chromatography–mass spectrometry (29). Residual sialic acid was <0.1 μg per mg IVIG for desialylated IVIG (NAsne IVIG) and 0.15 μg per mg Fc for desialylated Fc (NAsne Fc), as determined by HPLC (29).

CAbIA

Mouse monoclonal anti-type II collagen mAb mixture kit was purchased from Chondrex (Redmond, WA). On day 0 mice were injected i.p. with 2 mg mAb mixture followed by 50 μg LPS i.p. on day 3. For prophylactic treatment, mice were injected i.v. with different doses of IVIG (up to 1.0 g/kg) 1 h prior to the mAb mixture. For therapeutic protocols,arthritic mice were randomly assigned to treatment groups at day 5 or 6 and injected i.p. with IVIG (up to 2.0 g/kg) or IVIG derivatives. Mice were monitored for up to 12 d for clinical signs of arthritis. A clinical score was assigned to each paw as follows: 0, normal; 0.5, swelling confined to digits; 1, mild paw swelling; 2, marked paw swelling; 3, severe paw swelling and/or ankylosis.

K/BxN serum transfer arthritis

Disease was induced in B6 mice by i.p. injection of 0.16 ml pooled serum from arthritic K/BxN mice on day 0, as described (37). The volume was chosen based on in vivo titration of pooled serum. IVIG (2 g/kg) was injected i.p. prophylactically 2 h prior to the K/BxN serum. Clinical disease (maximum score of 12 per mouse) was monitored daily for up to 10 d, as previously described (39). Ankle widths of the hind paws (in millimeters) were measured at the widest point (the malleoli) with the legs fully extended daily for up to 10 d using a digital caliper.

Histology of arthritic joints

At termination of the experiment, mice were euthanized and the paws were fixed in 10% (w/v) neutral buffered formalin, decalcified, and embedded in paraffin. Frontal tissue sections of the right-side front and rear paws were stained with H&E and scored in a blinded fashion with respect to the treatment groups. The multiple joints of the front (carpal) and rear (tarsal) paws were globally scored for three features (exudate, presence of inflammatory cells within the joint space; synovitis, the degree of synovial membrane thickening and inflammatory cell infiltration; and tissue destruction, cartilage and bone erosion and invasion), each out of three (0, normal; 1, mild; 2, moderate; 3, severe), and these were tallied for a total score out of nine.

Basophil depletion

To examine the effect of basophil depletion in CAbIA, mice were given twice-daily i.p. injections (8 h apart) of 5 μg anti-CD123 mAb (MAR-1; eBioscience) or isotype control (Armenian hamster IgG; eBioscience) on days 1–3 after CAbIA induction. In some experiments, basophils were instead depleted by i.p. injection with 50 μg anti-CD123 mAb (4G12; CSL Ltd) 1 d prior to the induction of CAbIA. For the K/BxN serum transfer studies, basophils were depleted by 10 consecutive daily i.p. injections of 10 μg MAR-1, beginning 2 d prior to the K/BxN serum.

Flow cytometry

To evaluate basophil numbers in peripheral blood, single-cell suspensions of RBC-depleted peripheral blood were resuspended in PBS containing 1% (w/v) BSA. Cells (~1×10^7) were stained with allophycocyanin-conjugated anti-CD123 (5B11; eBioscience) and PE-conjugated anti-CD49b mAb (DX5; BD Pharmingen). Propidium iodide (1 μg/ml Sigma-Aldrich) was used to exclude dead cells, and live cells were analyzed on a BD LSR II (BD Biosciences) using FlowJo software (Tree Star, Ashland, OR).

Statistical analyses

Data are shown as means ± SEM. ANOVA with Dunnett’s or Tukey’s post hoc tests were used for clinical scores and ankle width measurements. The Mann–Whitney two-tailed test was used to compare histological scores. A p value of ≤0.05 was considered statistically significant. Statistics were determined using Prism 6 for Mac OS X (GraphPad Software).
Results

Prophylactic IVIG protects against disease in CAbIA

Using a prophylactic protocol, IVIG was previously reported to protect from K/BxN arthritis (35). To investigate whether IVIG is protective in other clinically relevant Ab-induced arthritis models, its effect in the CAbIA model was examined. IVIG, when given i.v. 1 h prior to the i.p. injection of arthritogenic Abs, was found to dose-dependently reduce disease severity with a significant effect (p < 0.01, ANOVA with Dunnett’s test) at the highest dose of 1 g/kg (Fig. 1A).

Therapeutic IVIG halts the progression of established CAbIA

Therapeutic rather than prophylactic protocols are more relevant to the treatment of human RA, and so the effect of IVIG on established disease in the CAbIA model was evaluated. As before, mice were induced to develop CAbIA by i.p. injection of anti-collagen mAb mixture at day 0 and i.p. injection of LPS at day 3. On the first day that significant clinical disease was apparent (day 5 or 6), arthritic mice were randomly assigned to treatment groups, such that the mean clinical score for each group was similar. A single injection of IVIG dose-dependently reduced the clinical severity of disease with maximum effect at 2 g/kg (p < 0.05, ANOVA with Dunnett’s test) (Fig. 1B). The effect was evident within 24–48 h of IVIG administration. IVIG injections were routinely given i.p., as we observed no difference in response between mice injected i.v. or i.p. (data not shown) and it also enabled higher dosing. Increasing the total IVIG dose to 3 g/kg, given as three consecutive injections of 1 g/kg at 2-d intervals, provided no further benefit over a single injection of 1 g/kg (data not shown).

Histological analysis and blinded scoring of joint sections was performed to confirm the clinical assessment. The joints of mice treated with therapeutic IVIG showed milder histopathology with reduced joint exudate, synovitis (black arrows) and cartilage (white arrows), and bone destruction (Fig. 1C). Mice treated with IVIG using the therapeutic protocol showed a significant reduction (p < 0.05, Mann–Whitney U test) in the total histological score, which combines all features of arthritis: joint exudate, synovitis, and cartilage and bone destruction (Fig. 1D).

Fc but not Fab is the active component of IVIG for protection from CAbIA

To investigate the active component of IVIG that provides therapeutic protection from CAbIA, IgG Fc and F(ab')2 fractions were evaluated at molar doses equivalent to the IgG levels in IVIG. Fc (0.67 g/kg) provided the same level of protection as an equivalent molar dose of IVIG (2 g/kg) (Fig. 2A). In contrast, purified IgG F(ab')2 (1.33 g/kg; molar equivalent to 2 g/kg IVIG) had no effect on CAbIA (Fig. 2A). Purified IgG Fc dose-dependently reduced disease progression (p < 0.05 for 0.67 g/kg and p < 0.01 for 1.0 g/kg doses, ANOVA, Dunnett’s test) (Fig. 2B).

Sialic acid residues are not required for the protective effect of IVIG in CAbIA

The mechanism of action of IVIG in protecting against arthritis has recently been reported to involve sialic acid side chains on the Fc portion of IgG (40). We investigated this claim using a therapeutic protocol in the CAbIA model. IVIG or purified IgG Fc were NAse treated to remove the sialic acid residues from IgG (29). We found that NAse pretreatment of either IVIG or Fc had no impact on the therapeutic protection from disease in CAbIA (Fig. 3A and 3B, respectively).

These results contrasted with previously published work that used a prophylactic protocol in the K/BxN model (40, 41). To account for this difference in procedure, we repeated our studies using a prophylactic protocol. We found that desialylation of IVIG (not shown) and Fc (Fig. 3C) by NAse pretreatment did not compromise the prophylactic benefit of IVIG/Fc in the CAbIA model, again suggesting that sialic acid residues were not a required component of the mechanism of action. Similar results were obtained when using NAse-treated Fc that had been purified by protein G–Sepharose to remove any residual NAse, which could potentially interfere with the disease readout (data not shown). Finally, both sialic acid–enriched Fc fragments (SNAFc) and Fc showed no benefit in the prophylactic CAbIA model when tested at a 10-fold lower dose (0.033 g/kg) (Fig. 3D). Importantly, SNAFc and untreated Fc bound to protein A in a similar manner (data not shown), suggesting that the SNAFc was not compromised by the lectin purification process.

Lack of requirement for IVIG sialylation for protection from K/BxN arthritis

To directly compare with published studies (40), we examined the requirement for sialylation of IVIG and Fc in the K/BxN model using a prophylactic protocol. Both IVIG (2 g/kg) and Fc (0.33 g/kg) potently suppressed arthritis; however, desialylation by NAse pretreatment did not compromise the prophylactic benefit of IVIG.
Basophils are not required for IVIG protection from Ab-induced arthritis

Finally, basophils have been proposed as key cell mediators of the protective activity of IVIG in the K/BxN model (35), and so the role of these cells in CAbIA was investigated. Mice were injected twice daily with basophil-depleting mAb MAR-1 (5 μg) or isotype control mAb over days 1–3. At day 5, there was no difference in disease response between the basophil-depleted and control mice. Diseased mice were further divided into IVIG and PBS treatment groups and disease was monitored for an additional 7 d. The data in Fig. 5A show that basophil depletion with MAR-1 did not impact on the therapeutic benefit of IVIG in CAbIA. Flow cytometry of peripheral blood from mice at day 12 confirmed basophil depletion by MAR-1 (CD49b+, CD123+ cells) (Fig. 5B). Similar results were obtained when mice were instead treated with a single injection of basophil-depleting mAb 4G12 (50 μg) 24 h prior to the induction of CAbIA (data not shown).

To determine whether these findings were unique to the CAbIA model, we also examined the need for basophils for IVIG protection from K/BxN arthritis using a prophylactic protocol. Mice were given 10 consecutive daily i.p. injections of MAR-1 (10 μg) beginning 2 d prior to the administration of IVIG (2 g/kg) and induction of disease on day 0. Prophylactic IVIG protected from K/BxN arthritis and this was unaffected by basophil depletion with MAR-1, whether assessed by clinical score or caliper measurement of ankle swelling (Fig. 5C and 5D, respectively).

Discussion

IVIG is used to treat a range of autoimmune, acute, and chronic inflammatory diseases, including investigational use in RA (12). However, the scientific basis of its effect has not been rigorously examined in animal disease models and remains controversial (34). In models of inflammatory arthritis, IVIG alleviated adjuvant-induced arthritis in rats (42) and K/BxN serum transfer arthritis in mice (43–45) but showed only a weak effect against murine collagen-induced arthritis (46). Detailed investigations into IVIG protection from inflammatory arthritis have focused on prophylactic studies in the K/BxN serum transfer model, and it is unclear how universal the proposed mechanisms of action are. To this end, we examined the effect of IVIG in two models of Ab-induced arthritis: CAbIA and K/BxN serum transfer arthritis. We found that high-dose IVIG (1-2 g/kg) alleviated disease in both models when given prophylactically before disease initiation. We also showed that IVIG suppressed CAbIA development when given therapeutically at the time of clinical appearance of disease symptoms. Our data are in line with a previous report showing that IVIG used in a therapeutic protocol was effective in the K/BxN model (43).

Based on a series of publications, Ravetch and colleagues (35, 40, 43) have attributed the mechanism of action of IVIG in inflammatory arthritis to a sequence of events beginning with the interaction of essential α2,6-linked terminal sialic acid residues in the Fc region of IgG with SIGNR-1 (DC-SIGN in human)–positive macrophages or dendritic cells. To investigate this paradigm in CAbIA we first compared IgG Fc and F(ab′)2 fractions and found that the protective activity was also confined to the Fc portion of IgG used in a therapeutic protocol was effective in the K/BxN model (43).
sialic acid–enriched Fc and found no additional benefit over unmanipulated Fc when tested at a 10-fold lower dose in CAbIA. Furthermore, sialic acid–enriched IVIG fractions did not show enhanced effects (data not shown). Taken together, these data do not support a role for sialic acid residues in the protective action of IVIG in Ab-induced arthritis. Critically, we were unable to confirm a role for sialic acid residues in IVIG protection from K/BxN arthritis using the published protocol (prophylactic i.v. injection) (40). This finding was confirmed in several additional experiments independently performed both at the Bio21 Institute (Melbourne, VIC, Australia) and at St. Michael’s Hospital (Toronto, ON, Canada).

Recently, Ravetch and colleagues (47) have proposed a mechanism whereby the sialylation of Fc induces a conformational change allowing binding to DC-SIGN and a reduced binding affinity to FcγRs. This hypothesis has been refuted by Crispin et al. (48) followed by a rebuttal from Sondermann et al. (49) questioning the physiological relevance of the analytical techniques used. Nevertheless, in this model the proposed structural re-orientation to an anti-inflammatory Fc remains dependent on the presence of sialic acid residues, which is not confirmed by our results. Additionally, a recent report (50) demonstrated that the interaction of DC-SIGN with sialylated IgG Fc is not the sole requirement for an anti-inflammatory effect of IVIG because both whole IVIG and the F(ab′)2 fragment inhibited TLR-mediated activation of dendritic cells. However, in a reply, the relevance of the assay system used to study IVIG anti-inflammatory activity change allowing binding to DC-SIGN and a reduced binding affinity to FcγRs. This hypothesis has been refuted by Crispin et al. (48) followed by a rebuttal from Sondermann et al. (49) questioning the physiological relevance of the analytical techniques used. Nevertheless, in this model the proposed structural re-orientation to an anti-inflammatory Fc remains dependent on the presence of sialic acid residues, which is not confirmed by our results. Additionally, a recent report (50) demonstrated that the interaction of DC-SIGN with sialylated IgG Fc is not the sole requirement for an anti-inflammatory effect of IVIG because both whole IVIG and the F(ab′)2 fragment inhibited TLR-mediated activation of dendritic cells. However, in a reply, the relevance of the assay system used to study IVIG anti-inflammatory activity

![FIGURE 4. Lack of requirement for sialylation of IVIG or Fc for suppression of K/BxN serum transfer–induced arthritis. Mice were injected i.p. with reagents 2 h prior to the induction of arthritis by i.p. injection of K/BxN serum. Data show the clinical scores (mean ± SEM) with time for mice given untreated or NAse-treated (A) IVIG (2 g/kg) (n = 4 animals/group) or (B) Fc (0.33 g/kg) (n = 3), compared with controls. (C) Caliper measurements of ankle widths for the experiment in (B). **p < 0.01, ***p < 0.001 compared with control; two-way ANOVA with Tukey’s test on days 1–10. (D) Confirmation of desialylation of IVIG and Fc by NAse treatment. SNA lectin blot under nonreducing conditions. Lane 1, IgG; lane 2, IVIG; lane 3, NAse IVIG; lane 4, Fc; lane 5, NAse Fc. Molecular masses are indicated at left, and the positions of IgG and Fc are shown at right.](http://www.jimmunol.org/)

![FIGURE 5. Basophil depletion has no impact on IVIG protection from CAbIA (therapeutic protocol) or K/BxN serum transfer arthritis (prophylactic protocol). (A) Clinical response of mice induced to develop CAbIA and treated at day 5 with IVIG (mean ± SEM, n = 5–7). *p < 0.05, **p < 0.01, compared with control, two-way ANOVA with Tukey’s test on days 6–12. (B) Representative flow cytometry plots from day 12 of the experiment in (A) showing peripheral blood basophil levels following treatment of mice with MAR-1 or hamster IgG control Abs. Rectangles show basophil gate (CD49b+, CD123+) with numbers indicating the percentage of gated cells. (C) Mean (±SEM, n = 3–5 animals/group) clinical scores and (D) ankle width measurements of mice pretreated with IVIG and then induced to develop K/BxN serum transfer arthritis. ***p < 0.001, compared with control, two-way ANOVA with Tukey’s test on days 0–10. Treatment groups (see text for details): control, hamster IgG then PBS; IVIG, hamster IgG then IVIG; MAR-1 IVIG, MAR-1 then IVIG.](http://www.jimmunol.org/)
was questioned (51). These examples demonstrate that the functional role of IgG sialylation remains a controversial topic.

Furthermore, basophils have been implicated as key effector cells in the mechanism of action of IVIG in the K/BxN model (35), and so their role was re-evaluated in this model, as well as in CAbIA, using anti-FcεRI (MAR-1), as previously described (35). Basophil-depleted mice responded normally in both models and were not compromised in their IVIG-mediated protection from disease. These findings do not support basophils as having an essential role in the mechanism of action. IL-33 is considered another important component of the IVIG paradigm through its stimulation of basophils to produce IL-4. In studies using an IL-33Rα blocking mAb it was concluded that IVIG protection from disease was IL-33 dependent (35). In preliminary studies, we were unable to demonstrate an effect of the same mAb to IL-33Rα used by Anthony et al. (35) on IVIG protection from CAbIA (Supplemental Fig. 1). Similarly, more recent studies from a separate laboratory showed that IL-33Rα−/− mice, but not IL-33−/− mice, were protected from K/BxN arthritis (52). These data suggest that IL-33Rα, a member of the IL-1R family, binds ligands other than IL-33, which are important in promoting disease. Further support for a lack of a requirement for IL-33 in the mechanism of IVIG action comes from recent studies using an experimental mouse model of ITP where it was shown that IVIG does not induce detectable levels of IL-33 in BALB/c or B6 mice, despite amelioration of the ITP (53). The data are therefore at odds with the concept of IL-33Rα–associated ligands providing protection from K/BxN arthritis.

To potentially explain some of the disparities between our findings and those of Ravetch and colleagues, it might be speculated that differences inherent to the two mouse models will lead to different outcomes. Indeed, CAbIA and K/BxN serum transfer arthritis are each models of Ab-induced arthritis that differ in the mode of induction and in some aspects of the disease response (reviewed in reference Ref. 54). CAbIA is initiated by injection of a mixture of mAbs (IgG2a and IgG2b) to cartilage-specific type II collagen. An otherwise mild arthritis is exacerbated by LPS injection 3 d later, probably through the generation of a “cytokine storm.” K/BxN serum transfer arthritis is induced by polyclonal serum from arthritic K/BxN mice, the active component being Abs (IgG1) that target the ubiquitous Ag glucose-6-phosphate isomerase, forming immune complexes that have been identified on the articular cartilage surface. The unpurified K/BxN serum derived from arthritic mice is also likely to contain proinflammatory cytokines and mediators. Each model is dependent on the alternative complement pathway, Fc receptors (FcγRI, FcγRII), cytokines (IL-1 and TNF) but not IL-6, and myeloid-derived (neutrophils, macrophages) but not lymphoid cells. CAbIA, but not K/BxN arthritis, is additionally dependent on IL-4 and the classic complement pathway. Mast cells were reported to be essential for K/BxN arthritis but their role in CAbIA is unclear.

Despite some differences between the CAbIA and K/BxN arthritis models, detailed above, we obtained comparable results for the effects of IVIG in the two models. Although we concur that IVIG and IgG Fc, but not Fab, can protect from Ab-induced arthritis, our findings do not support a role for Fc sialylation or basophil involvement in IVIG protection from disease in either model. The choice of model is therefore unlikely to explain our disagreement with the published findings of Ravetch and colleagues. Our data are also derived from multiple laboratories, reducing the chance of laboratory-specific outcomes. Other reasons should therefore be considered including IVIG source, route and time of administration, mouse strain, and experimental setup.

It can be hypothesized that different IVIG sources might contribute to the variable disease responses and proposed mechanisms of action. We used Privigen (CSL Behring AG), whereas Ravetch and colleagues have listed the Bayer Corporation (Elkhart, IN) (43) and, more recently, Octagam (Octapharma AG) (35, 55) as IVIG sources. Previous studies (28, 29) reported no differences in either the degree of IgG sialylation or the efficacy of IVIG in alleviating immune thrombocytopenia in mice when IVIG was derived from different manufacturers. IVIG is produced from the pooled blood of >10,000 individuals, which minimizes variation between pools by dilution of individual donors. Both Privigen and Octagam were derived from United States blood donors. Differences in the IVIG manufacturing procedures of suppliers could only be addressed by a side-by-side comparison of the products in the mouse disease models.

In terms of experimental setup, the published studies (35, 40, 41, 43, 55) indiscriminately employed both B6 and BALB/c mice in the K/BxN model with i.v. injection of IVIG in an exclusively prophylactic protocol. Clinical scores were usually reported only for days 5 or 6. We confined our studies to B6 mice and present kinetics data for both prophylactic and therapeutic treatment regimes, as this enables any delayed responses to be observed. IVIG was generally injected i.p. but was given i.v. in select experiments (i.e., prophylactic CAbIA studies). The i.v. injection of IVIG is reported to target splenic SIGH-R1+ (DC-SIGH+ in humans) myeloid cells by binding of sialylated Fc (35). Intraperitoneal injection of IVIG could result in most of the IgG Fc interacting with peritoneal macrophages through Fc sialylation-independent mechanisms, with less being available for binding to splenic myeloid cells. Despite this possibility, our data showed that i.p. IVIG was extremely effective in preventing K/BxN arthritis.

The consistent finding of the need for IgG Fc fragments for IVIG suppression of Ab-induced arthritis suggests FcR engagement or Ig aggregation may be part of the mechanism of action. Possible IgG Fc-driven mechanisms of action that are independent of a requirement for sialylation could include FcR blockade (13, 14), inhibition of complement deposition (17) or complement scavenging, increased regulatory T cell involvement (18, 19), enhancement of FcyRIIb on regulatory macrophages (20), and saturation of neonatal FcR to enhance autoantibody clearance (21, 22). As detailed above, each of the arthritis models used in this study is critically dependent on FcR engagement and the alternative complement pathway, which could account for how IVIG protects in a prophy- lactic protocol. In a therapeutic setting, where disease is already established, IVIG may be acting differently, for example, by dampening down the inflammatory cytokine response by engaging regulatory macrophages. Further studies are needed to clarify whether distinct mechanisms are involved under the different experimental conditions. The lack of protection by the F(ab′)2 fraction indicates that Fab-mediated mechanisms of action of IVIG are not essential in the models used in this study, including neutralization of autoantibodies, neutralization of proinflammatory cytokines, or direct interaction of Fab with dendritic cells.

In summary, our data confirmed that IVIG protects from Ab-induced arthritis by a mechanism that involves the Fc component of IgG. However, we were unable to corroborate a role for sialic acid residues or basophils, and so our data do not support the paradigm proposed by Ravetch and colleagues as a mechanism of action of IVIG in inflammatory arthritis. Where possible, we have controlled for experimental variations that might account for any disparity between the published work and our findings. In conclusion, it is likely that no single paradigm can entirely account.
for the mechanism of action of IVIG, even in a given disease model, such as K/BxN serum transfer arthritis. Our studies also highlight the importance of evaluating a mechanism of action using more than one disease model and treatment protocol.

Acknowledgments
We thank Monika Rüegsegger and Andreas Hofmann (CSL Behring, Bern, Switzerland) for producing various IVIG preparations and for excellent technical assistance.

Disclosures
I.K.C., S.M., A.W.Z.-E., B.S.M., and F.K. are employees of CSL Ltd. or CSL Behring AG, as indicated in the affiliations.

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