INFLAMMATION

Treating murine inflammatory diseases with an anti-erythrocyte antibody

Andrew R. Crow[1,2,3]*, Rick Kapur[1,2,3,4,5]*, Sandra Koernig[6], Ian K. Campbell[6], Chao-Ching Jen[2,3], Patrick J. Mott[2,3], Danielle Marjoram[2,3], Ramsha Khan[2,3], Michael Kim[2,3], Jennifer Brasseit[7], Yoelys Cruz-Leal[1,2,3], Alaa Amash[2,3], Simrat Kahlon[2,3], Issaka Yougbare[1,2,3], Heyu Ni[1,2,3,8,9,10], Adrian W. Zuercher[7], Fabian Käsermann[7], John W. Semple[1,2,3,4,8,9,11], Alan H. Lazarus[1,2,3,8,9†]

Treatment of autoimmune and inflammatory diseases typically involves immune suppression. In an opposite strategy, we show that administration of the highly inflammatory erythrocyte-specific antibody Ter119 into mice remodels the monocyte cellular landscape, leading to resolution of inflammatory disease. Ter119 with intact Fc function was unexpectedly therapeutic in the K/BxN serum transfer model of arthritis. Similarly, it rapidly reversed clinical disease progression in collagen antibody-induced arthritis (CAIA) and collagen-induced arthritis and completely corrected CAIA-induced increase in monocyte Fcγ receptor II/III expression. Ter119 dose-dependently induced plasma chemokines CCL2, CCL5, CXCL9, CXCL10, and CCL11 with corresponding alterations in monocyte percentages in the blood and liver within 24 hours. Ter119 attenuated chemokine production from the synovial fluid and prevented the accumulation of inflammatory cells and complement components in the synovium. Ter119 could also accelerate the resolution of hypothermia and pulmonary edema in an acute lung injury model. We conclude that this inflammatory anti-erythrocyte antibody simultaneously triggers a highly efficient anti-inflammatory effect with broad therapeutic potential.

INTRODUCTION

Autoimmune disease has an estimated prevalence of 4.5% in the general population with generally 81 disorders considered to be an autoimmune disease or syndrome. Current effective treatments rely heavily on immunosuppression, and although immunosuppression is an effective treatment for many patients, a large number of patients are not able to tolerate long-term use of immunosuppressive therapy often due to side effects including serious risks of infection. Initial treatment of many autoimmune diseases generally starts with corticosteroids. Patients who cannot tolerate corticosteroid use are often switched to other immunosuppressive drugs such as methotrexate, hydroxychloroquine, tumor necrosis factor–α (TNF-α) inhibitors, abatacept, anakinra, rituximab, or tocilizumab depending on the autoimmune disease and previous experience.

For non-immunosuppressive regimes, patients can sometimes be effectively treated with infusion of large doses of intravenous immunoglobulin (IVIg). The mechanism of effect of IVIg in autoimmunity remains controversial with a variety of mechanisms proposed (*1*–*3*). The use of IVIg requires the collection of large amounts of plasma for the manufacture of the product, and its considerable yearly increase in use for treating autoimmune and inflammatory diseases is a major challenge.

Rhesus immunoglobulin (anti-D) is a polyclonal immunoglobulin G (IgG) antibody directed against the Rhesus D (RhD) factor present in red blood cells (RBCs). It is thus a type of IVIg collected from the blood of men intentionally immunized with RhD^+ RBCs (*4*).

Immune thrombocytopenia (ITP) is an autoimmune disorder characterized by the production of platelet-reactive autoantibodies in most patients (*5*). Antibody-sensitized platelets then interact with activating Fcγ receptor (FcγR) on splenic macrophages followed by phagocytosis resulting in thrombocytopenia. This pathophysiology is thought to operate in most patients with ITP (*6*). Anti-D is an effective first-line therapy for ITP (*7*–*12*) and was used in ITP on the basis of the observation that IVIg caused anemia in RhD^+ patients with ITP and the speculation that anti-D works by overloading macrophages with erythrocytes and in effect competing out the destruction of platelets in ITP (*13*–*15*).

Anti-D is highly effective in about 65 to 85% of patients with ITP and works at a 5-log–fold lower dose than IVIg (50 to 75 μg/kg versus 1 to 2 g/kg) and can achieve a clinical response faster than IVIg (*16*). When compared to typical immune-inhibitory monoclonal antibodies, it is used at a ≥2-log–fold lower dose than rituximab or ofatumumab (anti-CD20), tocilizumab [anti–interleukin-6R (IL-6R)], belimumab [anti–B cell activating factor (BAFF)], epratuzumab (anti-CD22), abatacept (CTLA-4-Ig), certolizumab (anti–TNF-α), and canakinumab (IL-1 block) for other autoimmune diseases (*17*, *18*). Thus, anti-D is a high-activity antibody that would be attractive to use more widely. However, the proposed mechanism of action of anti-D in autoimmunity and its failure in a patient with chronic inflammatory demyelinating polyneuropathy (CIDP) (*19*) has limited its consideration to only ITP. Using murine models of ITP, we and others have shown that monoclonal antibodies to erythrocytes can also successfully ameliorate thrombocytopenia (*20*–*25*).

The antibody Ter119 reacts with a glycophorin A–associated protein on murine erythrocytes and has been used as a model antibody

[1]Canadian Blood Services Centre for Innovation, Ottawa, Ontario K1G 4J5, Canada. [2]Department of Laboratory Medicine and Keenan Research Centre for Biomedical Science of St. Michael's Hospital, University of Toronto, Toronto, Ontario M5B 1W8, Canada. [3]Toronto Platelet Immunobiology Group, Toronto, Ontario, M5B 1T8 Canada. [4]Department of Hematology and Transfusion Medicine, Lund University, Lund 221 84, Sweden. [5]Department of Experimental Immunohematology, Sanquin Research and Landsteiner Laboratory, Amsterdam UMC, University of Amsterdam, 1066 CX Amsterdam, Netherlands. [6]CSL Limited, Bio21 Institute, University of Melbourne, Parkville, Victoria 3010, Australia. [7]CSL Behring, Research, CSL Biologics Research Center, Bern, Switzerland. [8]Department of Medicine, St. Michael's Hospital, University of Toronto, Toronto, Ontario M5B 1W8, Canada. [9]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada. [10]Department of Physiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada. [11]Department of Pharmacology, University of Toronto, Toronto, Ontario M5S 1A8, Canada.
*These authors contributed equally to this work as co-first authors.
†Corresponding author. Email: lazarusa@smh.ca

to understand how anti-D may increase platelet counts in murine-passive ITP (*22*–*24*, *26*). We report here that Ter119 not only stimulated a number of inflammatory effects including the expected induction of anemia but also caused rapid changes in body temperature and systemic increases in inflammatory cytokines. This effect of the antibody to essentially fight inflammation with inflammation in multiple models of arthritis and transfusion-related acute lung injury (TRALI) could indicate that regimens to treat inflammatory disease may potentially go beyond just immunosuppression. RBC antibodies comprise a previously unexplored option to potentially consider in the treatment of inflammatory and autoimmune disorders.

RESULTS

Ter119, an antibody with inflammatory properties, can treat murine ITP uncoupled from anemia

The dogma regarding the mechanism of action of anti-D in ITP is the saturation of macrophages by anti-D–opsonized RBCs, which simply compete with sensitized platelets for splenic clearance (*27*–*32*). Mice treated with Ter119, however, underwent a rapid drop in body temperature measurable within 10 min after injection (Fig. 1A). Despite this inflammatory event, mice subsequently treated again with Ter119 24 hours later were protected against a second drop in body temperature (fig. S1).

Ter119 is known to induce anemia (*33*, *34*); to determine whether anemia was a prerequisite for the amelioration of murine ITP by Ter119 observed previously (*21*–*26*), mice were injected with Ter119 to induce anemia (*34*) versus a control rat IgG. To evaluate the ability of Ter119 to ameliorate ITP, murine-passive ITP was induced with an antiplatelet antibody (Fig. 1C). Ter119 marginally decreased RBC counts commencing 3 hours after administration (Fig. 1B) yet significantly increased platelet counts after ITP induction well before this time point ($P < 0.05$; Fig. 1C). Conversely, there was no amelioration of ITP coincident with maximal anemia on day 4 after administration (Fig. 1, B and C). These data indicate that the development of anemia did not correlate with the amelioration of ITP and led us to speculate that Ter119's inflammatory yet therapeutic activity in ITP might actually be broadly immune modulatory.

The Ter119 antibody with intact Fc domain function has therapeutic activity in the K/BxN serum transfer model of inflammatory arthritis

Rheumatoid arthritis is a common autoimmune disorder affecting roughly 1% of the world population and involves inflammation of the synovial joints (*35*). The murine K/BxN arthritis model captures many of the immunological mechanisms of human rheumatoid arthritis (*36*), and we used this model to test Ter119's potential broad therapeutic activity.

C57BL/6 mice injected with K/BxN serum developed inflammatory arthritis, evident 2 to 3 days after serum injection based on their ankle width (Fig. 2A) and clinical score (Fig. 2B). Disease severity increased with time, reaching a maximum at days 7 to 8. In comparison, mice prophylactically treated with Ter119 demonstrated significantly reduced arthritis scores ($P < 0.01$; Fig. 2, A and B). These data demonstrate that a monoclonal antibody to RBC can inhibit the development of inflammatory arthritis, suggesting that Ter119 might exert broad therapeutic activity beyond the treatment of only ITP.

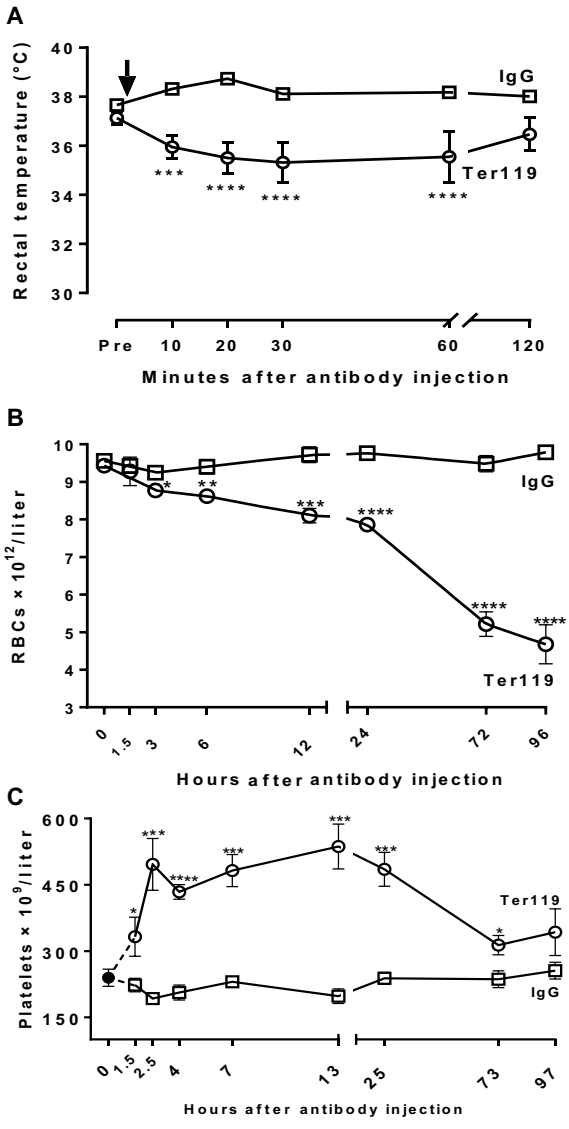

Fig. 1. The monoclonal RBC-specific antibody Ter119, an antibody with inflammatory properties, can treat murine ITP chronologically disparate from anemia. (**A**) CD-1 mice were assessed for rectal temperature (t = Pre), warmed for 5 min, and then injected with 40 μg of control rat IgG ($n = 6$ mice; squares) or Ter119 ($n = 6$ mice; circles), and rectal temperature was taken 10, 20, 30, 60, and 120 min after antibody injection. (**B**) Groups of CD-1 mice ($n = 5$ per data point) were independently assessed for anemia with terminal bleeds. Mice were treated with 40 μg of rat IgG or Ter119 and analyzed at the time points indicated. The time point represented as zero is a prebleed for the mice treated with IgG or Ter119. (**C**) To assess the ability of Ter119 versus control IgG to increase platelet counts in a passive ITP model over time, mice were first treated with Ter119 or IgG for the duration depicted on the x axis and terminally bled for platelet counts. Sixty minutes before this, terminal bleed mice were injected with 3 μg of antiplatelet antibody to induce thrombocytopenia. For clarity, the Ter119-treated mice at the 1.5-hour time point in (C) were injected with Ter119 (intravenously) for 30 min followed by antiplatelet antibody (intravenously) and then bled for platelet counts after a further 60 min. The time point represented as zero in (B) is a prebleed for all mice in the experiment. Data are presented as means ± SEM from five separate experiments with one mouse for each data point in each experiment (80 mice in total for the subpanel); each time point (except for time 0) is a terminal bleed and assesses different mice. Statistical analysis was performed using multiple t tests. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ in comparison to the same control IgG time point.

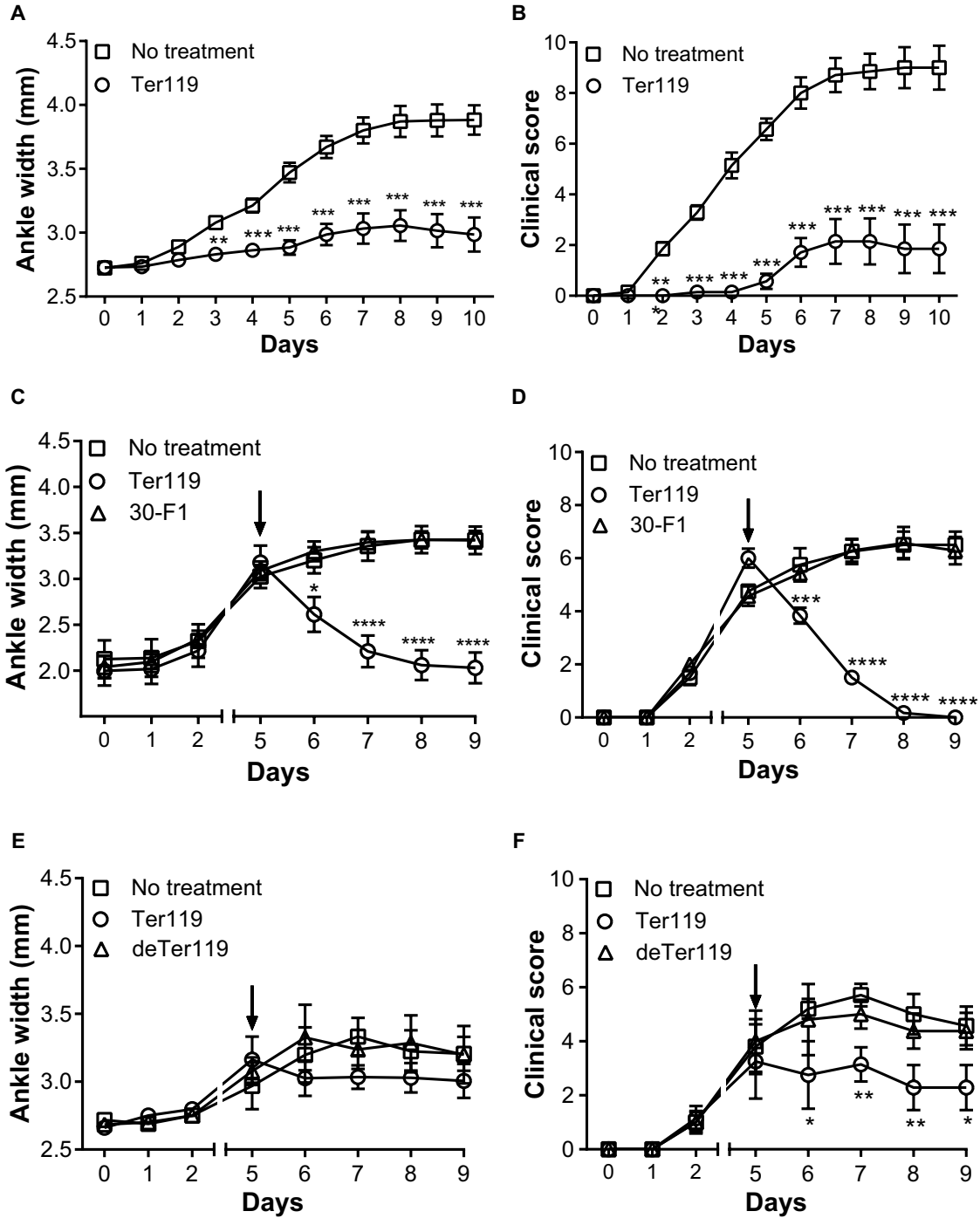

Fig. 2. Ter119 inhibits inflammatory arthritis in the K/BxN model. C57BL/6 mice were assessed for basal arthritis measurements on day 0 (**A** and **B**), and then, one group of mice received 45 μg of Ter119 (circles), the other group (squares) received nothing. Two hours later, all mice received an injection of K/BxN serum. Ankle measurements (A) and clinical score (B) were taken every day for 10 days. Data are expressed as means ± SEM divided across four separate experiments with ≥7 mice per group. Mice received an injection of K/BxN serum with no pretreatment (**C** and **D**). On day 5, arthritic mice were treated (arrow) with nothing (squares), 50 μg of control anti-RBC antibody 30-F1 (triangles), or 45 μg of Ter119 (circles). Ankle measurements (C) and clinical score (D) were measured on days 0 to 2 and days 5 to 9. Data are expressed as means ± SEM divided across four separate experiments. $n = 5$ (K/BxN serum alone); $n = 6$ (Ter119); $n = 7$ (30-F1). To assess the requirement for Fc region glycosylation in the therapeutic function of Ter119, C57BL/6 mice were injected with K/BxN serum for 5 days and then administered with either no treatment (squares), 45 μg of Ter119 (circles), or 45 μg of Ter119 that had been previously deglycosylated using PNGase F (triangles), and ankle measurements (**E**) and clinical score (**F**) were assessed. $n = 7$ (K/BxN serum alone); $n = 7$ (Ter119); $n = 8$ (degly-Ter119). Statistical analysis was performed using multiple *t* tests (A and B) and two-way analysis of variance (ANOVA) test with Dunnett's multiple comparisons test compared to the control arthritis group (no treatment group) (C to F). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

The initiation of arthritis in the K/BxN serum transfer model involves a number of early immune events including the accumulation of autoimmune immune complexes in the joint, activation of neutrophils and mast cells, increases in vascular permeability, and release of vasoactive mediators (*37*). To determine whether Ter119 therapeutic activity could bypass disease induction events, mice were injected with K/BxN serum, and arthritis scores were allowed to develop to near-maximal arthritis (Fig. 2, C and D). Arthritic mice that were treated with Ter119 on day 5 showed a marked and rapid reduction in inflammation 1 day after treatment with ankle widths and clinical scores continuing to improve over the course of the experiment. Mice receiving the control RBC antibody 30-F1 [a rat IgG2c, which has no therapeutic activity in murine ITP (*21*) and is a rat subclass not known for interactions with FcγRs (*38*)] showed no improvement in inflammation, similar to untreated mice. These data demonstrate that Ter119 can reverse established arthritis and potentially suggest IgG Fc region function in this activity.

To evaluate the requirement for a functional Fc region of Ter119, the antibody was fully deglycosylated using peptide *N*-glycosidase F (PNGase F) as described (*23*), impairing its FcγR interactions, complement activation, and antibody-dependent cellular cytotoxicity (*39*, *40*), as well as abrogating its therapeutic activity in ITP (*23*). Deglycosylated Ter119 did not exert therapeutic activity in K/BxN inflammatory arthritis (Fig. 2, E and F).

The Ter119 antibody has therapeutic activity in collagen antibody-induced arthritis

Although the K/BxN serum transfer model is considered a valuable tool for understanding human inflammatory arthritis, the predominant IgG1 autoantibodies are thought to form soluble immune complexes with the ubiquitous glucose-6-phosphate isomerase in sera followed by deposition in the joints (*37*). To evaluate a different model of inflammatory arthritis reliant on IgG2a/b subtype autoantibodies, which directly target collagen in the joints, we used the collagen antibody-induced arthritis (CAIA) model. Mice injected with the CAIA cocktail and lipopolysaccharide (LPS) were examined for clinical score and injected with Ter119 or an isotype control antibody after presentation of arthritic symptoms on day 5 (Fig. 3A). Ter119 again ameliorated disease activity within 1 day of its injection, leading to markedly improved paw redness and swelling on day 8 (Fig. 3B) and improved histological scores on day 12 (Fig. 3, C to E).

The Ter119 antibody rapidly and selectively corrects the arthritis-induced increase in FcγRIIB/IIIA expression in CAIA

Because Ter119 had a requirement for Fc region glycosylation in the K/BxN model, and this glycosylation is critical for driving interactions with FcγRs and complement, we evaluated the effect of different doses of Ter119 (Fig. 4, A and B) on FcγR and complement receptor expression on monocytes in CAIA mice (on day 6), 24 hours after Ter119 injection (Fig. 4C). In comparison to arthritic mice treated with only the isotype control antibody (isotype), Ter119 treatment demonstrated a selective reduction in FcγRIIB/IIIA (CD16/32) to a point indistinguishable from naïve mice (Fig. 4C). In contrast, expression of FcγRI (CD64), CR1/CR2 (CD21/35), and the C5a receptor (CD88) was not decreased by Ter119 but rather increased (Fig. 4C) commensurate with an inflammatory response (*41*, *42*).

Ter119 affects inflammatory cells and mediators in CAIA

Ter119 can ameliorate murine ITP independent of FcγRIIB (*22*), suggesting that it may work through FcγRIII. FcγRIIIA (but not FcγRI or II) cross-linking of human blood mononuclear cells has been shown to lead to the induction of CCL2 (*43*), which can initiate innate cell migration. To examine the induction of CCL2 and other key chemokines, we examined plasma concentrations of chemokines involved in monocyte (CCL2 and CXCL10), neutrophil (CXCL5 and CXCL9), lymphocyte (CCL5 and CXCL10), and eosinophil (CCL11) migration (Fig. 5A) after Ter119 administration. With the exception of CXCL5, Ter119 induced dose-dependent increases in these chemokines (Fig. 5A), suggesting an effect on cell migration. Because Ter119 increases platelet counts in murine ITP independent of the presence of B cells and T cells (*21*), we focused on migration of monocytes (Fig. 5B). Ter119 completely normalized the CAIA-induced decrease in monocytes in the blood (Fig. 5B) and increased monocytes in the liver as well (Fig. 5B).

Ter119 reduced or prevented the accumulation of inflammatory cells in the joint 24 hours after its injection (Fig. 5C). Examination of cytokines/chemokines from synovial fluid showed substantial dose-dependent decreases in the inflammatory cytokines/chemokines CCL2, TNF, CXCL5, and CCL5 demonstrating a significant decrease in chemokine/cytokine secretion in the joints ($P < 0.05$; Fig. 5D). CAIA results in increases in both C3 and C5a in the joints (*44*), and these complement components, as well as C1q, were all elevated in the joints of arthritic mice treated with the isotype control IgG (naïve versus control; fig. S2) but were all significantly and dose dependently decreased by Ter119 in the joints ($P < 0.01$; fig. S2).

Ter119 with impaired Fc domain function retains its ability to induce anemia but does not exhibit therapeutic activity in CAIA

To assess the anemia and verify whether deglycosylated Ter119 has therapeutic activity in CAIA, mice with established disease on day 5 were treated with either Ter119 or deglycosylated Ter119 (1.5 mg/kg) and assessed for subsequent clinical scores (Fig. 6, A and B). The deglycosylated Ter119 had no effect on the progression of arthritis (Fig. 6A); in comparison, wild-type Ter119 did statistically reduce arthritic clinical scores (Fig. 6B). To determine whether this difference was potentially due to a lack of in vivo binding to erythrocytes, mice on days 6 and 8 of the experiment were bled, and erythrocytes were assessed for ex vivo sensitization using anti-rat–phycoerythrin (PE) antibody. Deglycosylation of Ter119 does not affect RBC sensitization (*23*). Mice injected with deglycosylated Ter119 actually had higher erythrocyte sensitization as compared to mice treated with wild-type Ter119 on day 6 (Fig. 6C) and day 8 (Fig. 6D; days 1 and 3 after treatment, respectively) . To examine the effect on anemia, erythrocyte counts were assessed from days 6 to 12; both Ter119 and deglycosylated Ter119 induced anemia at all of the time points shown (Fig. 6E). The Ter119-induced anemia was marginally but significantly different from deglycosylated Ter119 on day 1 ($P < 0.05$) and day 3 ($P < 0.01$) after treatment only (fig. S3). In contrast to Ter119, deglycosylated Ter119 had no observable effect on the accumulation of inflammatory cells in the joint 24 hours after its injection (Fig. 6F) nor any effect on CCL2 concentration in the plasma (Fig. 6G) or synovial fluid (Fig. 6H). Thus, although deglycosylated Ter119 was able to bind to erythrocytes in vivo and induce anemia, it had no observable effect on arthritis.

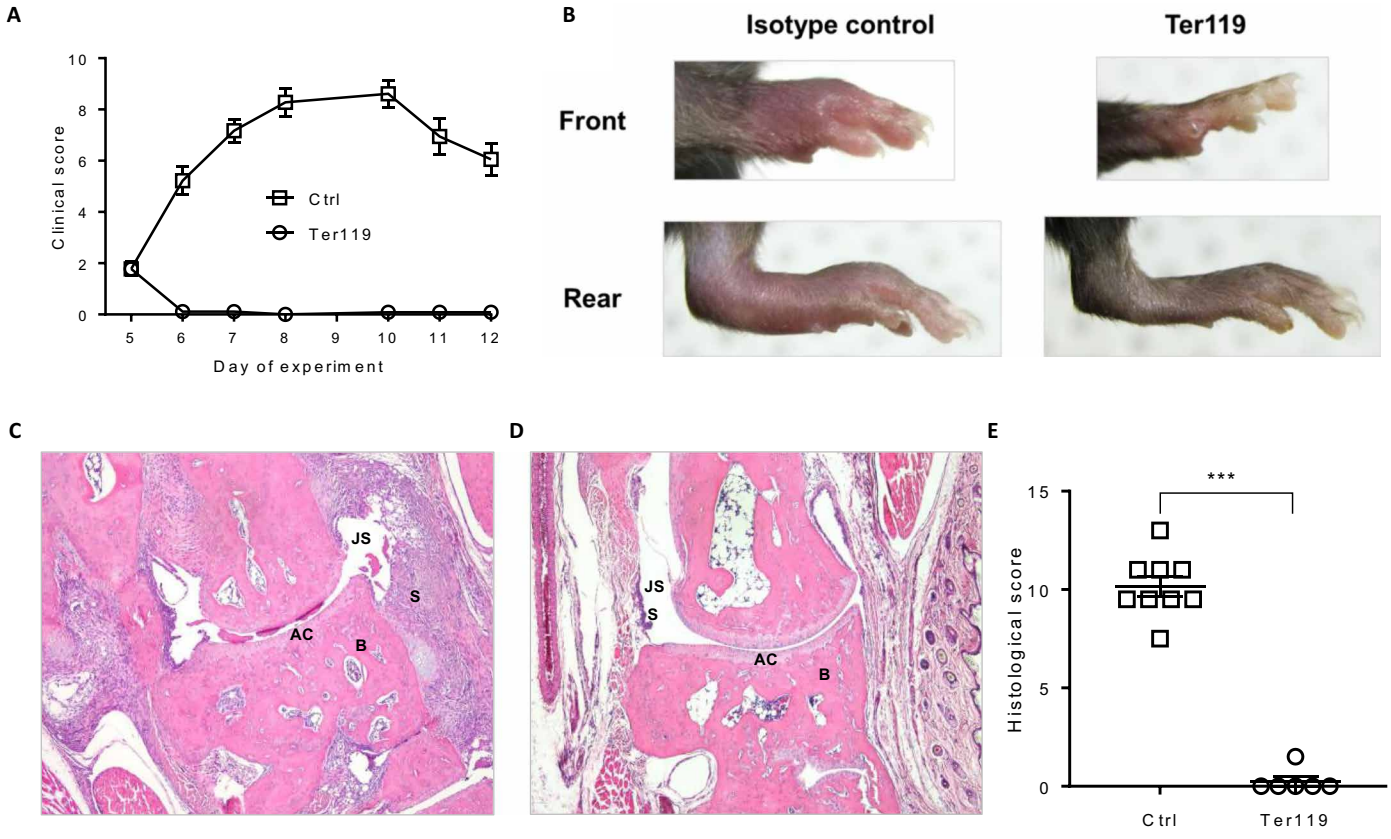

Fig. 3. Ter119 inhibits the progression of inflammatory arthritis in the CAIA model. (**A**) C57BL/6 mice injected with the collagen antibody cocktail (day 0) and LPS (day 3) were allowed to develop arthritis and then injected (day 5) with either Ter119 or an IgG2b isotype control antibody (2 mg/kg), and the clinical scores were evaluated until day 12. (**B**) Representative paws from mice on day 8 of the experiment are shown. The mice were assessed for histological score on day 12. Representative histology from the paws of mice therapeutically treated with the IgG2b isotype control antibody (**C**) or Ter119 (**D**) are shown. JS, joint space; AC, articular cartilage; S, synovium; B, bone. (**E**) The histological scores out of 15 for individual mice are shown with two sections taken per paw and one paw per mouse, always the rear right ankle cut at similar midsection depth. ***$P < 0.001$, Mann-Whitney two-tailed test.

The Ter119 antibody switched to murine IgG variants ameliorated collagen-induced arthritis

To verify disease-ameliorative activity in a B cell– and T cell–dependent chronic disease model independent of the infusion of disease inducing antibodies or sera, we assessed therapeutic responses in collagen-induced arthritis (CIA). In addition, to assess murine versions of Ter119, we replaced the rat IgG2b constant regions with murine IgG1 or IgG2a and injected each of these antibodies (2 mg/kg) into different groups of DBA/1 mice 7 days after the final immunization with type II collagen, as described in the Materials and Methods (*45*). Both murine IgG subtypes could decrease clinical scores in arthritic mice within 1 day of injection, and the clinical effects were significant for 3 days after therapy followed by an eventual return to full arthritis ($P < 0.05$; Fig. 7). Thus, disease amelioration extends beyond just antibody-induced arthritis models.

The Ter119 antibody prevents murine TRALI

TRALI is the leading cause of transfusion-related mortality and has no effective specific therapeutic interventions other than supportive therapy (*46*, *47*). Infusion of an anti–major histocompatibility complex class I antibody (34-1-2s) (*48*) into severe combined immunodeficient (SCID) mice induces symptoms approximating human TRALI (*49*), a highly inflammatory disease with a pathophysiology disparate from ITP and arthritis. We therefore explored the ability of Ter119 to inhibit the induction of murine TRALI. Mouse rectal temperatures were monitored to evaluate hypothermia, induced by 34-1-2s, indicative of systemic shock that occurs in mice undergoing TRALI (*49*). Mice receiving 34-1-2s displayed a decrease in rectal temperature at 30 min after injection (Fig. 8A), which decreased until 90 min, staying stable until the end point of 120 min. In contrast, mice receiving a pretreatment of Ter119 24 hours before 34-1-2s injection displayed a less pronounced drop in body temperature at 30 min, which became significant at 60 min ($P < 0.05$), 90 min ($P < 0.0001$), and 120 min ($P < 0.0001$) (versus 34-1-2s alone). These data demonstrate that Ter119 treatment can prevent 34-1-2s–induced hypothermia.

Postmortem determination of pulmonary edema, the hallmark of acute lung injury, was measured by lung wet/dry (W/D) weight ratios. Mice that received 34-1-2s after pretreatment with Ter119 displayed lung W/D ratios similar to those treated with Ter119 alone but significantly lower ($P < 0.05$) than mice injected with 34-1-2s only, which suffered from TRALI as shown by their increased lung W/D weight ratios and drop in body temperature (Fig. 8B). Ter119 did not have a significant effect ($P > 0.05$) in reducing pulmonary neutrophil infiltration (Fig. 8C) similar to IVIg (*50*).

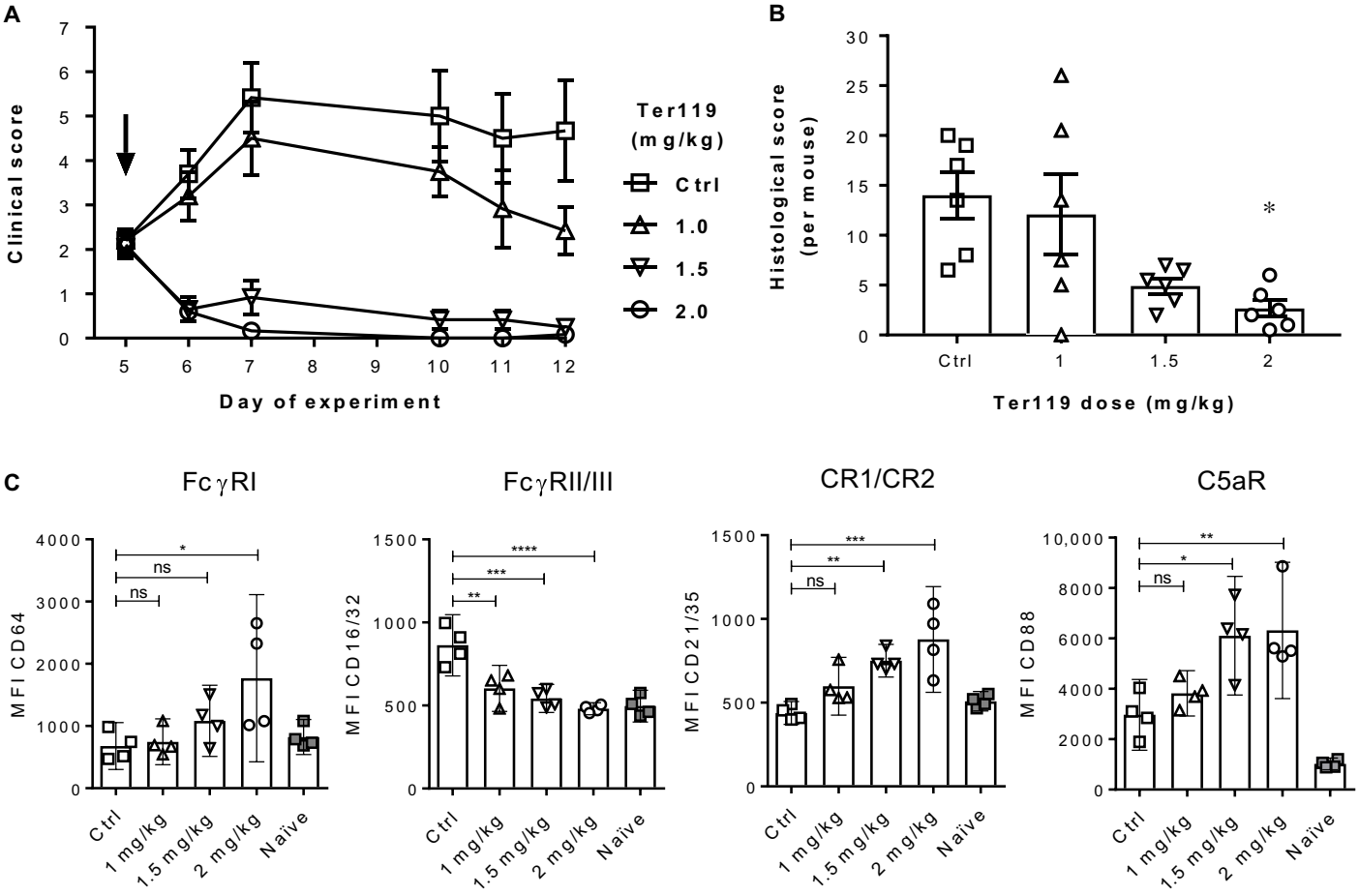

Fig. 4. Ter119 induces dose-dependent changes in CAIA and monocyte surface receptors. (**A**) C57BL/6 mice (10 per dosage group) were injected with the collagen antibody cocktail (day 0) and LPS (day 3), allowed to develop arthritis for 5 days, and injected (arrow) with the IgG2b isotype control antibody (2 mg/kg; Ctrl, square) or Ter119 (1, 1.5, or 2 mg/kg). Mean clinical scores for treatment period (days 6 to 12) (essentially, the area under the curve): 1.0 mg/kg (not significant), 1.5 mg/kg ($P < 0.05$), 2.0 mg/kg ($P < 0.001$), compared to the control; Kruskal-Wallis with Dunn's multiple comparisons test. (**B**) Mice were evaluated for histological score per mouse (maximum score, 36 per mouse) on day 12 for each dosage. One day after the isotype control or Ter119 injection (day 6), four mice from each group were used to assess the parameters displayed in (C), and the remaining mice allowed to develop arthritis for the 12 day duration of the experiment. A single-cell suspension for each mouse was made from the spleen, and monocyte FcγRI (CD64), FcγRIIB/IIIA (CD16/32), CR1/2 (CD21/35), and C5aR (CD88) mean fluorescence intensities (MFIs) were quantitated by flow cytometry (**C**). Naïve mice were not induced to develop arthritis. One-way ANOVA test with Dunn's multiple comparison as compared to the control arthritis group. Not significant (ns),*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

DISCUSSION

Autoimmune and inflammatory diseases together afflict a large cross section of the population. In the absence of a cure for many of these now chronic diseases, the major focus has been on therapy. In the case of autoimmune disease, therapies that have been applied to the management of autoimmunity focus on the induction of immunosuppression. Immunosuppressive therapy appears to be the cornerstone of treatment of autoimmune disease.

The first speculation that anti-D could be used in ITP was based on the postulation that anti-D–sensitized erythrocytes would competitively inhibit free FcγRs on macrophages in ITP-preventing opsonized platelet destruction (*13*), which was then convincingly demonstrated in patients with ITP (*14*). In murine models, the RBC-specific antibody Ter119 has also been used to treat passively induced murine ITP (*23*–*26*).

Anti-D is not known for inducing immunosuppression, which would make anti-D attractive as a treatment option for autoimmune or inflammatory diseases. The only autoimmune or inflammatory disease where anti-D is used, however, is ITP. In one study, anti-D was investigated in a patient with CIDP, but a response was not observed despite the ability of the patient to subsequently respond to IVIg (*19*). In this patient, the anti-D was given over 3 days rather than a single injection, as is common in ITP. Although it is difficult to know why the patient may not have responded to anti-D, it is possible that the initial injection of antibody prevented the ability of the subsequent injections to induce a response, analogous to the primary injection of Ter119 blocking the hypothermia mediated by a second injection of the same antibody a day later. It is also possible that the RhD antibody–sensitized erythrocytes require access to the tissue (for example, neural macrophages/glial cells), which may not occur across the blood-brain barrier in CIDP. Alternatively, there is evidence that IVIg and anti-D work by different mechanisms and that the patient was simply a nonresponder to anti-D.

Comparing IVIg to anti-D, a number of studies suggest that the mechanism of action may in fact be different. The spectrum of cytokines induced by IVIg versus anti-D have been shown to be different

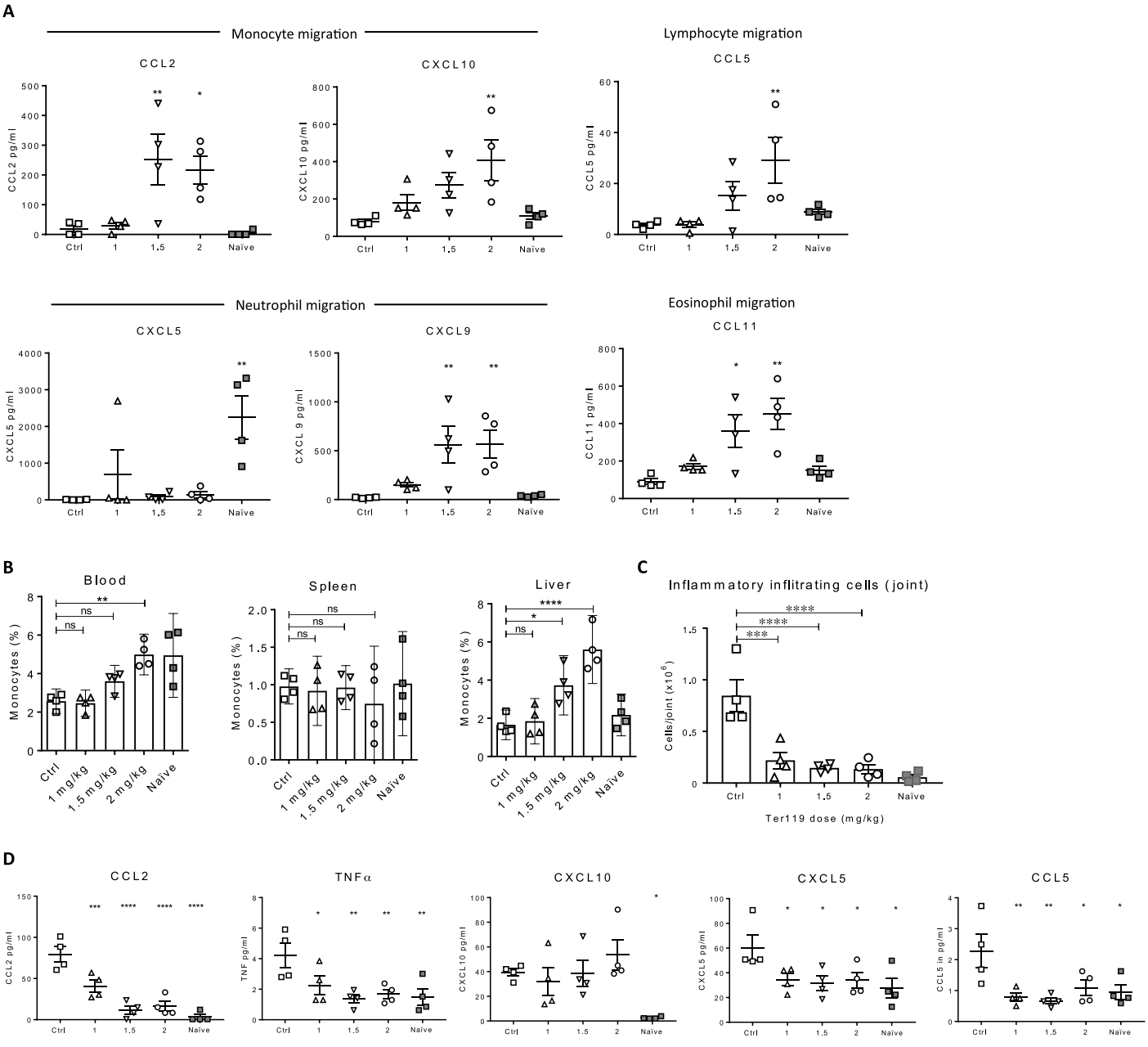

Fig. 5. Ter119 induces changes in chemokines and cellular migration in CAIA. (**A**) The same mice that were injected with the collagen antibody cocktail (day 0) and LPS (day 3) in Fig. 4 were used to assess the parameters in this figure 1 day after Ter119 or isotype control injection (day 6). Naïve mice were unmanipulated. To assess plasma chemokines CCL2, CXCL10, CXCL5, CXCL9, CCL5, and CCL11 (Eotaxin-1), plasma from each mouse was collected 1 day after isotype control or Ter119 injection (day 6 of the experiment) and chemokines were assessed by Luminex. (**B**) To evaluate changes in monocyte percentages in the blood, spleen, and liver, single-cell suspensions were made and monocytes were enumerated by flow cytometry. (**C**) To assess the number of infiltrating cells in the joints, the patellas from each mouse were collected and digested, and infiltrating leukocytes were enumerated by visual count. (**D**) To assess chemokines and cytokines from the joints, synovial fluid from each mouse was assessed by Luminex. One-way ANOVA test with Dunn's multiple comparison as compared to the control arthritis group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

(*51*). IVIg induced the production of IL-10, whereas anti-D also induced IL-10 but in addition stimulated the production of the inflammatory cytokines CCL2 (MCP-1), IL-6, and TNF-α, and the patients who displayed elevated concentrations of these inflammatory cytokines also displayed chills (*51*), similar to Ter119 here. Although both IVIg and anti-D induced a common increase in IL-10, we previously found that IVIg responses in murine ITP with mice genetically deficient in IL-10 responded normally to IVIg (*52*) decreasing our interest in IL-10 as a direct disease modifying cytokine in passive murine ITP. In the current study, we also looked in the plasma for IL-10 and found marginally elevated concentrations that did not reach significance at 24 hours after antibody administration. Whether IL-10 and the other cytokines/chemokines might have been stimulated at earlier time points was not evaluated in the current study, but changes in these cytokines/chemokines could have occurred earlier than what was seen in ITP (*51*).

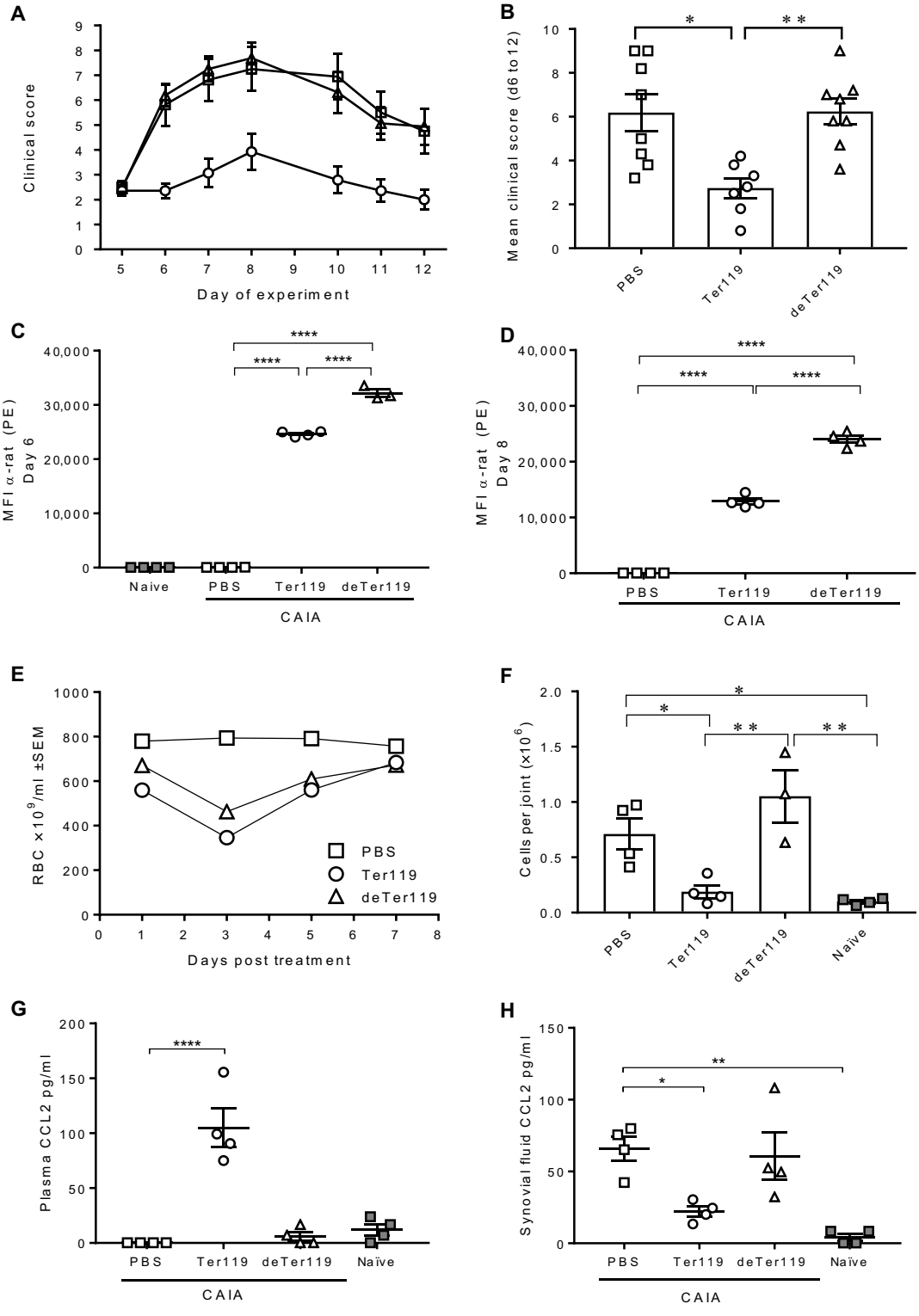

Fig. 6. Ter119 but not deglycosylated TER119 ameliorates CAIA. (**A** and **B**) Arthritis was induced in 12 mice per group on day 0 followed by LPS on day 3, and all 12 mice from each group were injected with either phosphate-buffered saline (PBS; squares), Ter119 (1.5 mg/kg; circles), or deglycosylated Ter119 (1.5 mg/kg; triangles) on day 5, and clinical scores were assessed. Erythrocyte sensitization was evaluated on day 6 (**C**) and day 8 (**D**) by staining the ex vivo erythrocytes with anti-rat IgG-PE, and MFIs of the erythrocytes were assessed. Erythrocytes were enumerated from the blood by a MACS Quant flow cytometer (**E**) with individual mice and statistics shown in fig. S3. Four mice from each group were sacrificed on day 6 to assess inflammatory cell infiltration from the patellas (**F**) and assess CCL2 concentrations from the blood (**G**) and from the synovial fluid (**H**). Statistics are as follows: (B) Kruskal-Wallis with Dunn's test; (C, D, and F to H) one-way ANOVA with Tukey's multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$.

The expectation in the ITP literature has classically been that IVIg and anti-D work by a similar mechanism; however, published observations report that there is no relationship between patients who responded to anti-D and IVIg (*15*), and in another study, five of six patients who did not respond to IVIg or anti-D given as single therapeutic agents responded when both were combined (*53*).

Although somewhat controversial, IVIg appears to have a requirement for the expression of the inhibitory Fc receptor (FcγRIIb) for a clinical effect in some strains of mice (*54*). In contrast, anti-RBC antibodies in general (*55*) and Ter119 in particular (*22*) had absolutely no requirement for this receptor in ITP on susceptible mouse backgrounds, again suggesting a different mechanism than IVIg (*55*).

Ter119 has broad therapeutic activity in three models of inflammatory arthritis and in an immune inflammatory model of acute lung injury. Ter119 could not only attenuate disease induction but also effectively treat inflammatory arthritis in all three models studied. The therapeutic activity required the presence of the Fc glycans in

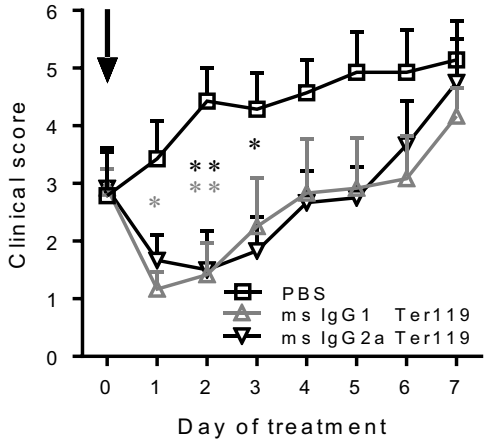

Fig. 7. Ter119 expressed as murine IgG switch variants can treat a chronic model of CIA independent of passive-antibody transfer. DBA/1 mice immunized against chick type II collagen were allowed to develop arthritis and then treated (timing as denoted by the arrow) with PBS ($n = 7$ mice; square) and Ter119 (2 mg/kg) expressed as a murine IgG1subtype ($n = 6$ mice; gray triangles) or expressed as a murine IgG2a subtype ($n = 6$ mice; inverted triangles), and arthritis clinical score was evaluated over the course of the experiment. Statistical comparison was performed using two-way ANOVA for repeated measures (assuming normality) with Tukey's multiple comparisons test. *$P < 0.05$ and **$P < 0.01$ (gray, PBS versus IgG1; black, PBS versus IgG2a).

both the K/BxN and CAIA models, and the presence of the glycans was also critical for stimulating increases in plasma CCL2. The ability of the IgG Fc region to function via interactions with Fc receptors or complement is greatly dependent on the glycan profile of the IgG Fc region. A complete lack of Fc glycosylation (as performed here) substantially reduces interactions with Fc receptors and complement (*39*, *40*). The complete loss of therapeutic activity due to deglycosylation could therefore indicate that one of these interactions is likely necessary for disease-modifying activity. Although not explored in this study, modifications to the Fc glycan including removal of the core fucose of the glycan can increase interactions with human FcγRIII or murine FcγRIV, whereas the addition of a terminal sialic acid residue has been suggested to increase anti-inflammatory activity with IVIg (*3*). Ter119 Fc glycan structures previously contained both fucosylated and nonfucosylated variants, as well as a small percentage of molecules containing terminal sialic acid residues (*23*), and thus, these potential interactions could be of interest to investigate.

In patients with rheumatoid arthritis, monocytes express increased FcγRIII, and immune complex engagement of FcγRIII$^+$ monocytes led to TNF production (*56*). Upon FcγRIII engagement, this receptor can be down-modulated or can also be shed from the cell surface (*57*). Analyzing the effect of Ter119 on splenic monocyte FcγRI, FcγRII/III, CR1/2, and C5aR demonstrated that FcγRII/III increased in CAIA arthritis (similar to patients with rheumatoid arthritis) (*56*), and its expression was selectively and fully decreased by Ter119. Because we have previously determined that Ter119 can ameliorate murine ITP independent of the inhibitory FcγRIIB and could down-regulate the expression of activating FcγRIIIA in splenic macrophages (*22*), our data here in arthritis match murine ITP, hinting at a similar therapeutic mechanism in both models. Thus, rather than Ter119 having different mechanisms of therapeutic activity in different models, we provide evidence for similar or overlapping mechanisms.

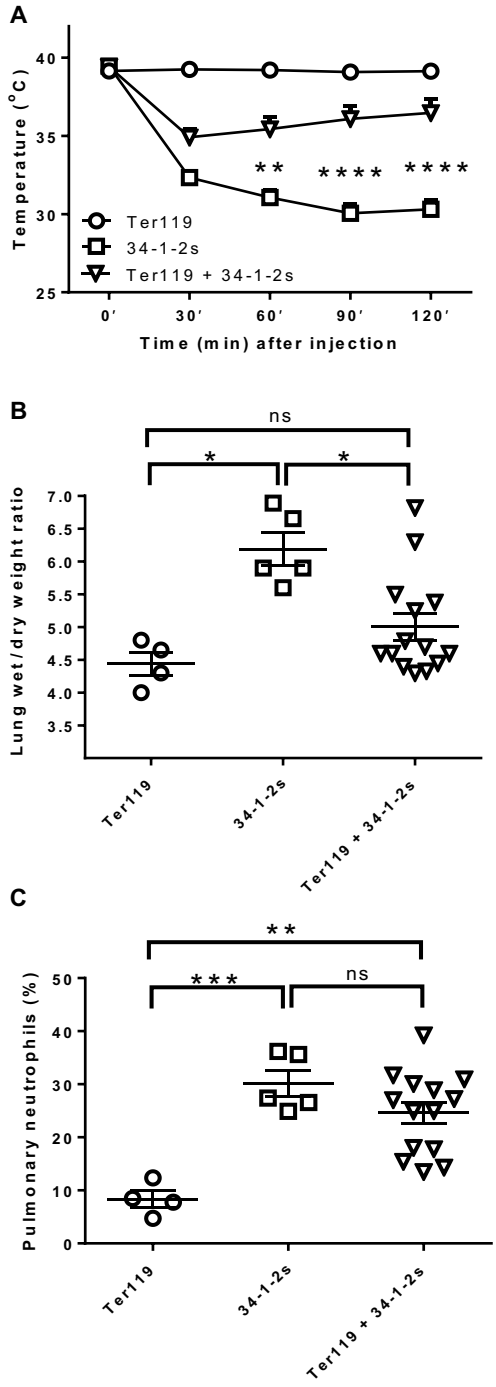

Fig. 8. Ter119 inhibits TRALI. (**A**) SCID mice were injected with 40 μg of Ter119 (circles and triangles) or left untreated (squares) for 24 hours. Mice were then injected with 50 μg of 34-1-2s (triangles and squares) or nothing (circles). Rectal temperatures were measured every 30 min for 2 hours after the second injection (A), and mice were subsequently sacrificed at 2 hours to assess pulmonary edema (**B**) and pulmonary neutrophil accumulation (**C**). Data are expressed as means ± SEM from four separate experiments. $n = 4$ (Ter119); $n = 5$ (34-1-2s); $n = 14$ (Ter119 + 34-1-2s). Statistical comparisons were made after testing for normality. A two-way ANOVA with Tukey's multiple comparison testing was performed in (A), a Kruskal-Wallis test with Dunn's multiple comparison testing was performed for (B), and a one-way ANOVA with Tukey's multiple comparison testing was performed for (C). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and **** $P < 0.0001$.

One of the effects of engagement of FcγRIIIA on human peripheral blood mononuclear cells is the induced secretion of the CCL2 chemokine (*43*), and we observed substantially elevated CCL2 in the plasma of CAIA mice treated with Ter119, and CCL2 only increased at doses inducing a clinical response. In addition, deglycosylated Ter119, which would not meaningfully react with FcγRIIIA, did not stimulate CCL2. CCL2 and several other plasma chemokines were increased including those that are known to drive migration of monocytes, T cells, and eosinophils but not CXCL5, a major driver of neutrophil activation and migration to sites of inflammation (*58*). Consistent with this, there were no meaningful differences in neutrophil percentages in the blood, spleen, and liver of Ter119-treated mice in the CAIA model. In contrast, there were differences in monocytes in the blood and liver and a reduction in inflammatory cell infiltration of the synovial fluid in CAIA mice treated with Ter119.

Reduced synovial fluid inflammatory cytokines/chemokines and complement components C1q, C3, and C5a likely contribute to the lack of cellular infiltration of the synovium. These decreases in joint cytokines/chemokines, with concomitant increases in the plasma, suggest an inflammatory distraction of the immune response away from sites of disease-specific inflammation and focused on the antibody-sensitized erythrocytes. Thus, an inflammatory response directed toward the erythrocytes appears to occur at the expense of continued inflammation directed to the disease of interest.

To examine a chronic disease model using both humoral and cellular immune mechanisms without passively transferring a disease-inducing antibody, we observed that Ter119 expressed as mouse IgG1 and mouse IgG2a subtypes could also ameliorate disease activity in CIA within 1 day of Ter119 injection. The IgG1 and IgG2a class-switched versions of Ter119 displayed equivalent therapeutic activity in the model. This finding does not support a role for interactions of Ter119 with complement C1q as the driver of therapeutic activity because murine IgG1 is not known to bind C1q (*59*). This is in contrast to murine IgG2a antibodies that bind well to C1q, which could trigger erythrocyte hemolysis (*59*). If a complement C1q interaction were involved, then the expectation would be that the murine IgG2a antibody would have superior therapeutic activity.

An FcγR blockade mechanism could potentially explain Ter119 activity across different diseases. All of the disease models studied do have some requirement for the major signaling component downstream of activating FcγRs, the FcRγ chain (*48*, *60*–*62*). Unfortunately, the activity of the FcRγ chain extends beyond interactions with only activating FcγR, and the requirements of selected FcγRs for disease physiology in the disease models have yet to be fully established.

The most insightful information with regard to FcγRs comes from the CIA model where, although activating FcγRIII is essential for the development of arthritis (*63*), destructive arthritis can nevertheless occur in the absence of both FcγRI and FcγRIII (*64*). Whereas the IgG2a switch variant of Ter119 would be theoretically able to interact and therefore block all activating Fc receptors (FcγRI, FcγRIII, and FcγRIV), the IgG1 switch variant would only be capable of theoretically blocking FcγRIII allowing disease pathology to potentially continue via FcγRIV (*64*). Because it has been suggested that murine IgG2a contributes substantially more to disease pathology in CIA as compared to IgG1 (*65*) and IgG2a antibodies can interact with any activating FcγR but have a higher affinity for FcγRIV (and FcγRI) as compared to FcγRIII (*66*), the equal therapeutic activity of the IgG1 and IgG2a variants of Ter119 seems at odds with simple FcγR blockade.

Whether an interaction with a single FcγR class is sufficient to drive therapeutic activity or even whether activating FcγR is centrally involved in the therapeutic activity of Ter119 remains to be established. There are also a number of interactions possible between IgG antibodies and other cellular receptors aside from classical FcγRs and complement (*67*, *68*), which could potentially mediate therapeutic activity alone or in concert with classical FcγRs or with other receptors.

To validate that a therapeutic response can extend beyond ITP and models of arthritis, prophylactic Ter119 treatment was administered before TRALI. We observed a rescue of TRALI-induced hypothermia and pulmonary edema—a hallmark of TRALI reactions. There are no therapeutic interventions in the clinic for TRALI other than supportive therapy such as oxygen supplementation, ventilatory assistance, and conservative fluid practices. The inability of Ter119 to effectively modulate CXCL5 and neutrophil numbers in the blood, spleen, and liver is matched in the TRALI model where neutrophil infiltration in the lung was not attenuated.

Important limitations of this study include that all of the work has been performed in murine models of disease with an antibody that does not react with human erythrocytes. Thus, a human erythrocyte–reactive antibody capable of triggering therapeutic effects would need to be developed. The diseases studied here are also all relatively acute models of disease activity, whereas most patients seeking experimental treatments for autoimmunity generally have chronic disease and are also most typically refractory to a number of previously attempted therapeutic interventions. In addition, whereas this work profiles a highly inflammatory antibody injected as a single dose without premedication, the goal going forward will be to actually treat disease without triggering, or at least only minimally triggering, adverse events.

IVIg, which itself has a number of adverse events in up to 81% of patients, can be successfully mitigated by slowing the infusion rate, and this is common practice with IVIg (*69*); this could also be used with an anti-RBC antibody. Additional successful measures with IVIg to minimize adverse events have included premedicating the patient with analgesics, nonsteroidal anti-inflammatory drugs, antihistamines, or intravenous glucocorticoids (*69*).

An interesting aspect of the present work, however, is that anti-D itself remains a first-line treatment option for nonsplenectomized children and adults with ITP (*70*), and a recent systematic review and meta-analysis in children with ITP concluded that there were slightly less frequent general symptoms after anti-D administration as compared to IVIg treatment, not including hemolysis, which was mild in most patients and not an expected effect of anti-D (*71*). In summary, we conclude that Ter119 and its murine variants have substantial therapeutic activity and suggest that RBC-specific antibodies such as anti-D may have ameliorative potential in autoimmune and inflammatory disorders.

MATERIALS AND METHODS

Study design

The overall objective for this study was to understand whether the disease-ameliorative activity of an erythrocyte-specific antibody goes beyond only the treatment of a passive murine model of ITP. Because the ITP model involves platelet clearance in the spleen, we

originally sought a model with very different disease pathophysiology, K/BxN, and we investigated prevention, as well as reversal of symptoms. Follow-up collaborative work was done in the CAIA and CIA mouse models, as well as mechanistic investigations involving FcγR, complement receptors, chemokines, monocytes, and anemia. To assess the contribution of the IgG Fc region in ameliorative effects, the Ter119 antibody was deglycosylated and tested in the K/BxN and CAIA models. In addition, Ter119 was also expressed as murine IgG1 and IgG2a subtypes and examined in the CIA model. Early observations of disease-ameliorative activity in the K/BxN model of arthritis also prompted us to evaluate a completely different disease with a pathophysiology different from both ITP and arthritis, TRALI. This inflammatory model was also performed in SCID mice to determine contributions of B cells and T cells to the ameliorative activity of Ter119. All animal work performed in Toronto was approved by the local animal care committee of St. Michael's Hospital, whereas all animal work performed in Melbourne was approved by the local animal care committee at CSL Ltd. For the CAIA and CIA studies, all clinical scoring of mouse paws and histological scoring of joint sections were performed by an investigator who was blinded to the experimental groups. Primary data are reported in data file S1.

Mice and antibodies

CD-1, C57BL/6, and SCID mice used in Toronto were from Charles River Laboratories. Monoclonal anti-platelet antibody (MWReg30, rat IgG1) was purchased from BD Biosciences. The 30-F1 (rat IgG2c) antibody recognizing mouse RBC was from BioLegend. Antibodies 34-1-2s (rat IgG2a) and Ter119 (rat IgG2b) were from Bio X Cell. Mouse monoclonal anti–type II collagen 5 clone antibody mixture was purchased from Chondrex. The control rat IgG used in Fig. 1 (ChromPure) was from Jackson ImmunoResearch. The Ter119, deglycosylated Ter119, mouse IgG1 Ter119, and mouse IgG2a Ter119 used in Figs. 1A, 2 (E and F), and 3 to 8 and figs. S1 to S3 were all still available at the time of writing the manuscript and were tested together in a single batch for endotoxin levels using the Lonza "Kinetic-QCL" Kinetic Chromogenic LAL Assay (catalog no. 50-650 U). These antibodies either had an endotoxin level below the limit of detection of the assay or were present at a level below the United States Pharmacopeia (USP)–recommended endotoxin limit of 5 EU/kg body weight for parenteral drugs, as well as below the same recommended limit for preclinical research (*72*, *73*). The Ter119 used in Figs. 1A and 7 (the temperature experiments) had an endotoxin level below the limit of detection providing a maximal theoretical in vivo dose of <0.76 EU/kg body weight.

Anemia/ITP/body temperature

The effect of 40 μg of rat IgG or Ter119 on body temperature was assessed in CD-1 mice. Mice were removed from their normal group housing and assessed for body temperature using a rectal temperature probe followed by placing the mice in a cage without bedding that had been prewarmed using a heating pad (low setting) placed under half the cage for 5 min, and the mice were allowed to freely roam the cage. This was necessary to allow visualization and access of the tail vein for injection. Mice were then injected by the tail vein with 40 μg of control rat IgG or 200 μl of Ter119, and rectal temperature was taken 10, 20, 30, 60, and 120 min after injection. Anemia and ITP were induced in CD-1 mice by the injection of 40 μg of Ter119 (anemia) versus a control rat IgG followed by the injection of 3 μg of antiplatelet antibody (ITP) 60 min before a terminal bleed for platelet and RBC enumeration as described (*33*,*74*).

Arthritis models

K/BxN arthritis was induced and scored as described (*75*). Mice were pretreated with nothing or 45 μg of Ter119 before injection of K/BxN serum. Mice were monitored daily for arthritis progression. In separate experiments, mice were rendered arthritic and treated on day 5 with 45 μg of Ter119 or a non-inflammatory anti-RBC antibody (30-F1). For the CAIA model, C57BL/6 mice in Melbourne were injected with mouse anti–type II collagen (2 mg, intraperitoneally; Arthrogen-CIA 5-Clone Cocktail Kit, Chondrex) on day 0 followed by 50 μg of LPS on day 3 to initiate arthritis. Mice were treated with Ter119 or an IgG2b isotype control on day 5 after induction. Clinical scores were evaluated up until day 12. The CIA model was performed as described (*45*). Briefly, DBA/1 mice were injected twice with 100 μg of chick type II collagen (Sigma) in complete Freund's adjuvant (containing 250 μg of heat-killed *Mycobacterium tuberculosis*) 21 days apart and treated at disease onset (7 to 12 days later) with isotype-switched (2 mg/kg; mouse IgG1 and mouse IgG2a) Ter119 by the intravenous route, and clinical scores were assessed. Clinical scores for all arthritis models were assigned 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. Mice were euthanized at the time points indicated in the CAIA model, the paws were fixed in formalin, decalcified, and embedded in paraffin and scored as assessed in (*76*).

Cytokines, complement, and flow cytometry

Joint fluid and sera were evaluated for cytokine concentrations by a Luminex-Multiplex assay (Millipore) according to the manufacturer's directions. Flow cytometric analysis of blood and joint digests was performed as described (*44*). Briefly, single-cell suspensions were stained with the following: Ly6C (HK1.4, eBioscience), Ly6G (1A8, BioLegend), CD11b (M1/70, eBioscience), CD16/32 (93, eBioscience), CD64 (X54-5/7.1.1; BD Pharmingen), and CD45 (30-F11, BD Pharmingen). The cells were fixed and acquired on a BD LSRFortessa and analyzed with FlowJo software. Joint washes and sera were evaluated for C1q, C5a (R&D Systems), and C3 (GENWAY) enzyme-linked immunosorbent assays. The ability of Ter119 and deglycosylated Ter119 to passively sensitize erythrocytes in vivo on days 1 and 3 after antibody injection was assessed ex vivo by flow cytometry using an anti–rat-PE–conjugated antibody.

Transfusion-related acute lung injury

TRALI was induced as described (*77*). Briefly, SCID mice were injected with 40 of μg Ter119 24 hours before injection of 50 μg of the TRALI-inducing antibody 34-1-2s. Rectal temperatures were recorded every 30 min for 2 hours, and mice were then euthanized to determine lung W/D weight ratios (*77*). Pulmonary neutrophils were enumerated as previously described (*77*).

Data analysis

Data are represented as means ± SEM. Point-to-point connecting lines indicate mice that have been sequentially sampled for the test displayed with the exception of Fig. 1 (B and C). Repetitive bleeds can decrease erythrocyte counts and platelet counts by depletion of these populations from the blood due to each sampling. In addition, because the primary function of a platelet is the repair of an injured

blood vessel, repetitive bleeds could lead to decreases in platelet count due to their hemostatic function in binding to the injured blood vessel from a previous bleed. Thus, to avoid these effects and limit the assessment of anti-inflammatory measurement to the point shown on the graph (Fig. 1B) or 1 hour before the point on the graph (Fig. 1C), each data point is an independent group of mice sampled at time displayed on the *x* axis.

Statistical analysis

Statistical analyses, as indicated in each figure legend, were performed using GraphPad Prism 6.02 or a later version. To compare the experimental groups, if only two groups were compared, then the multiple *t* test or Mann-Whitney two-tailed test were used. Data of multiple comparisons used analysis of variance (ANOVA) with the multiple comparison test as indicated in each figure legend. Significance was set at less than 0.05.

SUPPLEMENTARY MATERIALS

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Fig. S1. Priming with Ter119 can protect mice from Ter119-induced hypothermia.

Fig. S2. Ter119 substantially reduces the detection of complement components in the joint of arthritic mice.

Fig. S3. Ter119 and deglycosylated Ter119 induce anemia over time in the CAIA model.

Fig. S4. Ter119 class-switched to murine IgG1 and murine IgG2a sequences.

Data file S1. Primary data.

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Treating murine inflammatory diseases with an anti-erythrocyte antibody

Andrew R. Crow, Rick Kapur, Sandra Koernig, Ian K. Campbell, Chao-Ching Jen, Patrick J. Mott, Danielle Marjoram, Ramsha Khan, Michael Kim, Jennifer Brasseit, Yoelys Cruz-Leal, Alaa Amash, Simrat Kahlon, Issaka Yougbare, Heyu Ni, Adrian W. Zuercher, Fabian Käsermann, John W. Semple and Alan H. Lazarus

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Fighting inflammation with inflammation

Immune thrombocytopenia (ITP) can be treated with IVIg or even anti-D, which is immunoglobulin directed against red blood cells (RBCs). To investigate whether an anti-RBC antibody could confer benefits in other inflammatory models, Crow *et al.* turned to the mouse anti-RBC antibody Ter119, known to be not only inflammatory but also therapeutic in ITP models. They now show that the beneficial effects in ITP are not due to induction of anemia. Ter119 also successfully treated multiple mouse models of arthritis and prevented symptoms in an acute lung injury model. These effects were dependent on a functional Fc portion and involved modulation of monocytes and chemokines. Their results suggest that anti-RBC antibodies might be able to replace IVIg for many inflammatory indications.