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Intravenous immunoglobulin ameliorates ITP via activating Fcγ receptors on dendritic cells

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Despite a more than 20-year experience of therapeutic benefit, the relevant molecular and cellular targets of intravenous immunoglobulin (IVIg) in autoimmune disease remain unclear. Contrary to the prevailing theories of IVIg action in autoimmunity, we show that IVIg drives signaling through activating Fcγ receptors (FcγR) in the amelioration of mouse immune thrombocytopenic purpura (ITP). The actual administration of IVIg was unnecessary because as few as 10^5 IVIg-treated cells could, upon adoptive transfer, ameliorate ITP. IVIg did not interact with the inhibitory FcγRIIB on the initiator cell, although FcγRIIB does have a role in the late phase of IVIg action. Notably, only IVIg-treated $CD11c^+$ dendritic cells could mediate these effects. We hypothesize that IVIg forms soluble immune complexes *in vivo* that prime dendritic-cell regulatory activity. In conclusion, the clinical effects of IVIg in ameliorating ITP seem to involve the acute interaction of IVIg with activating FcγR on dendritic cells.

Administration of high-dose IVIg rapidly increases platelet numbers in individuals with the platelet autoimmune disease ITP. Although also of benefit in other autoimmune disorders, including Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy and myasthenia gravis[1,2], IVIg has, in contrast, been generally ineffective in individuals with systemic lupus erythematosus (SLE) and rheumatoid arthritis[1,2]. Why individuals with some autoimmune states benefit from IVIg and others do not remains unclear.

ITP is an autoimmune disease characterized by platelet clearance mediated by pathogenic platelet-specific antibodies[3–5]. The clearance is mediated by Fcγ receptor (FcγR)-bearing macrophages. Although several mechanisms have been proposed[6–10], the actual mechanism(s) of action of IVIg in autoimmunity remains unclear. In mouse models it has been shown that IVIg ameliorates ITP by a mechanism dependent upon the expression of inhibitory FcγR (FcγRIIB)[11,12], inducing increased surface expression of FcγRIIB on macrophages, which counterbalances the phagocytic activity, reducing platelet clearance.

We recently showed that small soluble immune complexes (sICs) could recapitulate the therapeutic effects of IVIg in mouse ITP. We showed that, like IVIg, sICs ameliorated ITP in a manner dependent upon expression of inhibitory FcγRIIB and independent of complement[13]. Using the K/BxN serum-induced arthritis model[14,15], we further showed that these sICs could mimic the ameliorative effects of IVIg in inflammatory arthritis[13].

We have now investigated whether IVIg and IVIg-mimetic activity (mediated by sICs) ameliorate autoimmunity by interacting directly with either activating or inhibitory FcγRs. We developed a model of IVIg treatment based upon adoptive transfer of IVIg-primed cells to examine the involvement of FcγRs in the mechanism of action of IVIg. We report that the effects of IVIg and sICs in ITP involve the acute interaction of IVIg with activating FcγRs on dendritic cells.

To determine whether the therapeutic effects of IVIg could be recapitulated by the passive transfer of IVIg-primed cells, we treated leukocytes from normal mice and mice with severe combined immune deficiency (SCID) with IVIg. We passively transferred the cells (washed free of extraneous IVIg) into normal mice, which we then injected with platelet-specific antibody to induce ITP. Both normal and SCID leukocytes treated with IVIg protected the mice from ITP. As only cells of the innate immune system are present in SCID mice, this indicates that IVIg-primed innate cells can mediate the *in vivo* clinical effects of IVIg (**Fig. 1**). Purified B and T cells individually subjected to IVIg did not ameliorate ITP, confirming that cells of the adaptive immune system do not mediate this effect (**Supplementary Fig. 1** online).

IVIg requires the expression of the inhibitory FcγR FcγRIIB (encoded by *Fcgr2b*) in the amelioration of both ITP and inflammatory arthritis[11,12,16]. To determine whether expression of FcγRIIB is required on the IVIg 'initiator' cell, we treated $Fcgr2b^{+/+}$ versus $Fcgr2b^{-/-}$ splenic leukocytes with IVIg *in vitro* and injected them into recipient $Fcgr2b^{+/+}$ and $Fcgr2b^{-/-}$ ITP mice (**Fig. 2**). Expression of FcγRIIB on donor leukocytes was not required for the amelioration of ITP. Conversely, IVIg-primed leukocytes from either $Fcgr2b^{+/+}$ or $Fcgr2b^{-/-}$ mice only ameliorated ITP when the recipient mouse expressed the inhibitory FcγRIIB. This establishes that IVIg does not require any direct interaction with FcγRIIB on the 'initiator' cell.

To determine whether the effects of IVIg are dependent upon activating FcγR signals, we treated leukocytes from mice lacking

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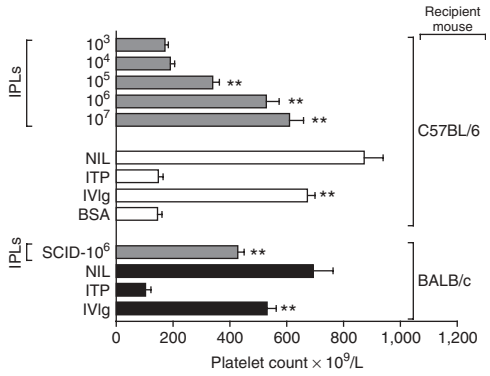

Figure 1 IVIg-primed leukocytes inhibit mouse ITP. C57BL/6 (right y-axis label): splenic leukocytes from C57BL/6 mice were treated with IVIg for 30 min, washed and the indicated number of IVIg-primed leukocytes (IPLs, gray bars, 10^3–10^7) injected intravenously into recipient C57BL/6 mice, followed 24 h later by injection of 2 μg platelet-specific antibody. Platelet counts were performed after a further 24 h. NIL (white bar), unmanipulated C57BL/6 mice; ITP (white bar), C57BL/6 mice injected with platelet-specific antibody only; IVIg, BSA, (white bars) C57BL/6 mice that received standard therapy with IVIg (50 mg/mouse, intraperitoneally) or BSA (as an IVIg treatment control) followed 24 h later by platelet-specific antibody. BALB/c (right *y*-axis label): Experiments performed in BALB/c mice. SCID-10^6: IPLs from donor CB17 severe combined immune deficient (SCID) mice (gray bar) treated with IVIg as described above and injected (10^6/mouse) intravenously into recipient BALB/c mice followed 24 h later by injection of platelet-specific antibody. NIL, ITP, IVIg (black bars) were as above except using BALB/c mice. The *y*-axis denotes the treatments given to mice; the *x*-axis denotes platelet counts taken 24 h after injection with platelet-specific antibody. $n = 6$ mice for each group from three independent experiments. $^{**}P < 0.001$ versus ITP mice. Data are mean ± s.e.m.

expression of the FcR γ chain with IVIg and passively transferred these cells into normal mice, after which we injected them with a platelet-specific antibody (**Fig. 3a**). IVIg-primed leukocytes from FcR γ chain–deficient mice did not inhibit ITP. This suggests that signals from activating FcγRs may be primarily involved in IVIg action. To substantiate this, we attempted to completely bypass the need for IVIg itself by directly inducing FcγR-dependent signaling using an FcγRIIIA-specific antibody (2.4G2) plus a crosslinking antibody that induces FcγR-dependent signaling events[17–19]. When leukocytes treated with 2.4G2 and the crosslinking antibody were injected into mice followed by platelet-specific antibody, the protective effect of IVIg was completely recapitulated (**Fig. 3b**). Hence, it is not only IVIg that can induce an IVIg-like primed leukocyte effect. Because 2.4G2 binds FcγRIIIA as well as FcγRIIB, we evaluated 2.4G2 passive-transfer effects using cells from *Fcgr2b*$^{-/-}$ mice; these also ameliorated ITP (**Fig. 3c**), indicating that the crosslinking or co-crosslinking of FcγRIIB on the initiator cells is not required.

To verify that FcR γ chain–dependent signaling has a central role in mediating the effects of IVIg, we induced γ chain–dependent signaling independently of the presence of the various FcγRs on leukocytes. The paired immunoglobulin-like receptor A (PIR-A) is expressed on innate immune system cells, inducing FcR γ chain–dependent cellular activation. PIR-A and its inhibitory counterpart, PIR-B, act as physiologic receptors for major histocompatibility complex (MHC) class I molecules[20]. Although not an immunoglobulin receptor, PIR-A associates with the FcR γ chain and induces cellular activation. Passively transferred leukocytes pretreated with antibody 6C1 together with a crosslinking antibody ameliorated mouse ITP (**Fig. 3b**). Thus, bypassing FcγRs and stimulating FcR γ chain–dependent signaling in leukocytes was sufficient to ameliorate ITP. Although the above regimen would also engage PIR-B, which drives negative signaling, leukocytes from FcR γ chain–deficient mice treated with antibody 6C1 plus a crosslinking antibody did not ameliorate ITP (**Fig. 3d**). Furthermore, PIR-B preferentially associates with SHP-1 in mouse macrophages[20], and we have previously reported that IVIg-mediated amelioration of mouse ITP occurs in a SHP-1–independent manner[12].

We recently reported that IVIg can be replaced with either polyclonal or monoclonal antibodies that form sICs, and antibodies forming these sICs ameliorate both ITP and inflammatory arthritis[13]. These antibodies, like IVIg, do not require complement to mediate their therapeutic effects[11,13] and function at a 3-log lower dose than IVIg[13]. We found that sICs also stimulated passively transferred amelioration of ITP (**Supplementary Fig. 2** online), again indicating that IVIg is not the only entity that can induce an IVIg-like primed leukocyte effect.

To determine which innate cells mediate these effects of IVIg, we subjected splenic leukocytes to selection using a CD11c-specific antibody. IVIg-treated CD11c$^+$ dendritic cells (DCs), but not CD11c$^-$ cells, ameliorated ITP (**Fig. 4a**). In contrast to DCs, CD11b$^+$ myeloid cells did not mediate the effects of IVIg (**Fig. 4b**). Furthermore, treatment of thioglycollate-elicited peritoneal macrophages or bone marrow–derived cultured macrophage lineage cells with IVIg did not ameliorate ITP (**Fig. 4c**). Conversely, bone marrow–derived GM-CSF–cultured DCs, when treated with IVIg, could ameliorate ITP (**Fig. 4c**).

Hence, the beneficial effect of IVIg in mouse ITP can be explained by the interaction of IVIg with activating FcγRs on DCs. When adoptively transferred into mice, IVIg-primed DCs inhibited immune platelet destruction. We show that the primary functional target of IVIg in this effect is activating FcγRs. This is consistent with a recent study[21] reporting that human immature monocyte-derived DCs treated with IVIg *in vitro* undergo a selective downregulation of surface expression of the activating FcγRIIA but not FcγRIIB[21]. We

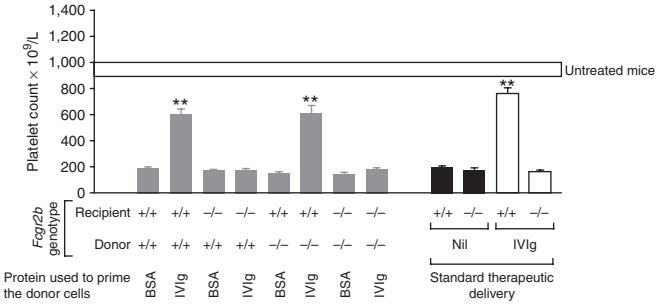

Figure 2 IVIg does not interact with the inhibitory FcγRIIB in the amelioration of mouse ITP. Splenic leukocytes from donor *Fcgr2b*$^{+/+}$ or *Fcgr2b*$^{-/-}$ mice were treated with IVIg or BSA *in vitro* for 30 min, washed and injected (10^6/mouse) intravenously into recipient *Fcgr2b*$^{+/+}$ mice or *Fcgr2b*$^{-/-}$ mice (gray bars), followed by platelet-specific antibody as in **Figure 1**. The *Fcgr2b*$^{+/+}$ and *Fcgr2b*$^{-/-}$ genotypes are represented as +/+ and –/–, respectively. The horizontal bar represents the mean platelet count ± 1 s.e.m. of *Fcgr2b*$^{-/-}$ mice that were left untreated. Mice receiving platelet-specific antibody only (black bars) or standard IVIg therapy plus platelet-specific antibody (white bars) are also shown. The graph shows platelet counts taken 24 h after injection with platelet-specific antibody. $n = 6$ mice for each group from three independent experiments. $^{**}P < 0.001$ versus ITP mice. Data are mean ± s.e.m. The platelet counts of untreated *Fcgr2b*$^{+/+}$ and *Fcgr2b*$^{-/-}$ mice were not significantly different.

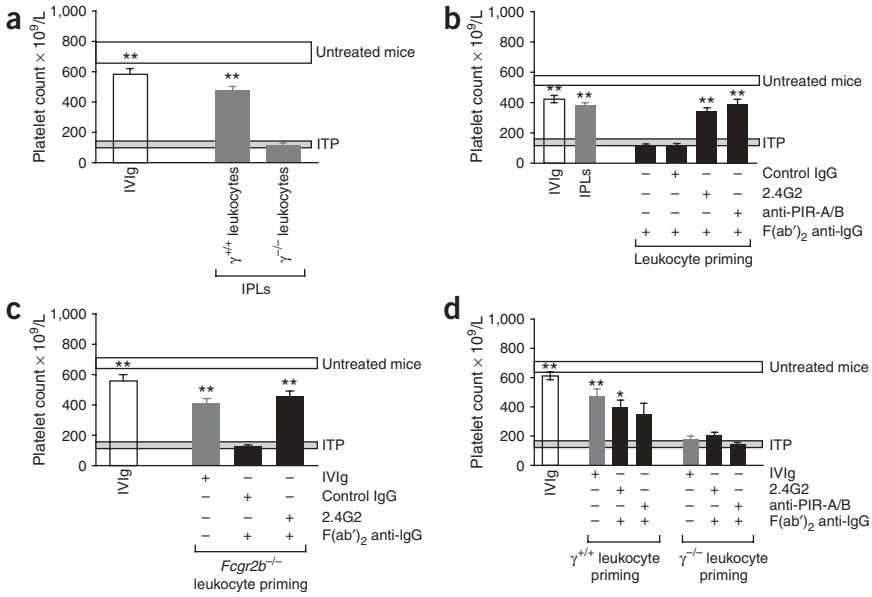

Figure 3 IVIg-primed leukocytes require FcR γ chain–dependent signals to ameliorate mouse ITP. (**a**) C57BL/6 recipient mice were injected with IVIg (white bar) or with IVIg-primed leukocytes (IPLs, 10^6/mouse) from donor C57BL/6 ($\gamma^{+/+}$) mice or IVIg-primed leukocytes from $\gamma^{-/-}$ mice followed by platelet-specific antibody at 24 h. $n = 9$ mice for each group from three independent experiments. (**b**) C57BL/6 mice were injected with IVIg (white bar), IVIg-primed leukocytes (gray bar), or with leukocytes pretreated with control rat IgG, FcγRIIIA-specific antibody (2.4G2) or PIR-A/B-specific antibody (6C1) followed by a crosslinking antibody, as indicated (black bars). (**c**) C57BL/6 (*Fcgr2b*$^{+/+}$) mice were injected with IVIg (white bar), or leukocytes from *Fcgr2b*$^{-/-}$ mice pretreated with IVIg (gray bar), control rat IgG or FcγRIIIA-specific antibody (2.4G2), followed by a crosslinking antibody, as indicated (black bars). $n = 6$ mice for each group from two independent experiments. (**d**) C57BL/6 recipient mice were injected with IVIg (white bar), leukocytes from donor C57BL/6 ($\gamma^{+/+}$) mice or $\gamma^{-/-}$ mice pretreated with IVIg (gray bars), FcγRIIIA-specific antibody (2.4G2), or PIR-A/B–specific antibody (6C1) followed by a crosslinking antibody as indicated (black bars), followed by platelet-specific antibody at 24 h. $n = 6$ mice for each group from two independent experiments. $^*P < 0.01$, $^{**}P < 0.001$ versus ITP mice. ITP (gray horizontal bar), indicates mean platelet count (± 1 s.e.m.) of mice injected with platelet-specific antibody only. Untreated mice (white horizontal bar) indicates mean platelet count of unmanipulated mice (± 1 s.e.m.); Data are mean ± s.e.m.

also showed that surface expression of activating FcγRIIIA on splenic macrophages was not downregulated in IVIg-treated mice[22], supporting the concept that the effect of the IVIg on DCs is selective and specific. Although the IVIg-primed DCs did not require the expression of the inhibitory FcγRIIB, this was required in the adoptively transferred mice. Thus, although FcγRIIB is not itself the relevant physical target of IVIg, it does have a role in a downstream phase of IVIg function.

DCs orchestrate immune activation or tolerance; these effects are primarily mediated through interactions between DCs and T cells. In ITP, however, both IVIg[23] and IVIg-primed DCs can mediate their effects in SCID mice, which lack functional B or T cells. We conclude, therefore, that the IVIg-primed DCs function independently of the adaptive immune system, hypothesizing that DCs either directly downregulate the function of phagocytic macrophages or use an intermediary cell or pathway.

These results may elucidate why IVIg has variable benefit in different autoimmune states. IVIg seems to be effective in autoimmune diseases in which sICs are not a common feature of the disease (for example, ITP, chronic inflammatory demyelinating polyneuropathy and Guillain-Barré syndrome), but is of questionable benefit in diseases in which sICs are a common feature (for example, SLE and rheumatoid arthritis). Although generally ineffective in human rheumatoid arthritis, IVIg is effective in experimental inflammatory arthritis, in which sICs are presumed not to be present. Similarly, individuals with concurrent SLE and ITP (in which sICs are present)

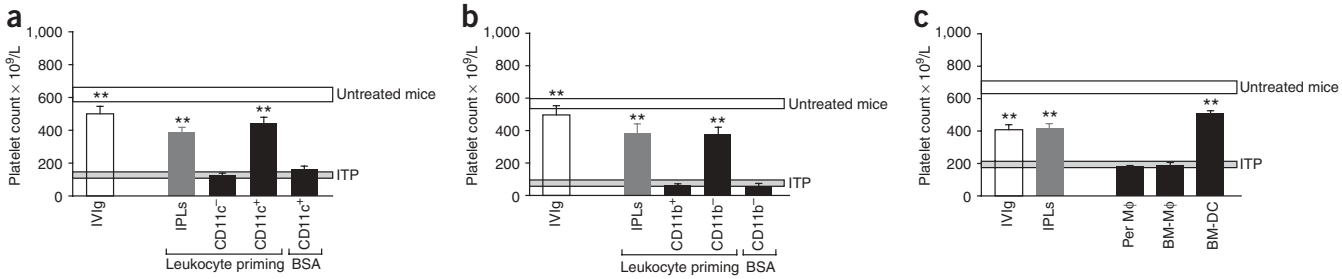

Figure 4 IVIg-primed dendritic cells ameliorate mouse ITP. (**a**) C57BL/6 recipient mice were injected with IVIg (white bar), IVIg-primed leukocytes (IPLs, 10^6/mouse) from donor C57BL/6 mice (gray bar), IPLs depleted of CD11c$^+$ cells (CD11c$^-$, 10^6/mouse), IVIg-primed CD11c$^+$ cells (10^5/mouse) or BSA-treated CD11c$^+$ cells (10^5/mouse) as indicated (black bars), followed by platelet-specific antibody at 24 h. (**b**) C57BL/6 recipient mice were injected with IVIg (white bar), IPLs (10^6/mouse) from donor C57BL/6 mice (gray bar), IVIg-primed CD11b$^+$ cells (CD11b$^+$, 10^5/mouse), IPLs depleted of CD11b$^+$ cells (CD11b$^-$, 10^6/mouse) or BSA-treated CD11b$^-$ cells (CD11b$^-$, 10^6/mouse) as indicated (black bars), followed by platelet-specific antibody at 24 h. $n = 6$ mice for each group from two independent experiments. (**c**) Mice were injected as in **a** with IVIg (white bar), IPLs (dark gray bar) or IVIg-primed peritoneal macrophages (MΦ), IVIg-primed bone marrow–derived cultured macrophages (BM-MΦ) or IVIg-primed bone marrow–derived cultured DCs as indicated (black bars). $n = 6$ mice per group from three independent experiments. ITP (gray horizontal bar) indicates mean platelet count (± 1 s.e.m.) of mice injected with platelet-specific antibody only. Untreated mice (white horizontal bar) indicates mean platelet count of unmanipulated mice (± 1 s.e.m.). $^{**}P < 0.001$ versus ITP mice. Data are mean ± s.e.m.

do not generally benefit from IVIg therapy[24,25]. We found that IVIg treatment of SLE-ITP mice did not ameliorate immune thrombocytopenia (**Supplementary Fig. 3** online). We therefore speculate that in autoimmune states where endogenous sICs are present, chronic stimulation of dendritic cells via FcγRs occurs, and IVIg therefore does not provide significant benefit.

We suggest that infusion of high-dose IVIg itself is not necessary but can be replaced with a low dose of a monoclonal antibody that engages activating FcγRs. Given the costs and potential for disease transmission of IVIg, the use of a monoclonal antibody could be a major benefit. Furthermore, IVIg may be bypassed using DCs that have been pretreated with an IVIg mimetic (or with IVIg), followed by reinfusion of these DCs back into the individual. Delivery of therapy with *in vitro*–manipulated leukocytes (including DCs[26]) may provide a new, highly effective treatment for some autoimmune diseases.

METHODS

Mice and reagents. We purchased C57BL/6 and BALB/c mice (females, 6–8 weeks of age) and *Fcgr2b*$^{-/-}$ (B6;129S4-*Fcgr2b*tm1Rav/J) mice from the Jackson Laboratories, SCID (CB17/lcr-*Prkdc*scid/Crl) mice from Charles River Laboratories and *Fcer1g*$^{-/-}$ (B6.129P2-*Fcer1g*tm1Rav) mice from Taconic Laboratory. All methods were approved by the Animal Care Committee of St. Michael's Hospital. IVIg was Gamimune N, 10% (Bayer Corporation). We purchased normal rat IgG and goat F(ab′)$_2$ fragments of IgG-specific rat antibody from Caltag, CD41-specific (integrin α_{IIb}) antibody and PIR-A/B–specific rat antibody was from BD PharMingen. We dissolved BSA in 0.2 M glycine (pH 4.25). We freshly dialyzed IVIg and BSA in PBS (pH 7.2).

Preparation of IVIg-primed leukocytes. We mechanically disrupted spleens in 5 ml complete RPMI-1640 (Sigma; 'complete' indicates RPMI-1640 supplemented with 10% heat-inactivated FCS, 80 μg/ml streptomycin sulfate, 80 U/ml penicillin G, 0.2 μg/ml amphotericin B) and filtered cells through a 70-μm strainer (BD Falcon). We lysed erythrocytes using 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2 EDTA (ACK) buffer, washed leukocytes two times in RPMI-1640 and incubated 1.4×10^6 cells/ml with 18 mg/ml IVIg or BSA for 30 min at 37 °C, then washed them two times in RPMI-1640, resuspended the cells to 5×10^6/ml and injected 200 μl into the mouse tail vein. In the FcγRIIIA and PIR-A/B experiments, we first treated leukocytes (2×10^7/ml and 4×10^6/ml, respectively) with antibody 2.4G2 (2.5 μg/ml), 6C1 (10 μg/ml) or normal rat IgG for 15 min at 22 °C. We then washed the cells, reacted them with goat F(ab′)$_2$ fragments of IgG-specific rat antibody (25 μg/ml) for 30 min at 37 °C, washed them two times in RPMI-1640, resuspended the cells to 5×10^6/ml and injected 200 μl into the mouse tail vein.

Purification of CD11c$^+$ cells. We prepared the CD11c$^+$ and CD11c$^-$ cell fractions from splenic leukocytes by magnetic separation using the mouse CD11c positive-selection kit (StemCell Technologies). Briefly, we disrupted spleens, treated them with collagenase IV for 20 min at 37 °C, added EDTA (1 mM final concentration). We then filtered and washed the cells with Ca^{2+}- and Mg^{2+}-free PBS (pH 7.22) containing 2% FCS and 1 mM EDTA. We incubated splenocytes (2×10^8/ml) with CD11c-PE at 15 μl/ml for 15 min at 22 °C. After washing, we incubated the splenocytes in the PE selection cocktail for 15 min at 22 °C, added magnetic particles for 10 min at 22 °C, followed by magnetic separation. The CD11c$^-$ cell fraction was collected in the supernatant. We incubated the CD11c$^+$ (1.4×10^5/ml) and CD11c$^-$ (1.4×10^6/ml) cells with 18 mg/ml dialyzed IVIg or BSA for 30 min at 37 °C, washed the cells two times with RPMI-1640, and injected them into recipient mice.

Purification of CD11b$^+$ cells. We prepared CD11b$^+$ and CD11b$^-$ cell fractions using CD11b immunomagnetic beads (MACS; Miltenyi Biotec). Briefly, splenic cells (10^8/ml) were depleted of red cells (ACK), which we suspended in 0.5% BSA, 2 mM EDTA, in PBS (pH 7.22) containing CD11b microbeads. After incubation for 15 min at 4 °C, we washed cells and applied them to a MACS column. We collected the effluent as the CD11b$^-$ cell fraction. We incubated CD11b$^+$ (1.4×10^5/ml) and CD11b$^-$ (1.4×10^6/ml) cells with 18 mg/ml dialyzed IVIg or BSA as above.

Bone marrow cultures. We flushed C57BL/6 mice femurs and tibiae with complete RPMI-1640 to isolate cells. After red-cell lysis and washing, we cultured cells in 10 cm sterile tissue culture dishes at 37 °C, 5% CO_2 at 2×10^5/ml in 10 ml complete RPMI-1640 with 50 ng/ml mouse recombinant macrophage colony-stimulating factor (rM-CSF, R&D Systems) to propagate macrophages, or with 20 ng/ml mouse recombinant granulocyte macrophage colony-stimulating factor (rGM-CSF, R&D Systems) to propagate dendritic cells. On day 3, we added 10 ml fresh complete RPMI-1640 containing 50 ng/ml rM-CSF or 20 ng/ml rGM-CSF to the appropriate plates. On days 6 and 8, we resuspended the cultures, collected one-half the culture from each plate, washed the collected cells and resuspended them in 10 ml complete RPMI-1640 supplemented with 50 ng/ml rM-CSF or 20 ng/ml rGM-CSF and added the cells back to the original plates. On day 10, we harvested cells, primed them with IVIg and injected 10^6 cells into mice as above.

Induction and treatment of ITP. We injected mice intraperitoneally with 50 mg (2 g/kg) IVIg, BSA or IVIg-primed cells. After 24 h, we rendered mice thrombocytopenic by intraperitoneal injection of 2 μg CD41-specific (integrin α_{IIb}) antibody in 200 μl PBS. Twenty-four hours later, we bled mice by the saphenous vein and counted platelets using a flow rate–calibrated FACScan flow cytometer (Becton Dickinson)[13,23,27].

Statistical analysis. We analyzed data using the Student *t* test. $P < 0.05$ was considered significant.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

V.K.S. and A.R.C. helped conceptualize and design the study, co-wrote the manuscript, performed the research and analyzed the data. D.B. and S.S. helped conceptualize and design the study and performed the research. J.F. helped conceptualize the study and co-wrote the manuscript. A.H.L. conceptualized and designed the study, obtained grant support and co-wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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