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Research Article **Vascular biology**

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a life-threatening disease in which intracranial hemorrhage (ICH) is the major risk. Although thrombocytopenia, which is caused by maternal antibodies against β3 integrin and occasionally by maternal antibodies against other platelet antigens, such as glycoprotein GPIbα, has long been assumed to be the cause of bleeding, the mechanism of ICH has not been adequately explored. Utilizing murine models of FNAIT and a high-frequency ultrasound imaging system, we found that ICH only occurred in fetuses and neonates with anti–β3 integrin–mediated, but not anti-GPIbα–mediated, FNAIT, despite similar thrombocytopenia in both groups. Only anti–β3 integrin–mediated FNAIT reduced brain and retina vessel density, impaired angiogenic signaling, and increased endothelial cell apoptosis, all of which were abrogated by maternal administration of intravenous immunoglobulin (IVIG). ICH and impairment of retinal angiogenesis were further reproduced in neonates by injection of anti–β3 integrin, but not anti-GPIbα antisera. Utilizing cultured human endothelial cells, we found that cell proliferation, network formation, and AKT phosphorylation were inhibited only by murine anti–β3 integrin antisera and human anti–HPA-1a IgG purified from mothers with FNAIT children. Our data suggest that fetal hemostasis is distinct and that impairment of angiogenesis rather than thrombocytopenia likely causes FNAIT-associated ICH. Additionally, our results indicate that maternal IVIG therapy can effectively prevent this devastating disorder.

Maternal anti-platelet β3 integrins impair angiogenesis and cause intracranial hemorrhage

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Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a life-threatening disease in which intracranial hemorrhage (ICH) is the major risk. Although thrombocytopenia, which is caused by maternal antibodies against β3 integrin and occasionally by maternal antibodies against other platelet antigens, such as glycoprotein GPIbα, has long been assumed to be the cause of bleeding, the mechanism of ICH has not been adequately explored. Utilizing murine models of FNAIT and a high-frequency ultrasound imaging system, we found that ICH only occurred in fetuses and neonates with anti–β3 integrin–mediated, but not anti-GPIbα–mediated, FNAIT, despite similar thrombocytopenia in both groups. Only anti–β3 integrin–mediated FNAIT reduced brain and retina vessel density, impaired angiogenic signaling, and increased endothelial cell apoptosis, all of which were abrogated by maternal administration of intravenous immunoglobulin (IVIG). ICH and impairment of retinal angiogenesis were further reproduced in neonates by injection of anti–β3 integrin, but not anti-GPIbα antisera. Utilizing cultured human endothelial cells, we found that cell proliferation, network formation, and AKT phosphorylation were inhibited only by murine anti–β3 integrin antisera and human anti–HPA-1a IgG purified from mothers with FNAIT children. Our data suggest that fetal hemostasis is distinct and that impairment of angiogenesis rather than thrombocytopenia likely causes FNAIT-associated ICH. Additionally, our results indicate that maternal IVIG therapy can effectively prevent this devastating disorder.

Introduction

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a severe bleeding disorder in which maternal antibodies cross the placenta and destroy fetal/neonatal platelets (1–3). FNAIT occurs in approximately 0.5 to 1.5 per 1,000 live-born neonates, but this does not include miscarriages in utero, as has been reported (4, 5). There are 33 reported human platelet antigens (HPAs) that can be recognized by maternal antibodies in FNAIT, and approximately half are located in the different regions of the extracellular domain of GPIIIa (β3 integrin [β3] subunit) (1, 6). At least one antigen (HPA-2) is located on GPIbα (6, 7). In people of mixed European descent, 80% to 90% of cases are due to antibodies targeting HPA-1a on β3. Severe bleeding is a hallmark of FNAIT, particularly intracranial hemorrhage (ICH), which can occur in fetuses as early as the 14th week of pregnancy and in 10% to 20% of neonates and can lead to neurological impairment or death in both fetuses and neonates (8–10). The pathogenic mechanism leading to the devastating ICH has, however, never been adequately explored.

Thrombocytopenia has generally been considered to be the cause of ICH. However, mice deficient in the hematopoietic subunit of the transcription factor NF-E2 (*Nfe2*$^{-/-}$), which lack circulation platelets, can survive in utero with no significant bleeding observed in fetuses or neonates after delivery by Caesarean section (11). NF-E2–deficient mice have a diminutive fraction (1%–3%) of platelet-like particles in their blood, which respond poorly to platelet agonists and have a markedly decreased binding ability for fibrinogen. This suggests that thrombocytopenia may not be essential for the development of ICH in fetuses. Consistently, in a murine model, antibody-mediated platelet depletion alone did not result in hemorrhage (12, 13). Interestingly, fibrinogen-deficient mice also did not experience bleeding in utero, and no obvious hemorrhage was found in their brains, despite a lack of fibrin clots in the fetuses (14–16). More strikingly, mouse fetuses with a combined deficiency in platelets (*Nfe2*$^{-/-}$) and fibrinogen exhibited normal embryonic development and were morphologically indistinguishable from their WT controls at 18.5 days postcoitum (17). Therefore, it is likely that neither thrombocytopenia nor deficiency in blood coagulation is crucial for the development of ICH in FNAIT, particularly in utero.

On the other hand, several pieces of evidence suggest that impaired angiogenesis may contribute to hemorrhage during embryogenesis, particularly in the brain (18, 19). Several coagulation factors (e.g., tissue factor, thrombin, etc.) also play important

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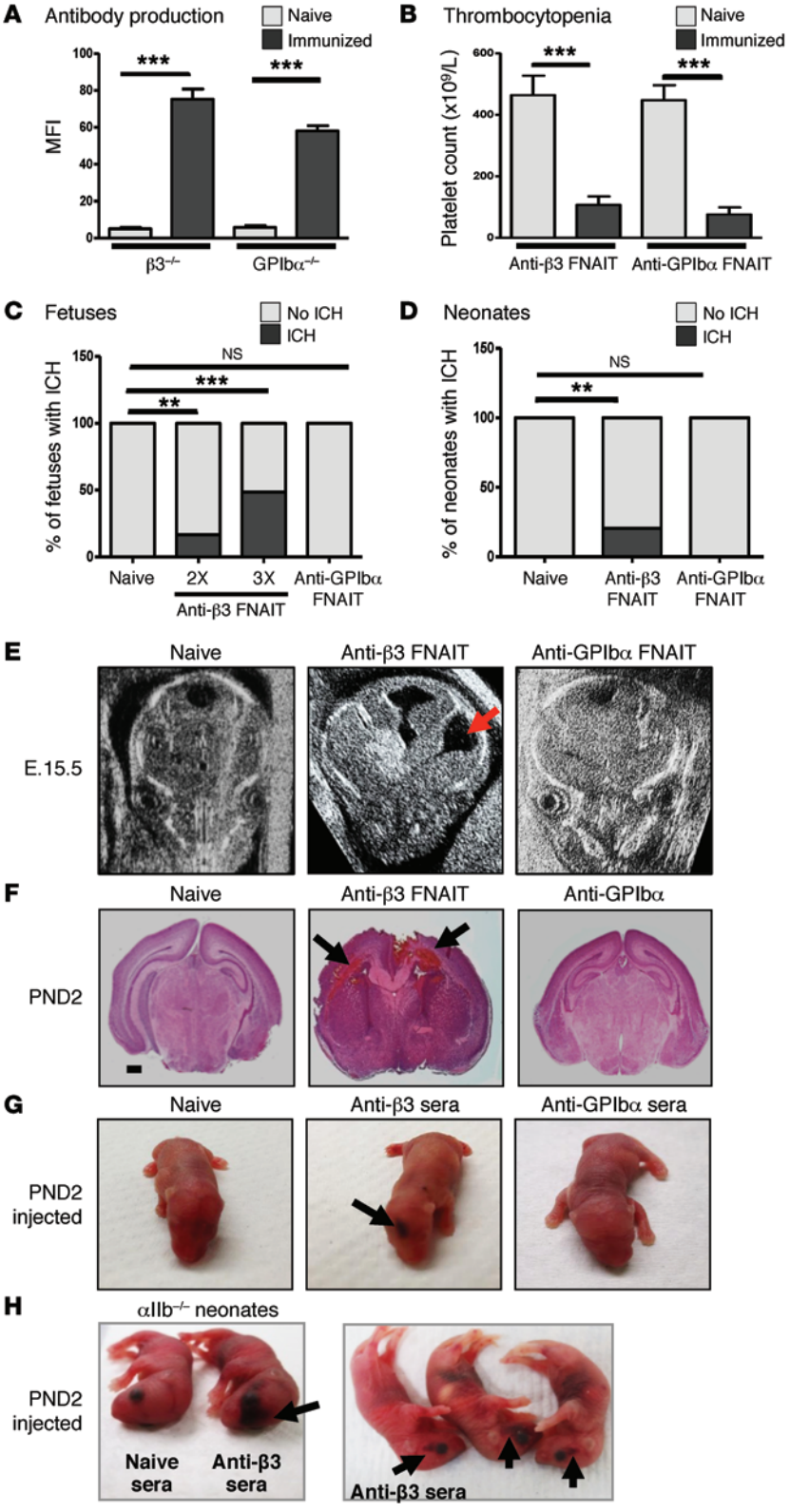

Figure 1. ICH in anti-β3–mediated FNAIT neonates and pups. (**A**) Anti-platelet antibodies were detected in both immunized $\beta3^{-/-}$ and $GPIb\alpha^{-/-}$ mice. (**B**) Platelet counts were lower in neonates delivered from immunized $\beta3^{-/-}$ and $GPIb\alpha^{-/-}$ mice compared with those delivered from naive mice. (**C**, **D**, and **F**) ICH was observed in anti-β3–mediated FNAIT pups (black arrows), but not in naive controls or anti-GPIbα–mediated FNAIT pups. Frequency of ICH increased with the number of maternal immunizations in anti-β3–mediated FNAIT fetuses. 2× and 3× indicates the number of immunizations. (**E**) Representative images of high-frequency ultrasound of ICH (red arrow) in utero at E15.5. (**G** and **H**) P2 pups were injected with naive sera and anti-sera. ICH was only observed in β3 heterozygote and αIIb pups after anti-β3 sera injection. Statistical analysis was performed using unpaired, 2-tailed Student's *t* test (**A** and **B**) and 1-way ANOVA followed by Bonferroni's post-hoc test (**C** and **D**). Data were collected from more than 5 pregnancies per group. Mean ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$. Original magnification, ×1 (H&E staining of brain sections). Scale bar: 100 μm.

roles in angiogenesis (20, 21), which may explain why hemorrhage was observed in fetuses deficient in these genes, but not in fibrinogen and/or NF-E2 (17, 20). It has been reported that development of the central nervous system required vascularization through angiogenesis, impairment of which led to cranial hemorrhage (22, 23). In a zebra fish model, impaired angiogenic signaling that affects vascular stability resulted in development of severe hemorrhage in the brain without obvious bleeding in other organs during embryogenesis (23). In a rabbit model of germinal matrix hemorrhage, it was shown that enhanced angiogenesis caused vascular immaturity and led to ICH (24). However, whether abnormalities in angiogenesis contribute to ICH in FNAIT has not previously been addressed.

The integrin αIIbβ3 is the most abundant glycoprotein on platelets. The β3 subunit is also coexpressed with the αV subunit (i.e., αVβ3) on proliferating endothelial cells (ECs) during angiogenesis (25–28). However, the exact role of αVβ3 integrin in angiogenesis is still controversial (29, 30). Earlier studies demonstrated that αVb3 was required for angiogenesis (25) and that αVb3 antagonists promoted tumor regression by inducing apoptosis of angiogenic blood vessels (26). Evidence also showed that integrin αVβ3 cooperated with VEGFR-2 in proangiogenic signaling (31, 32) and that AKT phosphorylation was essential in VEGF-mediated postneonatal angiogenesis (33). Interestingly, enhanced pathological angiogenesis and tumor growth were also observed in mice lacking β3 (30). These studies clearly demonstrate that β3 plays an important role in angiogenesis, although whether it is supportive or suppressive remains debatable. Further investigation of the role of β3 in angiogenesis is important, not only for understanding its broad impact on vascular biology, but also potentially for understanding its effect on FNAIT and fetal development.

The brain is one of the most angiogenic organs in the developing fetus, and integrins containing αV subunits (e.g., αVβ3) have been shown to be required for proper capillary development within the central nervous system (34, 35). It may be that by crossreacting with ECs, anti-β3 antibodies can affect angiogenesis and ICH in FNAIT. To test this hypothesis, we employed

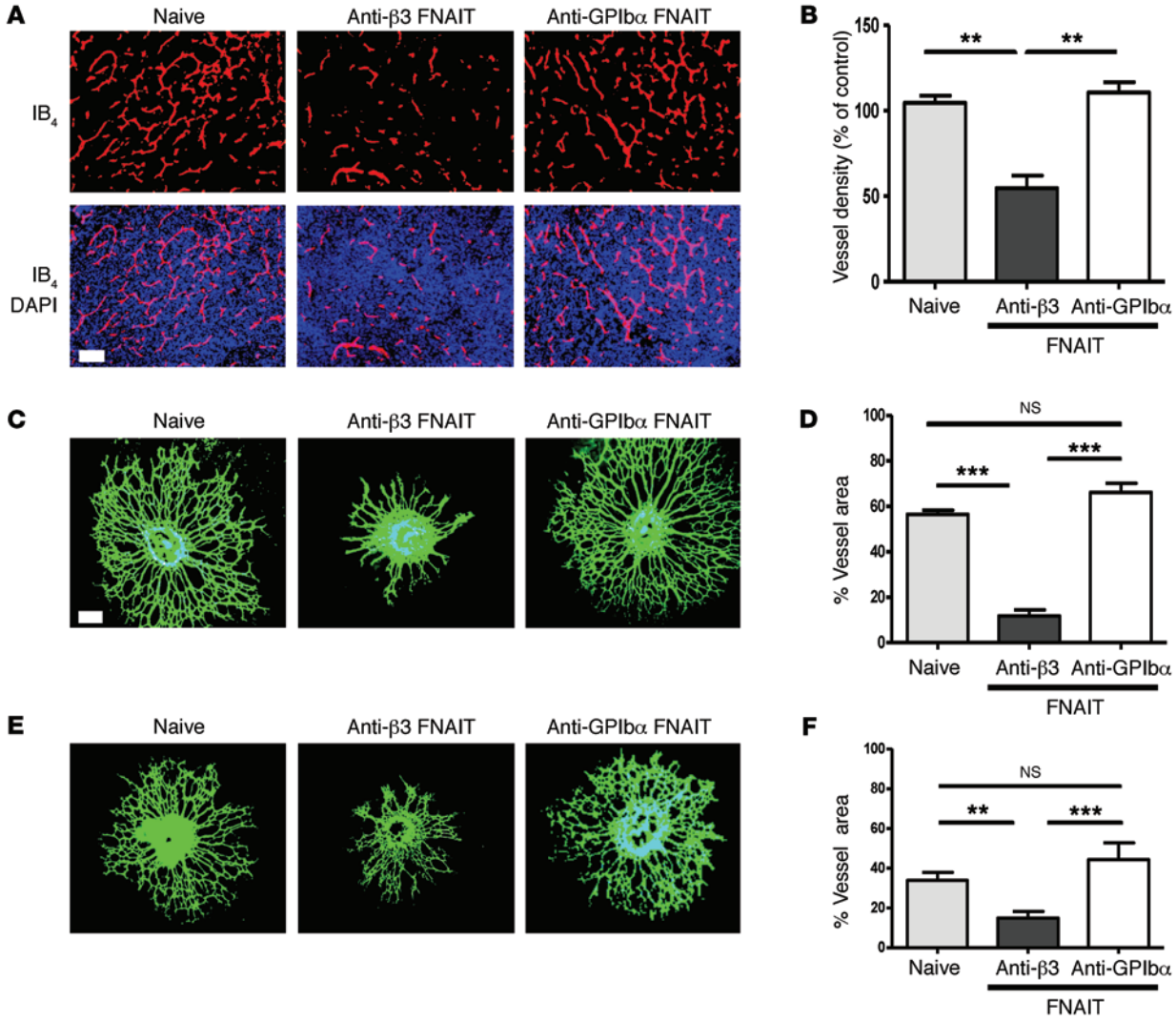

Figure 2. Severely impaired vascularization observed in brain and retina of anti-β3–mediated FNAIT pups. (**A**) Representative images and (**B**) quantification of IB4 staining of brain blood vessels are presented. Anti-β3–mediated FNAIT pups had reduced blood vessel density compared with anti-GPIbα–mediated FNAIT pups or naive controls. Representative images and quantification of FNAIT (**C** and **D**) or sera-injected (**E** and **F**) pup retinas immunostained by anti-collagen IV antibody and revealed with anti-rabbit conjugated to Alexa Fluor 488. Vessel development in the retina from anti-β3–mediated FNAIT and anti-β3 sera–injected pups was significantly impaired compared with that of naive controls and anti-GPIbα–mediated FNAIT and anti-GPIbα sera–injected pups. Results are representative of at least 4 independent experiments. All of the statistical comparisons were performed using 1-way ANOVA followed by Bonferroni's post-hoc test. Mean ± SEM. n = 4–6 mice per group. $^{**}P < 0.01$. Scale bars: 200 μm (**A**); 500 μm (**C**, **E**). Original magnification, ×10 (**A**); ×4 (**C**, **E**).

both active and passive (postnatal antisera injections) murine models of FNAIT. As GPIbα is not constitutively expressed on ECs and anti-GPIbα antibody (i.e., anti–HPA-2a) is involved in FNAIT (36, 37), we used our anti-GPIbα–mediated FNAIT model as a control (37–39). Our findings demonstrate that anti–platelet β3, but not anti-GPIbα antibodies, impaired angiogenic signaling, inhibited angiogenesis, and induced ICH in the brain of murine fetuses and neonates, and this could be prevented by administration of intravenous immunoglobulin (IVIG) to the mother. Anti-β3 antibodies also inhibited proliferation, angiogenic signaling, and network formation of HUVECs in vitro. Importantly anti–HPA-1a IgG purified from human mothers who gave birth to neonates with FNAIT also had similar effects on HUVECs. Thus, β3 plays an important role in angiogenesis in the context of fetal development, and this is targeted by maternal anti-platelet antibodies leading to ICH. These findings may shift the prevailing view that thrombocytopenia is the cause of ICH in FNAIT and may alter the current therapeutic strategy, which focuses on the improvement of platelet counts in patients. The present study may not only provide insights into developing new therapies to control ICH in FNAIT; it may also have an impact on other angiogenesis-related disorders.

Results

Anti-β3–, but not anti-GPIbα–mediated FNAIT caused ICH. We used our established murine models of FNAIT (2, 37, 40) to investigate the mechanism of ICH in affected fetuses and neonates. To mimic exposure to β3 or GPIbα during conception, *Itgb3*$^{-/-}$ and *Gp1ba*$^{-/-}$

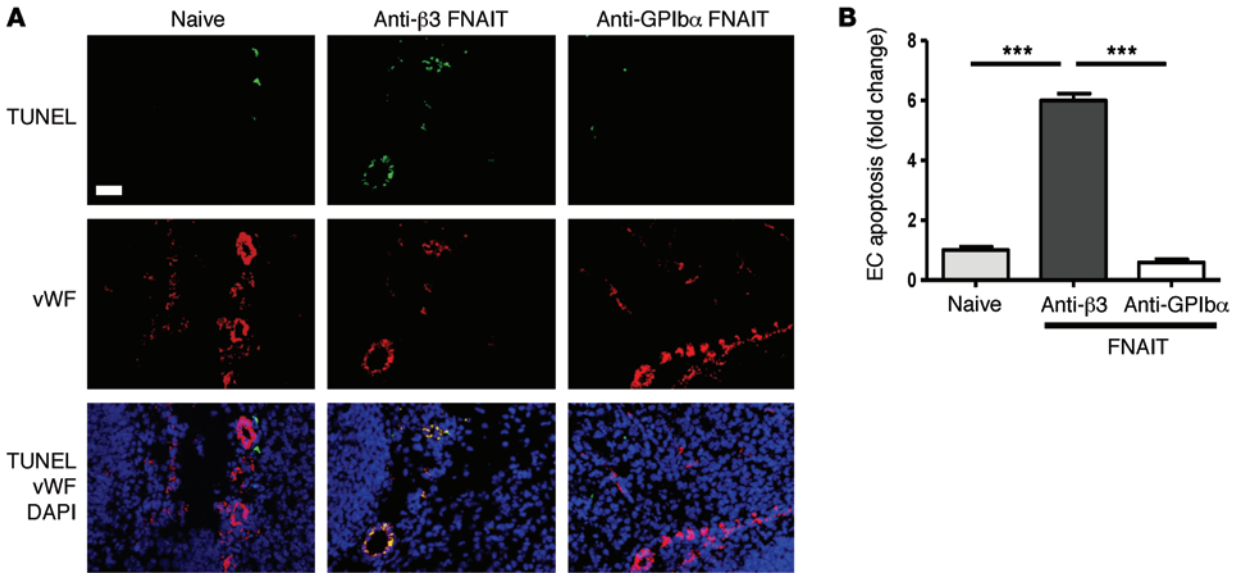

Figure 3. Increased apoptosis in the brain vessels of anti-β3–mediated FNAIT pups. (**A**) Representative images of TUNEL (green) and vWF (red) costaining are presented. (**B**) Area of colocalized fluorescence (yellow) was quantified using ImageJ. Increased apoptosis was detected in the brain blood vessels of anti-β3–mediated FNAIT pups compared with naive controls and anti-GPIbα–mediated FNAIT pups. Statistical analysis was performed using 1-way ANOVA followed by Bonferroni's post-hoc test. Mean ± SEM. n = 3–6 mice per group. ***$P < 0.001$. Scale bar: 50 μm. Original magnification, ×40.

mice (referred to hereafter as $\beta3^{-/-}$ and GPIbα$^{-/-}$) were transfused with WT platelets. Anti-β3 or anti-GPIbα antibodies were detected (Figure 1A), and these immunized mice were subsequently bred with WT males. We found similar severity of thrombocytopenia in the heterozygote (–/+) neonates delivered from immunized $\beta3^{-/-}$ and GPIbα$^{-/-}$ mice (Figure 1B).

Using a high-frequency ultrasound imaging system to detect in utero ICH in pregnant mice and performing H&E staining of brain sections to confirm the ICH, we found ICH in the $\beta3^{-/+}$ fetuses starting around E15.5 as well as in neonates. Hemorrhage was observed in different areas of the brain (Figure 1E), and the frequency of ICH increased in fetuses in accordance with the number of maternal immunizations (Figure 1C). We did not detect any abnormalities in fetuses or neonates from naive mothers without detectable antiplatelet antibodies (Figure 1D). In contrast, ICH was never found in anti-GPIbα–mediated FNAIT fetuses or neonates (Figure 1, C–F).

To confirm that ICH was indeed antibody mediated, $\beta3^{-/+}$ and GPIbα$^{-/+}$ neonates delivered from naive mice were passively injected with antisera at P2. As shown in Figure 1G, postnatal injection of anti-β3 sera into $\beta3^{-/+}$ neonates induced ICH, but anti-GPIbα sera did not induce any ICH in GPIbα$^{-/+}$ neonates ($P < 0.01$). To further elucidate whether platelet-mediated cytotoxicity (41, 42) might be involved in the mechanism of ICH, anti-β3 sera were injected into αIIb integrin–deficient pups that did not express αIIbβ3 integrin on their platelets. ICH was observed in *Itga2b*$^{-/-}$ (referred to hereafter as αIIb$^{-/-}$) pups with normal platelet counts (Figure 1H and Supplemental Figure 1). Furthermore, postnatal injection of anti-β3 sera into $\beta3^{-/-}$ neonates failed to induce ICH and impair retinal vascular development (Supplemental Figure 2) in these antigen-negative pups. These data demonstrate that anti-β3 antibodies, but not anti-GPIbα antibodies or thrombocytopenia alone (Supplemental Figure 1), were the cause of ICH.

Vessel density and proangiogenic signaling are reduced in the brain and retina of anti-β3–mediated FNAIT pups. Since impairment of angiogenesis may contribute to hemorrhage in brains during development and angiogenic ECs markedly increase the expression of αVβ3 integrin (25), we assessed whether antiangiogenic effects occur in anti-β3–mediated FNAIT. We measured vessel density in the neonate brains by immunofluorescent staining with isolectin IB4. Pups with anti-β3–mediated FNAIT exhibited a significant reduction of blood vessel density in the brain (Figure 2, A and B). Conversely, anti-GPIbα–mediated FNAIT neonates had blood vessel density similar to that of neonates delivered from naive mice (Figure 2, A and B).

To confirm the impairment of angiogenesis in affected fetuses, we assessed retinal vascular development (which begins postnatally in mice), staining the retinal vasculature with an anti-collagen antibody. Vascular development was impaired in the retinas of anti-β3–mediated FNAIT pups (Figure 2, C and D), but developed normally in both naive control pups and anti-GPIbα–mediated FNAIT pups (Figure 2, C and D). To demonstrate the direct effect of anti-β3 antibody on retinal vascular development, we injected anti-GPIbα or anti-β3 antisera into the respective heterozygote neonates shortly after delivery. We found that anti-β3, but not anti-GPIbα, antisera significantly impaired vessel development in neonates (Figure 2, E and F).

We then investigated EC apoptosis in the brains of developing neonates. Using TUNEL and immunofluorescent von Willebrand Factor (vWF) staining, we observed that anti-β3–mediated FNAIT neonates exhibited increased EC apoptosis in their brains, but did not detect any significant increase in apoptosis of ECs in the brains of heterozygotes delivered from naive mothers and those with anti-GPIbα–mediated FNAIT (Figure 3, A and B). These results were further confirmed by staining for CD31 and TUNEL (Supple-

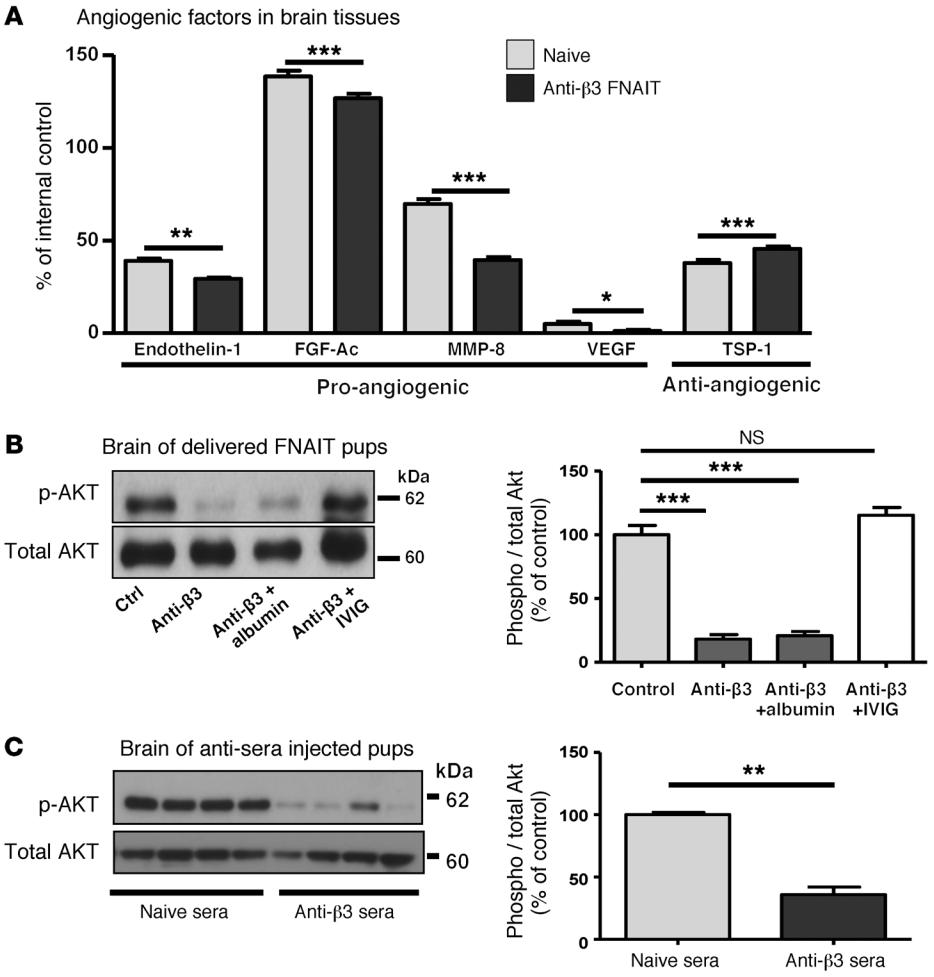

Figure 4. Anti-β3 antibody decreased proangiogenic and AKT signaling in brain. (**A**) Brain homogenates from heterozygote pups with FNAIT were analyzed with the mouse angiogenesis antibody cytokine array kit (R&D Systems). Anti-β3–mediated FNAIT significantly downregulated angiogenic growth factors, but upregulated angiogenic inhibitor TSP-1. Representative images and densitometry quantification of Western blotting of P2 brain lysates from FNAIT pups (**B**) and sera-injected pups (**C**) are shown. Phosphorylated AKT was normalized to total AKT and expressed as a percentage of the naive control group. Statistical analysis was performed using an unpaired, 2-tailed Student's *t* test (**A** and **C**) and 1-way ANOVA followed by Bonferroni's post-hoc test (**B**). Results are representative of at least 3 independent experiments of Western blots. Mean ± SEM. $n = 3$ mice per group. $^{**}P < 0.01$; $^{***}P < 0.001$.

mental Figure 3). We only observed a decreasing trend of vascular smooth muscle cells via staining of smooth muscle actin (ref. 43 and Supplemental Figure 2, $P > 0.05$), suggesting that maternal anti-β3 antibody–mediated antiangiogenic effects may mainly be restricted to ECs in less mature vessels. Immunoblot and Western blot assays of brain homogenates indicated that anti-β3 sera significantly downregulated AKT phosphorylation, although no significant change of ERK phosphorylation (44) was found (data not shown). We also found that the levels of proangiogenic factors (endothelin-1, acidic fibroblast growth factor, matrix metalloproteinase-8, and VEGF) were downregulated and the antiangiogenic factor thrombospondin-1 was upregulated (Figure 4, A–C). This indicates that anti-β3 antibodies have a potent antiangiogenic effect in vivo. Together, these data indicate that anti-β3 antibodies impair angiogenesis and vascular development, which may play a crucial role in the pathogenesis of ICH in FNAIT.

Maternal IVIG administration abrogated impairment of vascular development in the brain and retina in anti-β3–mediated FNAIT pups. We previously demonstrated that IVIG can decrease maternal and neonatal anti-platelet antibody titers and ameliorate FNAIT in mice (2, 37, 40), consistent with recent studies in human patients (3). We therefore examined whether IVIG can restore vessel density in this murine model of FNAIT. IVIG or albumin control was injected into the immunized mothers 2 times once a week starting at E.0.5. The pups delivered from the albumin-treated mothers with high anti-β3 antibody titer developed FNAIT and exhibited decreased vessel density in both the brain (Figure 5, A and C) and retina (Figure 5, B and D), whereas IVIG treatment decreased EC apoptosis and restored vascular development (Figure 5, A–D, and Supplemental Figure 3). These findings reveal a new mechanism of action of IVIG in FNAIT treatment and demonstrate that it not only improves thrombocytopenia, but also ameliorates vascular pathology in anti-β3–mediated FNAIT.

EC proliferation, network formation, and AKT phosphorylation are inhibited by human and mouse anti-β3 antibodies in vitro. In order to investigate how anti-β3 antibodies impair vessel development, we examined EC proliferation and network formation in vitro. Human anti–HPA-1a IgG bound to HUVECs (Figure 6A) and inhibited their proliferation (Figure 6B). HUVECs treated with murine anti-β3 sera also exhibited reduced proliferation (Figure 6C) with a dose-dependent impairment of network formation on Matrigel (Figure 6D). In contrast, with naive or anti-GPIbα sera treatment, no significant difference was found in HUVEC proliferation (Figure 6C) or capillary-like network formation (Figure 6D). Importantly, incubation with anti–HPA-1a IgG resulted in significantly decreased HUVEC network formation (Figure 6E) compared with the use of control IgG–treated cells. We found that anti-β3 IgG has a similar inhibitory effect, when compared with cyclic arginine-glycine-aspartic acid

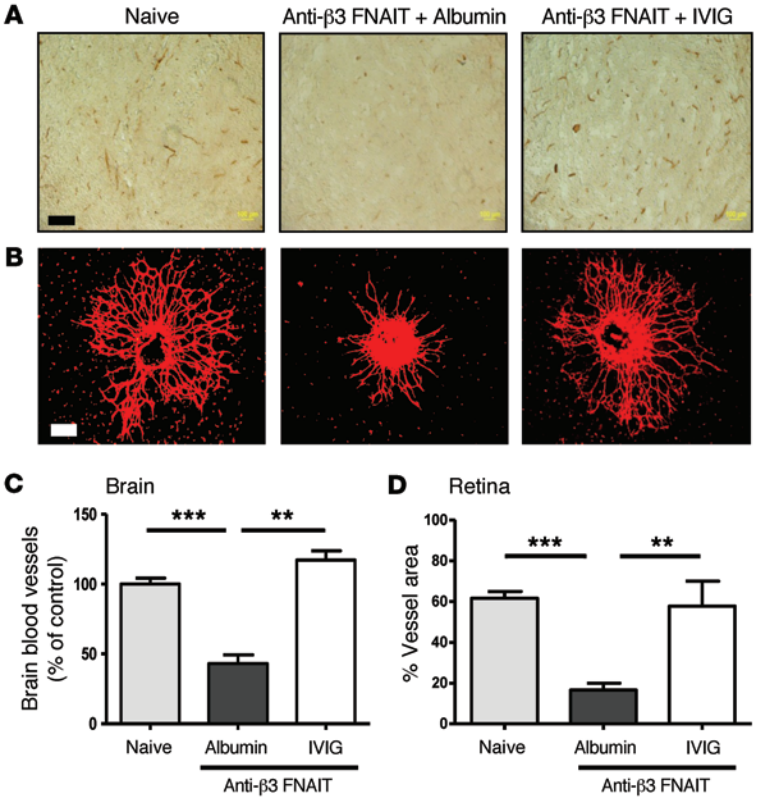

Figure 5. IVIG reverses the impairment in retinal and brain vascular development in anti- β3–mediated FNAIT pups. (**A**) Representative images and quantification of brain sections stained by peroxidase immunohistochemistry for vWF. (**B**) Representative fluorescent images and quantification of isolectin (IB4 conjugated to Alexa Fluor 594) immunostaining of retinas from P2 pups. (**C**) Brain and (**D**) retina vessel length were quantified. Blood vessel development in the retina and brain of pups from immunized $\beta3^{-/-}$ mice treated with albumin was significantly impaired compared with that in pups from naive $\beta3^{-/-}$ mice. IVIG significantly reversed the inhibition of vessel growth. Statistical analysis was performed using 1-way ANOVA followed by Bonferroni's post-hoc test. Mean ± SEM; n = 4–6 mice per group. $^{**}P < 0.01$; $^{***}P < 0.001$. Scale bars: 200 μm (**A**) 500 μm (**B**). Original magnification, ×10 (**A**); ×4 (**B**).

(RGD) peptides that target αVβ3 integrin, on HUVEC proliferation and invasion in Matrigel matrix (Supplemental Figure 4). Subsequent experiments with anti-β3 IgG and anti–HPA-1a IgG showed a significant reduction of EC adhesion to a fibronectin-coated surface (Supplemental Figure 4).

We performed Western blotting to assess the phosphorylation of AKT, a downstream target of VEGFR-2 that supports EC survival and is involved in EC network formation. We pretreated HUVECs with mouse anti-sera, anti–HPA-1a IgG, or human negative control IgG. Murine anti-β3 sera and human anti–HPA-1a IgG inhibited phosphorylation of AKT, whereas human negative control IgG or mouse anti-GPIbα did not (Figure 7A). In addition, human anti–HPA-1a IgG increased proapoptotic signaling through caspase-3 activation (Figure 7B). These results suggest that the reduced vessel density observed in the murine model of FNAIT is probably caused by impaired EC proliferation and network formation, both essential stages of angiogenesis.

Discussion

Thrombocytopenia caused by maternal anti-platelet antibodies has long been considered the cause of ICH in FNAIT. In this study, using both active and passive murine models of FNAIT, we clearly demonstrate that ICH only occurred in anti-β3–mediated, but not anti-GPIbα–mediated FNAIT despite similar thrombocytopenia induced by anti-GPIbα antibodies. Anti-β3 antibodies also induced ICH in $\alpha IIb^{-/-}$ pups without any thrombocytopenia. We further found that anti-β3 antibodies inhibited angiogenic signaling, induced EC apoptosis, and decreased the vessel density in affected brains and retinas. Both anti-β3 antisera and anti–HPA-1a IgG inhibited EC proliferation and network formation in vitro and induced impairment of AKT and other angiogenic signaling. These data suggested that impairment of angiogenesis rather than thrombocytopenia is the critical cause of the ICH in FNAIT, although we cannot exclude that thrombocytopenia may synergistically contribute to the hemorrhage. This information may shift the prevailing view of the mechanism of this bleeding disorder in FNAIT, particularly in fetuses. Furthermore, we demonstrated that maternal administration of IVIG can prevent this devastating disorder.

Bleeding usually occurs when the blood vessel is injured (45–47). Platelet adhesion and aggregation at the site of injury have been considered as the primary wave of hemostasis. Activation of the coagulation system that converts fibrinogen to fibrin is the subsequent event that further enhances hemostasis (48, 49). In addition to the primary wave, activated platelets can also provide a negatively charged surface (e.g., phosphatidylserine) for thrombin generation that can markedly accelerate blood coagulation and hemostasis (50). It has therefore been generally accepted that thrombocytopenia caused by maternal anti-platelet antibodies is the reason for ICH in FNAIT.

There are several pieces of evidence, however, that challenge this dogma. In NF-E2–deficient mice, which lack circulating platelets, no significant bleeding was detected in fetuses or neonates after delivery by Caesarean section (11). Fibrinogen-deficient mice, which completely lack fibrin, also did not develop bleeding or ICH in utero. Even more surprisingly, in mice with double deficiencies of NF-E2 and fibrinogen, the fetuses were still well developed and lacked signs of hemorrhage in utero. Interestingly, in contrast to NF-E2 or fibrinogen single-gene–deficient mice, no double-deficient mice survived beyond 2 to 3 days after birth due to bleeding caused by the trauma of vaginal delivery and/or minor trauma ex utero (17). This suggests that platelets and fibrin play important roles in hemostasis postnatally. In human patients, up to 80% of FNAIT cases occur in utero, indicating that ICH, in these cases at least, is not caused by the trauma of birth (51), and importantly, ICH has been observed in some patients whose platelet counts are within normal range (52, 53). Therefore, in contrast with bleeding disorders after birth, it is probable that neither platelets nor impaired fibrin clot is crucial for the maintenance of hemostasis in fetuses. This suggests that another mechanism or mechanisms (17, 20) may play an even more important role than these 2 classical hemostatic pathways in fetal hemostasis that protect fetuses from ICH.

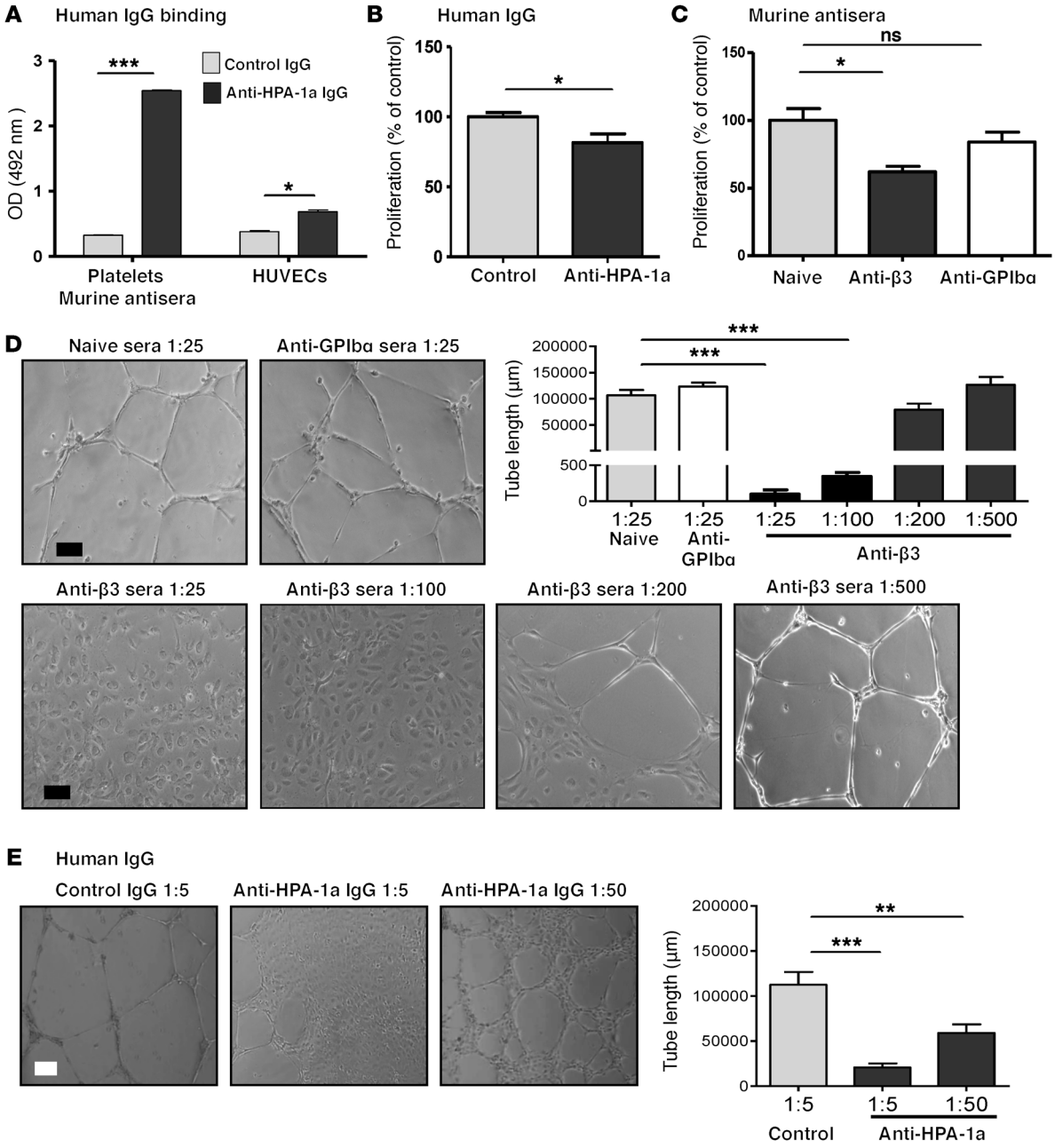

Figure 6. Mouse anti-β3 sera and human anti–HPA-1a IgG inhibit EC proliferation and network formation on Matrigel. (**A**) Anti–HPA-1a IgG binding to HUVECs was tested with MAIPA assay. (**B** and **C**) Anti-β3 sera significantly inhibited HUVEC proliferation compared with naive control sera. HUVECs seeded onto Matrigel-coated wells were incubated with EGM-2 media containing murine sera (**D**) or containing human IgG (**E**), as indicated. HUVECs treated with naive sera or anti-GPIbα sera and control IgG formed capillary-like networks, while anti-β3 sera and anti–HPA-1a inhibited this process in a dose-dependent manner. Results are pooled from 4 independent experiments. Statistical analysis was performed using unpaired, 2-tailed Student's *t* test. Mean ± SEM; $n = 3$ wells per group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Scale bars: 200 μm (**D**); 500 μm (**E**). Original magnification, ×10 (**D**); ×4(**E**).

Given the ethical difficulties in performing basic research on human fetuses and neonates in life-threatening FNAIT, animal models are necessary. Notably, there are at least 16 HPAs identified on the human β3 subunit. It is currently unknown which antigen has higher risk of causing ICH, although HPA-1a and HPA-4a (polymorphism of residue 33 and residue 143 in the β3 subunit) are the reported most common antigens to cause FNAIT in people of mixed European and Asian descent, respectively. Difficulties in studying the ratio of ICH cases to individual HPA are due to the lack of information on miscarriages caused by incompatibility of these HPAs and their related ICH; many of these are not reported, although miscarriage can occur in up to 50% in humans and a significant number of them are caused by FNAIT (3, 4, 54). Since these 16 HPAs are located throughout the extracellular β3 subunit (from residue 33 to residue 636), study of the immune response to the entire β3 subunit is important. We therefore used β3- and GPIbα-deficient female mice to develop murine models, which recapitulate human mothers who developed antibodies targeting

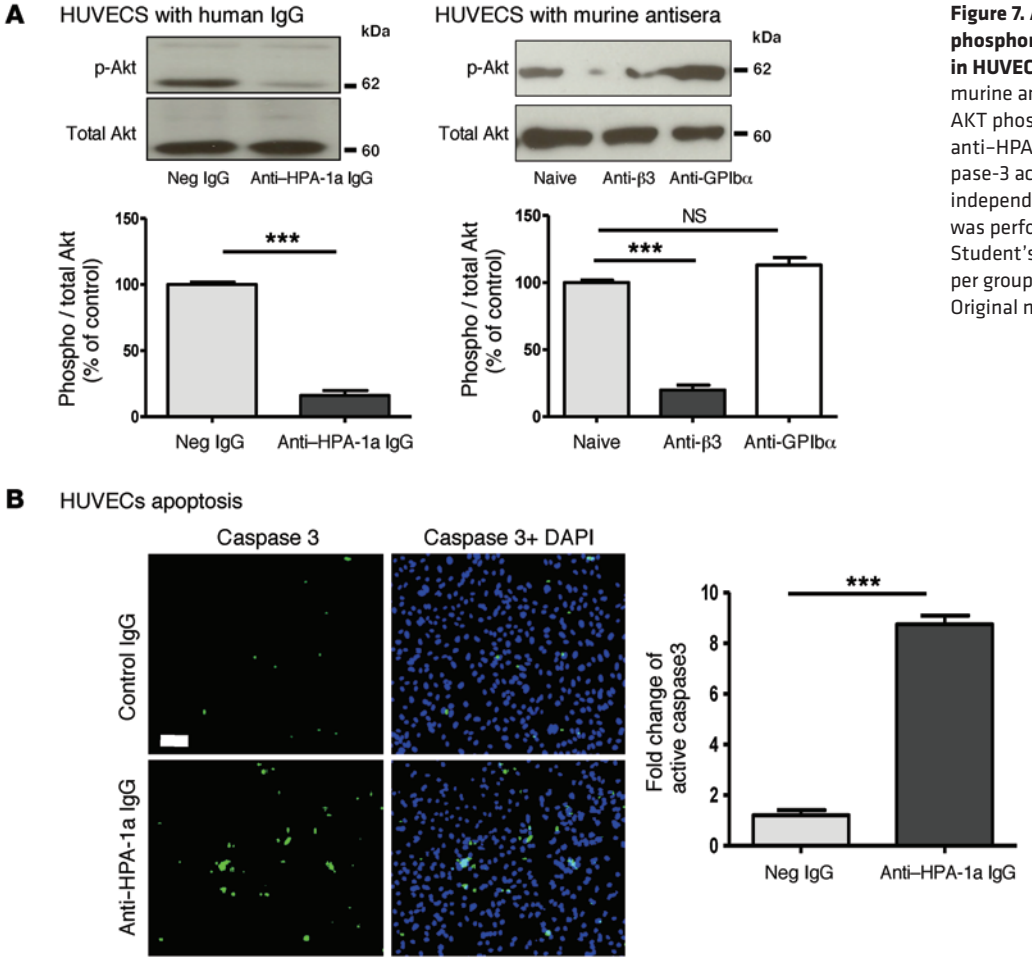

Figure 7. Anti-β3 antibody decreased AKT phosphorylation and increased apoptosis in HUVECs. (**A**) Human anti–HPA-1a IgG and murine anti-β3 sera significantly reduced AKT phosphorylation in HUVECs. (**B**) Human anti–HPA-1a IgG significantly increased caspase-3 activation. Results are pooled from 4 independent experiments. Statistical analysis was performed using an unpaired, 2-tailed Student's *t* test. Mean ± SEM. $n = 3$ wells per group. $^{***}P < 0.001$. Scale bar: 200 μm. Original magnification, ×10.

multiple alloantigens. To further confirm our observations in these murine models, we also used human anti–HPA-1a alloantibodies.

Our findings that impairment of angiogenesis is the mechanism, or at least the major mechanism, that causes ICH in fetuses are consistent with the earlier observations in zebra fish and rabbit models (23, 24). However, in contrast with these 2 genetically targeted animal models, our models were established with maternal antibodies against fetal platelet proteins. To exclude other possible pathogenic effects of anti-β3 antibodies, we examined complement activity and found no significant enhancement of C5a in the affected fetal brain tissues (data not shown). We also did not observe obvious antibody-dependent cell-mediated cytotoxic effect on ECs in the immunohistochemistry assays on the fetal brains. In contrast, antiangiogenic effects were observed by vessel density (Figures 2 and 3), EC apoptosis, alterations of angiogenic factors (downregulated VEGF, FGF-acid, MMP-8, and endothelin-1; upregulated TSP-1), and AKT signaling (Figure 4). These effects were reproduced in vitro in cultured HUVECs, in that EC proliferation, network formation, AKT phosphorylation, and proangiogenic signaling were inhibited by anti-β3 antisera. Importantly, these effects were also observed when HUVECs were treated with human anti–HPA-1a IgG (Figures 6 and 7).

We further studied the murine retinal vasculature. This model has been widely used for investigating angiogenesis, as retinal vessels develop de novo over the first 2 postnatal weeks (55). Reduced retinal vessel density was observed only in anti-β3–mediated, but not in anti-GPIbα–mediated, FNAIT. Retinal vascular development was similarly impaired when pups were passively injected with anti-sera, consistent with an earlier study with a peptide inhibitor of αVβ3 integrins (56). It is currently unknown how important this finding is, but our data may explain the visual impairment in some cases of FNAIT (57).

The role of endothelial β3 in angiogenesis remains unclear. Studies in chicken chorioallantoic membrane and murine tumor models demonstrated that αVβ3 integrin is required for angiogenesis and that αVβ3 antagonists (both antibody and cyclic RGD peptide) inhibited this process (25, 26, 28). Interestingly, β3-deficient mice have been successfully generated (58), and enhanced angiogenesis was also reported in arterial and tumor tissues of these genetically targeted mice (30). It is currently unknown whether the enhanced pathogenic angiogenesis is due to compensatory mechanisms (e.g., upregulated VEGFR-2) that occurred in gene-deficient mice (30). Using $\beta3^{-/+}$ fetuses/neonates, our data demonstrate that targeting β3 with antibodies inhibited angiogenesis. These data may have, at least partially, clarified a paradigm from a controversy and further demonstrated that integrin αVβ3 plays an important role in angiogenesis during development and that this process can be targeted by maternal anti-β3 antibodies.

In addition to being expressed on platelets and ECs, integrin αVβ3 is also expressed on the invasive trophoblast cells during placental growth (59). Trophoblast migration and invasion into

the maternal decidua is essential for placental angiogenesis during pregnancy. Invasive trophoblast cells replace existing vascular ECs in the placental spiral arteries, which lowers maternal vascular resistance and increases uteroplacental blood flow. Inadequate migration and invasion of trophoblast has been reported in complications of pregnancy, including miscarriage, preeclampsia, and intrauterine growth restriction (IUGR) (60). Therefore, in addition to ICH that may be caused by maternal anti-β3 antibodies, it is possible that these antibodies may also target αVβ3 integrin on trophoblasts (59) and impair placenta angiogenesis, leading to placental dysfunction and IUGR, or even miscarriage, in FNAIT.

Clinical management of FNAIT is still a challenge (1, 3, 4, 61), but IVIG has been demonstrated to be a useful therapy for this disease. We previously demonstrated that IVIG downregulated both maternal and neonatal circulating levels of anti-platelet antibodies (40), although IVIG did not show antiidiotype activity and did not significantly inhibit the binding of anti-β3 antibodies to platelets (40). Subsequently, we demonstrated that the neonatal Fc receptor (FcRn) is required for transplacental transport of maternal IgGs to the fetus, which can be blocked by IVIG. In addition, IVIG can downregulate maternal antibodies in both FcRn-dependent (likely via the enhancement of IgG clearance) and -independent pathways (probably T and B cell tolerance) (2). It is therefore conceivable that maternal IVIG administration may markedly decrease the level of circulating pathogenic antibodies available to bind to platelets and ECs, which ameliorates both thrombocytopenia and antiangiogenesis-mediated ICH. We cannot, based on the current study, reach a conclusion regarding whether IVIG can directly affect ECs and/or systemic platelet–mediated cytotoxicity (41, 42), which may also be important for its mechanisms of action against ICH. Future studies may be able to address these questions.

In summary, to the best of our knowledge, the current study provides the first direct evidence that vascular impairments occur in anti-β3–mediated FNAIT. The findings also demonstrate that αVβ3 integrin plays an important role in angiogenesis during fetal development. Our data suggest that impaired angiogenesis, instead of thrombocytopenia, is the key cause of ICH in FNAIT; although thrombocytopenia and coagulation-factor deficiencies may synergistically contribute to this disease process. We demonstrated that maternal administration of IVIG can rescue fetal and neonatal angiogenesis and prevent ICH (Figure 6). This suggests that therapies that decrease antiangiogenic effects induced by anti-β3 antibodies (e.g., IVIG, anti-FcRn, etc.) (2, 40) may be more efficient than fetal platelet transfusion. Platelet transfusion, however, may be critical to controlling bleeding after birth, as suggested from both clinical and animal studies (1, 3, 17, 62). These findings may shift the prevailing view and advance our understanding of the pathogenesis of FNAIT and may contribute markedly to improvements in the clinical management of this life-threatening disease.

Methods

Mice. $\beta3^{-/-}$, $\alpha IIb^{-/-}$, and $GPIb\alpha^{-/-}$ mice were provided by Richard O. Hynes (Massachusetts Institute of Technology, Boston, Massachusetts, USA) (40, 58), Mortimer Poncz (University of Pennsylvania, Philadelphia, Pennsylvania, USA) and Jon Frampton (University of Birmingham, Birmingham, United Kingdom), and Jerry Ware and Zaviero M. Ruggeri (The Scripps Research Institute, La Jolla, California, USA) (37). Both $GPIb\alpha^{-/-}$ and $\beta3^{-/-}$ mice were backcrossed to a BALB/c background 10 times and bred to generate syngeneic gene-deficient mice. Experiments were performed when mice were 7 to 10 weeks of age. BALB/c WT mice were purchased from Charles River.

Reagents. Rabbit anti-mouse phosphorylated (catalog 9271) and total AKT (catalog 4685) antibodies were obtained from Cell Signaling Technology. Rabbit anti-human (crossreacts with mouse) vWF (catalog AB7356), rabbit anti-mouse collagen IV (catalog AB2031), and rabbit anti-mouse active (cleaved) caspase-3 antibodies (catalog PC679) were purchased from Millipore. Alexa Fluor 488–conjugated goat anti-rabbit IgG (catalog A11094), Alexa Fluor 594–conjugated Isolectin GS-IB4, and Alexa Fluor 594–conjugated goat anti-rabbit IgG (catalog A11020) were acquired from Invitrogen. Growth factor–reduced Matrigel was obtained from BD Biosciences. Anti-phospho ERK1/2 (catalog 4370) antibodies were from Cell Signaling Technologies. Anti-CD31 (catalog Ab24960) and anti-smooth muscle actin (catalog Ab5694) antibodies were from Abcam. Thiazolyl blue tetrazolium bromide and DMSO were obtained from Sigma-Aldrich. The EGM-2 bullet kit and HUVECs were from Lonza. HPA-1a IgG was provided by Bjorn Skogen's group from University Hospital of North Norway. The angiogenesis array was purchased from R&D Systems. Flat-bottomed microtiter Costar plates for MAIPA assays were from Corning. Mouse anti-mouse mAbs against β3 were generated from β3-deficient mice immunized with BALB/c platelets and characterized using flow cytometry, Western blotting, and immunoprecipitation.

Murine model of FNAIT. Active FNAIT models were previously described (2, 37, 40, 63); briefly, $\beta3^{-/-}$ and $GPIb\alpha^{-/-}$ female mice were immunized twice weekly via tail-vein injections of 1×10^8 gel-filtered WT platelets (64–66). The immunized females were bred with WT males. The passive FNAIT model was established by postnatal intraperitoneal injection of 50 μl of antisera generated from naive and immunized $\beta3^{-/-}$ or $GPIb\alpha^{-/-}$ mice.

Platelet enumeration. Blood (10 μl) was collected from P2 neonates via carotid bleeding and immediately diluted to 1:25 in 240 μl of 1% (v/v) EDTA/PBS, pH 7.4. Platelet-rich plasma (PRP) was isolated by centrifugation at 220 *g* for 2 minutes, and 50 μl PRP was diluted 1:200 in 9.95 ml Isoton II Diluent. Platelet counts were determined with a Z2 Series Coulter Counter (Beckman Coulter). Thrombocytopenia was defined as a platelet count less than or equal to 100×10^9/l.

Detection of anti-β3 or anti-GPIbα antibodies via flow cytometry. Whole blood was collected from the immunized mice via the saphenous vein, both prior to and following platelet immunizations, and allowed to clot. Sera were obtained by centrifuging clotted blood at 9600 *g* for 5 minutes and were diluted in PBS. 10^6 WT platelets were incubated with diluted sera (1:100) for 1 hour. Samples were washed and incubated with FITC-conjugated anti-mouse IgG for 30 minutes and then analyzed on a FACSCalibur flow cytometer (BD). The value for anti-platelet IgG is expressed as mean fluorescence intensity (MFI).

High-frequency ultrasound imaging of fetuses in utero. Ultrasound imaging was performed at E.12.5, E.15.5, and E.17.5 in each mouse to determine embryo/fetus vitality and in utero ICH, as previously described (31, 67). Pregnant mice were anesthetized with 2% isoflurane (inhaled) and maintained on 1% during imaging. After abdomen shaving and skin clearing with hair removal cream (Nair), high-frequency ultrasound imaging was performed by nonlinear contrast imaging using a Vevo 2100 system and an MS550 transducer (32–56 MHz).

Histology, immunofluorescence, and TUNEL. For histological analysis, P2 mouse pup brain tissue was fixed in formalin, paraffin-embedded, sectioned (6–8 μm), transferred to glass slides, and stained with H&E using standard techniques. For immunohistochemistry staining, frozen tissue sections or ECs cultured on coverslips were fixed with paraformaldehyde (PFA) (4% w/v, 5 minutes), then incubated with primary antibody (anti-vWF and active form of caspase-3) overnight and secondary antibody (anti-rabbit IgG-Cy3 or Alexa Fluor 594–conjugated goat anti-rabbit IgG and Alexa Fluor 488–conjugated goat anti-rabbit IgG) for 1 hour, as previously described. Brain vessel density was stained with Isolectin IB4 conjugated to Alexa Fluor 594. Apoptosis in mouse brain tissue was detected via TUNEL staining with an In Situ Cell Death Detection Kit (Roche Diagnostics), according to the manufacturer's protocol. Briefly, frozen tissue sections were fixed in PFA (4%, 5 minutes, room temperature [RT]) and permeabilized. Positive controls were treated with 100 U DNase I for 30 minutes at 37°C. Slides were mounted in VECTASHIELD mounting medium containing DAPI for nuclear counterstaining. Fluorescent images were acquired using a Nikon E800 fluorescence microscope (Nikon) and analyzed by ImageJ software (http://imagej.nih.gov/ij/).

Postnatal retina vessel development. The globe of the eye was removed from P2 pups and fixed in PFA (4%, 30 minutes). The retina was extracted and stored in methanol at –20°C until staining. Retinas were incubated with anti-collagen IV in BSA/PBS overnight at 4°C, then washed, and incubated with 1:400 Alexa Fluor 488–conjugated anti-rabbit IgG or directly stained with Alexa Fluor 594–conjugated isolectin GS-IB4 (in BSA/PBS, 2 hours). Retinas were whole mounted, and images were acquired using a Nikon E800 fluorescence microscope. To measure the percentage of area covered by vessels, the pictures in red, green, and blue were split into 3 channels with ImageJ software, and only the green channel was considered for analysis. We first cleared the area covered by the optic nerve and inverted the image. The percentage of vessel area was measured in each picture accordingly with ImageJ. The mean percentage of area was calculated for each retina.

mAb immobilization of platelet antigens assay. mAb immobilization of platelet antigens (MAIPA) assay was performed as previously described (68, 69). Briefly, 96-well plates were coated with goat anti-mouse β3 antibody overnight. Human platelets or HUVECs were incubated with anti–HPA-1a IgG or negative IgG and a murine mAb against human β3 IgG (JAN D1) for 60 minutes. Opsonized platelets or HUVECs were solubilized with NP-40 lysis buffer overnight at 4°C. Complexes consisting of murine anti-human mAb, human anti–HPA-1a IgG, and platelet glycoproteins were captured on the ELISA plates. Human platelet antibodies were detected with a peroxidase-conjugated goat anti-human IgG (Beckman). Results were expressed in OD.

In vitro EC proliferation and tub-formation assays. HUVECs were cultured in endothelial basal medium plus growth supplements (EGM-2) and incubated in a humidified chamber at 37°C with 5% CO_2. Proliferation and tube-formation assays were conducted. For the proliferation assay, HUVECs were seeded into wells of a 96-well plate with 100 μl of EGM-2. After 24 hours, cells were incubated with fresh media containing naive sera, anti-β3 sera, or anti-GPIbα sera. After 12 hours, cells were incubated with thiazolyl blue tetrazolium bromide (MTT) (20 μl, 5 mg/ml, 3.5 hours). The supernatant was aspirated, and cells were lysed with DMSO for 15 minutes. Absorbance was read at 570 nm, with a reference wave length of 630 nm, on an optical plate reader, and proliferation was calculated as percentage of negative control. For the vascular-like network formation assay, wells of a 24-well plate were coated with 100 μl Matrigel. Cells were added to the wells and incubated with sera overnight. Images were captured with an inverted microscope under phase contrast. Network formation was quantified by measuring the total tube length.

Western blot and mouse angiogenesis antibody array analysis. In preparation for Western blotting, HUVECs were harvested from confluent flasks and treated with human IgG or mouse anti-sera. Cells were lysed in RIPA buffer and stored at –20°C until Western blotting. Protein (25 μg) from brain homogenate or cell lysate prepared in RIPA buffer was analyzed by Western blotting, as previously described (31). Briefly, protein samples were electrophoresed on 8% SDS polyacrylamide gels and electrotransferred onto PVDF membranes. Immunodetections were carried out with anti-mouse phospho-AKT and total AKT antibodies. For the angiogenic array, brain homogenates were subjected to immunoblotting, as recommended by the manufacturer (R&D System). Immobilized antigens were detected by chemiluminescence using horseradish peroxidase–conjugated secondary antibodies, an ECL kit (GE Healthcare), and autoradiography on Kodak film.

Statistics. Data shown are mean ± SEM. Statistical comparisons were made using an unpaired, 2-tailed Student's *t* test or 1-way ANOVA followed by Bonferroni's post-hoc test, as appropriate. Densitometry and immunofluorescence analysis was performed using ImageJ software, and values were normalized to internal control or total AKT. Differences were considered statistically significant at $P < 0.05$.

Study approval. All mice were housed in the St. Michael's Hospital Research Vivarium after the protocol was approved by the animal care committee.

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