PAD4 Deficiency Decreases Inflammation and Susceptibility to Pregnancy Loss in a Mouse Model[1]

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ABSTRACT

Inflammation is thought to play a critical role in the pathogenesis of placentation disorders such as recurrent miscarriages, growth restriction, and preeclampsia. Recently, neutrophil extracellular traps (NETs) have emerged as a potential mechanism for promoting inflammation in both infectious and noninfectious disorders. To investigate a pathogenic role for NETs in placentation disorders, we studied a model of antiangiogenic factor-mediated pregnancy loss in wild-type (WT) mice and in mice deficient in peptidylarginine deiminase 4 ($Padi4^{-/-}$) that are unable to form NETs. Overexpression of soluble fms-like tyrosine kinase 1 (sFlt-1), an antiangiogenic protein that is pathogenically linked with abnormal placentation disorders during early gestation, resulted in pregnancy loss and large accumulation of neutrophils and NETs in WT placentas. Interestingly, sFlt-1 overexpression in $Padi4^{-/-}$ mice resulted in dramatically lower inflammatory and thrombotic response, which was accompanied by significant reduction in pregnancy losses. Inhibition of NETosis may serve as a novel target in disorders of impaired placentation.

angiogenesis, inflammation, NETs, placenta, preeclampsia

INTRODUCTION

Inflammation is central to the pathogenesis of poor placentation disorders ranging from miscarriages to fetal growth restriction and preeclampsia. In particular, polymorphonuclear neutrophils (PMN) have been implicated in both reproductive health and adverse pathologies [1–3]. Although PMNs have been conventionally thought to play a key role in inducing inflammation by production of reactive oxygen species (ROS) or through release of toxic granules, it is being increasingly recognized that the inflammatory activity of PMNs is also promoted by extrusion of their DNA into the extracellular environment in the form of neutrophil extracellular traps (NETs). These strands of chromatin covered with antimicrobial peptides were first discovered as a novel defense mechanism to trap and kill pathogens [4] but also appear to be involved in a large range of noninfectious diseases [5, 6]. In particular, NETs have also been implicated in the pathogenesis of numerous vascular disorders such as thrombosis [7], atherosclerosis [8], and systemic lupus erythematosus [9] and reproductive disorders such as preeclampsia [10].

In preeclamptic women, a nonspecific, systemic (vascular) inflammatory response with an increase in neutrophil counts can be observed, as well as placental vasculopathy and neutrophil infiltration into the vasculature [11, 12]. Neutrophil extracellular traps have also been identified in preeclamptic placentas, suggesting a role in the pathogenesis of this disease [13, 14]. Preeclamptic women also have strongly increased markers of NETosis in their blood, such as an elevation of cell-free DNA [15, 16]. Interestingly, it has recently been shown that neutrophils can be activated by placental microparticles to form NETs [13]. It has also been speculated that NETs may be involved in spontaneous fetal loss induced by autoantibodies or by infectious agents, as there is strong evidence that neutrophil activation may play a crucial role in these events [10]. Moreover, NETs are implicated in antiphospholipid-associated pregnancy loss, a frequent cause of recurrent pregnancy loss [17, 18]. One may speculate that activation of neutrophils via C5a, as shown in a mouse model of antiphospholipid antibody-induced pregnancy loss, may lead to NETosis, which in turn may contribute to pregnancy loss [19, 20].

In order to determine the influence of NETosis during placentation, we used a model of overexpression of adenovirus-mediated soluble feline McDonough sarcoma-like tyrosine kinase 1 (sFlt-1) during the first trimester that leads to high percentage of spontaneous fetal losses. Overexpression of sFlt-1, a circulating antiangiogenic protein, in pregnant rodents causes symptoms in a dose-dependent manner that range from spontaneous fetal losses to a preeclampsia-like state [21]. Alterations in sFlt-1 and other angiogenic proteins such as placental growth factor have been documented in several reproductive disorders such as spontaneous miscarriages, idiopathic fetal growth restriction, and preeclampsia [22–25]. Here, we report the phenotypic consequences of sFlt-1 overexpression during early pregnancy in wild-type (WT) mice or in mice lacking the enzyme peptidylarginine deiminase

4 (PAD4, encoded by the *Padi4* gene in mice). This enzyme modifies histone charges through citrullination and thus enables chromatin decondensation, which is a crucial step in the formation of NETs [26–28]. We evaluated the rate of pregnancy loss resulting from sFlt-1 overexpression in WT or in *Padi4*$^{-/-}$ mice and histological markers of NETosis and inflammation. We also characterized NETs in placentas from preeclamptic and nonhypertensive pregnant women.

MATERIALS AND METHODS

Animals

The *Padi4*$^{-/-}$ and WT mice bred from a C57BL/6J background used in this study have been described elsewhere [26]. Female mice used for experiments were 8–10 wk old. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Boston Children's Hospital (protocol no. 14-03-2631R). Pregnant BALB/c mice (8–10 wk old) were used for additional studies under an animal protocol approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee (protocol no. 023-2014).

Model of sFlt-1 Overexpression

For timed pregnancies, female C57BL/6 WT or *Padi4*$^{-/-}$ mice in estrus were mated with experienced male breeders of the corresponding genotype for 1 night. Observation of a copulation plug the following morning was denoted gestational day (GD) 0.5. Pregnancy was confirmed by weight gain of the mice at Days 6.5 and 8.5 after mating. At GD 8.5, pregnant mice received lateral tail vein injections with 2.2×10^9 plaque-forming units (pfu) of adenovirus (cytomegalovirus) carrying sFlt-1 (Ad-sFlt-1) or empty cytomegalovirus (Ad-null) at equivalent doses. Both viruses had been generated at the Harvard Vector Core Laboratory, as described previously [21, 29, 30]. Mice were monitored for general well being and euthanized on Day 17.5 of pregnancy. Mice were scored as having undergone pregnancy loss/miscarriage if their entire litter had been lost.

Plasma and Whole-Blood Collection

Blood was collected from the retroorbital plexus of anesthetized mice (using 3.5% isoflurane) into sodium citrate anticoagulant (10% v/v). Whole blood was centrifuged at 6000 rpm for 5 min, and plasma was collected and again centrifuged at 13 200 rpm for 5 min to remove any remaining cellular components. Plasma samples were stored immediately at −80°C until analysis. Blood was also collected into EDTA-coated capillary tubes from the retroorbital sinus and was analyzed by a Hemavet 950FS (Drew Scientific) for complete blood counts.

Tissue Preparation and Analysis

Anesthetized mice were euthanized on Day 17.5 of pregnancy (GD = 17.5) by cervical dislocation. Placentas were isolated and preserved in zinc fixative (100 mM Tris-HCl containing 37 mM zinc chloride, 23 mM zinc acetate, and 3.2 mM calcium acetate) for paraffin sections or embedded in optimum cutting temperature (OCT) medium and snap-frozen in cryomolds. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E). For the quantification of necrotic areas within the decidua, placentas were cut into two equal pieces (mid-sagittal section) and serially sectioned at 4 μm. At 50-μm intervals, sections were evaluated, and images were taken of four placentas per sample at 25× magnification, and necrotic areas were delineated and measured using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland; http://imagej.nih.gov/ij). Frozen sections were fixed in zinc fixative overnight and then stained with primary antibodies against Ly6G (catalog no. 551459; Abcam) as a neutrophil marker or for citrullinated histone 3 (H3cit; catalog no. ab5103; Abcam) as a marker for NETs (BD Biosciences) at 4°C overnight. Sections were then incubated with antirat Alexa-555 and antirabbit Alexa-488 (both Invitrogen) as secondary antibodies. Sections were counterstained with Hoechst-33342 dye to visualize all nuclei, then mounted with Fluoro-gel (Electron Microscopy Sciences), and observed using epifluorescence microscopy (Axiovert; Zeiss). Confocal images were acquired using confocal microscopy (IX 81 FV1000 LSM unit using UPLAPO 20×/0.70 or UPLAPO 60×/1.20 water objective lens and Fluoview version 3.1.2.2 software; Olympus).

Mosaic images of placentas were generated using the ImageJ MosaicJ and TurboReg software (ImageJ). For quantification of placenta necrosis, necrotic

areas were quantified by ImageJ in 4 placentas per mouse and 3 sections per placenta. For quantification of Ly6G- and H3cit-positive areas, the size of the decidua basalis was determined in the blue color channel (showing all cell nuclei stained with Hoechst dye), Ly6G-positive areas were then measured in the red color channel and H3cit-positive areas in the green channel, and percentage of total decidual area was calculated for both markers.

Paraffin-embedded mouse placental tissue was also stained with an antibody against myeloperoxidase produced by neutrophils (MPO; catalog no. AF3667; R&D Systems) at room temperature for 40 min. Sections were then incubated with horseradish peroxidase (ImmPRESS) antigoat immunoglobulin G (IgG; peroxidase) polymer, as secondary antibody. Sections were counterstained with hematoxylin to visualize all nuclei, mounted (Permount; Electron Microscopy Sciences), and observed using brightfield microscopy (Olympus).

Human placental tissues (from 5 preeclamptic and 5 gestationally age-matched control nonhypertensive pregnancies), collected under an institutional review board-approved protocol at the Beth Israel Deaconess Medical Center, were used to characterize NETs. Preeclampsia phenotypes were confirmed as described previously [31]. Placental tissue collected in 10% neutral buffered formalin was used for histology and immunohistochemistry studies. Consecutive sections were stained for CD11b (catalog no. 52478; Abcam) as a marker present on neutrophils or for H3cit as a marker for NETs using a standard immunohistochemistry protocol, as described [32]. Slides were then rinsed in phosphate-buffered saline and developed according to the manufacturer's directions (Vectastain Impress Elite ABC kit; Vector Laboratory). Slides were counterstained with H&E, dehydrated, and mounted.

ELISAs

Plasma sFlt-1 levels were measured using mouse sVEGF R1/Flt-1 Quantikine ELISA kit (R&D Systems) in mouse plasma on GD 17.5 [32]. Plasma thrombin-antithrombin (TAT) complexes were quantified using ELISA with the TAT mouse ELISA kit (Abcam).

Statistical Analysis

Data are mean ± SEM if parametric tests were used and amedian ± interquartile range if nonparametric tests were used. For comparisons between two groups, either a two-sided Student *t*-test or Mann-Whitney *U*-test was used, depending on whether data were normally distributed. For comparison of more than two groups, one-way ANOVA test with Bonferroni adjustment was used. For the correlation between plasma DNA and necrotic areas, nonparametric Spearman correlation and linear regression analysis were performed. The numbers of miscarriages versus intact pregnancies were compared using the chi-squared test. All *P* values below 0.05 were considered significant. For all statistical analyses, Prism version 6.0d software (GraphPad) was used.

RESULTS

sFlt-1 Overexpression Induces Neutrophil Invasion and Formation of NETs in the Decidua Basalis of WT Mice

Overexpression of sFlt-1 in rodents induces endothelial damage with variable phenotypes ranging from pregnancy loss to new-onset hypertension and proteinuria, depending on the dose and timing of injection [21]. In our model of sFlt-1–induced pregnancy loss, pregnant WT mice were injected with either Ad-sFlt-1 or corresponding amounts of Ad-null as control on GD 8.5 of pregnancy and euthanized on Day 17.5. A large percentage of the mice subjected to sFlt-1 overexpression lost their litters in the course of the experiment. The placentas of those mice that retained the pregnancy were examined for damage secondary to sFlt-1 overexpression. The WT mice overexpressing sFlt-1 showed severe vasculopathy with necrotic areas within the lower decidua basalis of the placentas, whereas little or no necrosis was evident in the WT Ad-null mice (Fig. 1A). A similar phenotype was seen in pregnant BALB/c mice, suggesting that the sFlt-1 effects are not limited to C57BL/6J mouse strain (Supplemental Fig. S1; available online at www.biolreprod.org). In addition, the necrotic areas of the WT placentas showed a massive invasion of leukocytes. To identify the invading cells and to address the question of whether NETs were to be found within the necrotic areas,
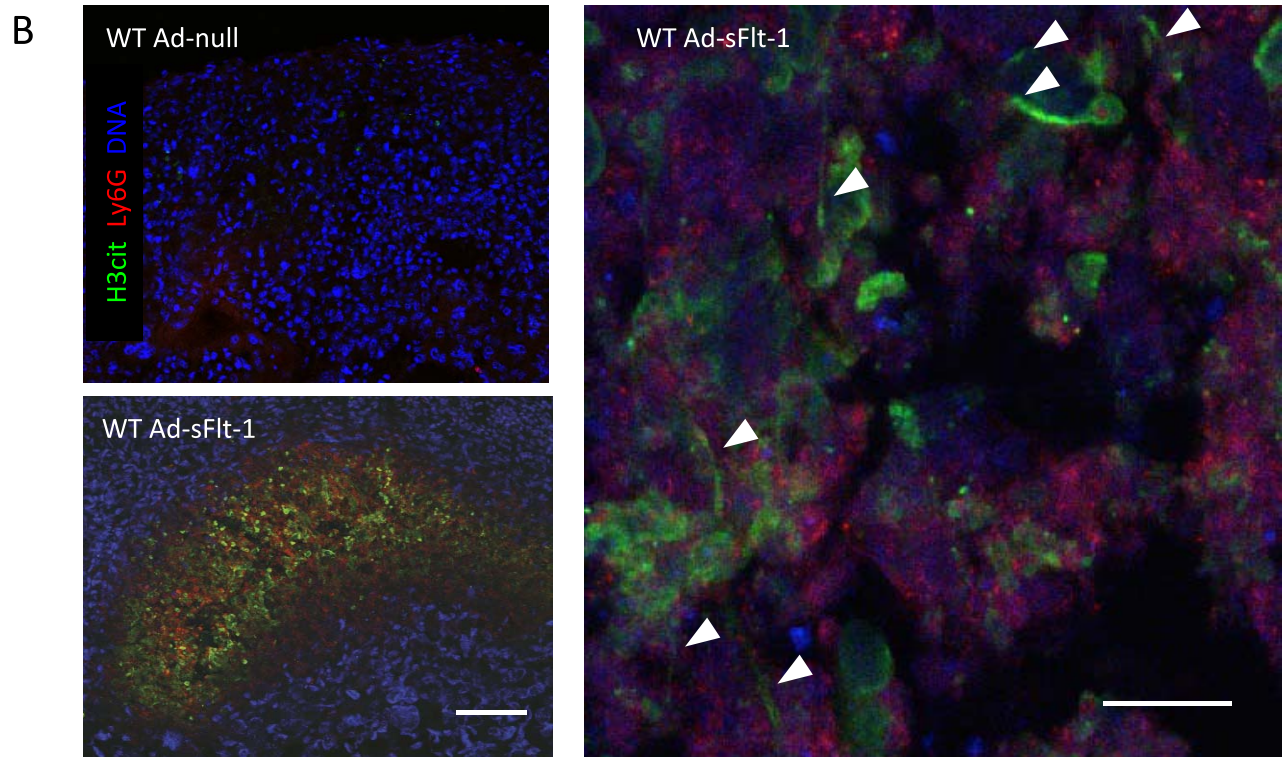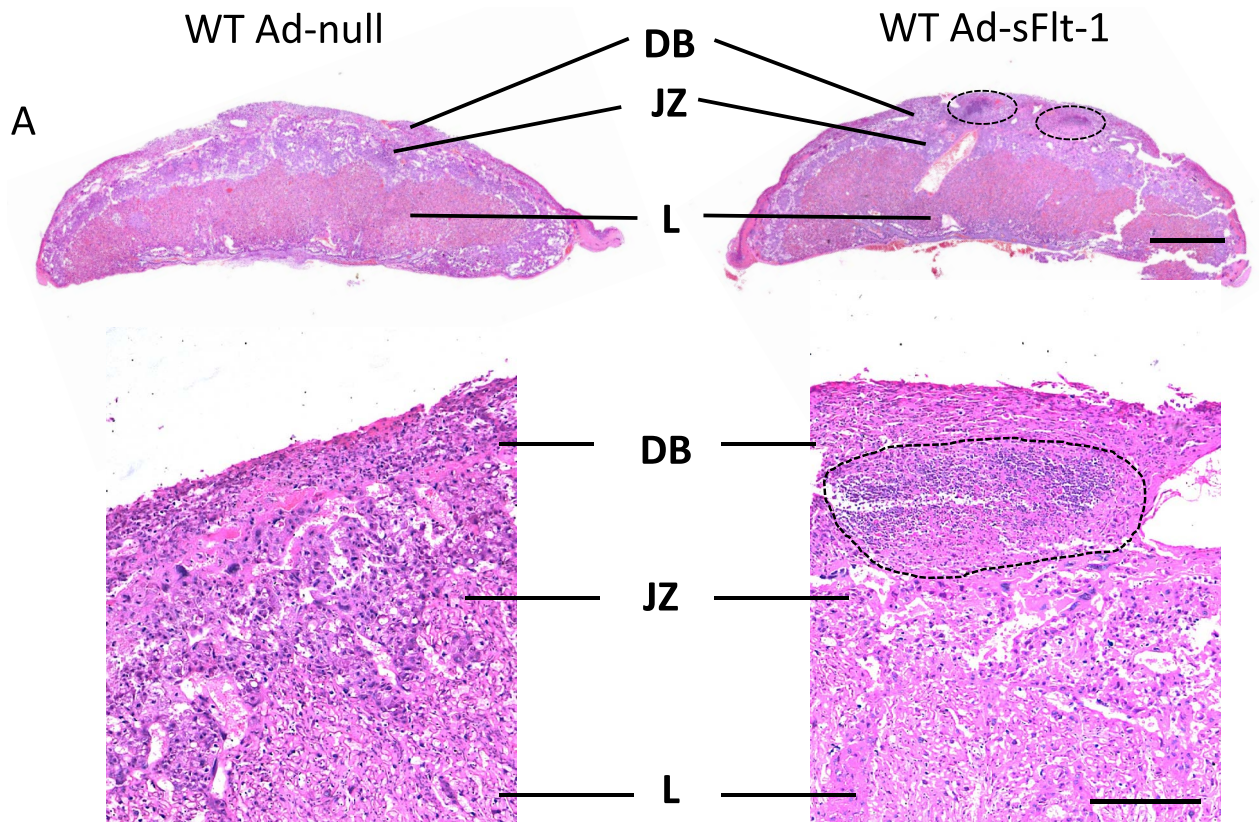
FIG. 1. sFlt-1 overexpression induces necrosis in the decidua basalis with neutrophil and NETs accumulation. Placentas from pregnant WT mice that had received either Ad-sFlt-1 or Ad-null virus at Day 8.5 of pregnancy were harvested at Day 17.5 of pregnancy. **A**) Placentas were stained with H&E; the major placental zones (DB, decidua basalis; JZ, junctional zone; L, labyrinth) are indicated. Large necrotic areas were visible in the WT Ad-sFlt-1 mice but not the Ad-null controls. Necrotic areas are circled by black dashed lines. The upper row shows a mosaic of the entire placentas. Bar = 1 mm. Bar in the second row = 100 µm. **B**) Immunofluorescence staining for Ly6G as a neutrophil marker (red) and H3cit (green) to indicate NETs showed accumulations of both in the necrotic areas in WT placentas treated with Ad-sFlt-1 but not Ad-null virus in confocal images. Bars = 20 µm (lower power), 100 µm (higher power). Arrows = NETs.

immunofluorescence staining for the specific neutrophil marker Ly6G and for citrullinated histone 3 (H3cit) as a marker for NETs was performed. Confocal images of the decidua basalis of WT sFlt-1 mice revealed that the necrotic areas contained large accumulations of neutrophils. In addition, these areas were strongly positive for H3cit, indicating ongoing NETosis (Fig. 1B). At higher magnification (Fig. 1B, right), it was possible to identify not only H3cit-positive neutrophils but also single H3cit-positive strands characteristic for NETs within tissue, corroborating the observation of the presence of NETs in these necrotic areas. In contrast, no neutrophil accumulations could be found in the WT Ad-null control mice. Additional stains with MPO supported the presence of NETs in the decidua basalis of animals overexpressing sFlt-1 (Fig. 2) [33, 34].

Neutrophil Invasion Is Reduced in the Placentas of Padi4$^{-/-}$ Mice Compared with WT Mice

We previously demonstrated that, in a model of myocardial ischemia/reperfusion injury, $Padi4^{-/-}$ mice have reduced neutrophil infiltration and are protected from extensive tissue injury [6]. Therefore, we wanted to address the question of whether the placentas of $Padi4^{-/-}$ mice that could not form NETs would likewise be protected from neutrophil infiltration and whether the necrotic area could be reduced by PAD4 deficiency. To this end, the placentas of WT and $Padi4^{-/-}$ mice with intact pregnancies by Day 17.5, previously injected either with Ad-sFlt-1 or Ad-null virus, were compared. Plasma levels of sFlt-1 were not different between the two groups (2135.6 ± 389.9 ng/ml for the WT Ad-sFlt-1 mice and 2567.8 ± 438.4 ng/ml for $Padi4^{-/-}$ Ad-sFlt-1 mice). Areas of neutrophil infiltration as well as H3cit-positive areas, as an indicator of NET formation, were quantified using ImageJ (Fig. 3B). As expected, H3cit staining was virtually absent in the $Padi4^{-/-}$ mice, as such citrullination does not occur in these animals (Fig. 3, A and B) [26]. Interestingly, neutrophil invasion of the placenta tissue was also much reduced in the $Padi4^{-/-}$ mice (Fig. 3C). No neutrophil accumulation was visible at low magnification of the placentas, whereas higher magnifications showed low numbers of neutrophils in the decidua basalis, which were not H3cit-positive (Fig. 3A). Quantification of Ly6G-positive areas of the decidua basalis corroborated the fact that, although neutrophils were not entirely absent, they were rare within $Padi4^{-/-}$ placentas (Fig. 3C). Similar to what we showed in ischemia reperfusion injury [35], PAD4 deficiency appears to limit the influx of inflammatory cells into tissue.

PAD4 Deficiency Reduces Necrosis in the Decidua Basalis and Limits Systemic Inflammation

A strong inflammatory response within the placenta together with the formation of large amounts of NETs would be expected to be injurious to the surrounding tissue, as NETs are strongly cytotoxic [36]. Therefore, we quantified the overall necrotic area within the decidua basalis of WT and $Padi4^{-/-}$ mice that had received Ad-sFlt-1 or Ad-null and had retained their pregnancies until Day 17.5 of pregnancy. The necrotic area increased significantly after sFlt-1 treatment in both WT and $Padi4^{-/-}$ mice compared with Ad-null injected mice that showed only very small necrotic areas within the decidua basalis (Fig. 4, A and B). However, this increase was higher in the WT Ad-sFlt-1 mice than it was in the sFlt-1-overexpressing $Padi4^{-/-}$ mice. This led us to hypothesize that sFlt-1 causes a certain amount of tissue damage independently of PAD4 but

that secondary damage and further tissue necrosis are limited by an inhibition of NETosis.

This hypothesis is further supported by the peripheral neutrophil counts of the sFlt-1-overexpressing mice (Fig. 4C). Injection of Ad-sFlt-1 caused an increase in peripheral neutrophil counts in both WT and $Padi4^{-/-}$ mice compared with mice that received Ad-null virus. This is in line with the nonspecific, systemic inflammatory response seen in pre-eclamptic women which results largely from vascular injury [37]. Yet again, similar to the local inflammatory response, in $Padi4^{-/-}$ Ad-sFlt-1 mice systemic inflammation was more contained compared to WT mice (Fig. 4C).

Additionally, double-stranded plasma DNA was measured in WT and $Padi4^{-/-}$ mice (Fig. 4D). An increase of cell-free double-stranded DNA is often used as a surrogate marker for NETosis, as the extrusion of chromatin by neutrophils in vessels leads to an elevation of plasma DNA. However, an increase of DNA is not specific for NETosis and can also be a sign of other forms of cell death such as necrosis.

The mice of each genotype were divided into those mice that had retained their pregnancy and those that had suffered miscarriage. In the Ad-null group, all pregnancies had remained intact until Day 17.5. The pregnant Ad-sFlt-1-treated WT mice showed the highest plasma DNA levels, followed by the WT Ad-sFlt-1 mice that had lost their pregnancies. Pregnant WT Ad-null mice had the lowest DNA levels (Fig. 4D).

Interestingly, plasma DNA levels in $Padi4^{-/-}$ Ad-sFlt-1 mice were significantly lower than in the corresponding WT groups, respectively (Fig. 4D). The $Padi4^{-/-}$ mice that had been treated with Ad-sFlt-1 or Ad-null virus showed a tendency toward a distribution of plasma DNA values similar to that in WT mice in the respective groups, yet differences between pregnant and nonpregnant Ad-sFlt-1 mice or Ad-null mice were not significant in the $Padi4^{-/-}$ mice.

Together with the previously shown absence of NETs, reduced inflammatory response and less tissue damage in the $Padi4^{-/-}$ mice, the lower DNA levels in the $Padi4^{-/-}$ mice were thus likely a result of a combination of less NETosis and necrosis.

To test whether a correlation could be established between plasma DNA and necrotic area, a correlation analysis for the WT and the $Padi4^{-/-}$ mice treated with Ad-sFlt-1 or Ad-null, was performed (Fig. 4E). In the WT mice, there was a clear correlation between plasma DNA and necrosis with a positive Spearman coefficient of r = 0.9 (P = 0.002). In the $Padi4^{-/-}$ mice, no correlation could be established between plasma DNA and necrotic area (r = 0.191, P = 0.58).

As NETs are known to be strongly pro-coagulant, we were interested in whether WT Ad-sFlt-1 mice would show increased thrombin generation compared to the $Padi4^{-/-}$ Ad-sFlt-1 group (Fig. 4F). The majority of mice in all groups had comparable values of TAT complexes. However, in the WT Ad-sFlt-1 group, 4 (of 13) mice showed a dramatic increase in TAT complexes, indicating a thrombotic reaction and leading to a significant increase of TAT complexes in the WT Ad-sFlt-1 group compared to that in the $Padi4^{-/-}$ Ad-sFlt-1 mice (P = 0.048).

Padi4$^{-/-}$ Mice Are Less Susceptible to sFlt-1–Induced Miscarriage

In our model of sFlt-1 overexpression, the incidence of miscarriage in either WT or $Padi4^{-/-}$ mice injected with Ad-sFlt-1 or Ad-null was quantified (Fig. 5A). Wild-type Ad-sFlt-1 mice had miscarriages in 62% of all pregnancies in contrast
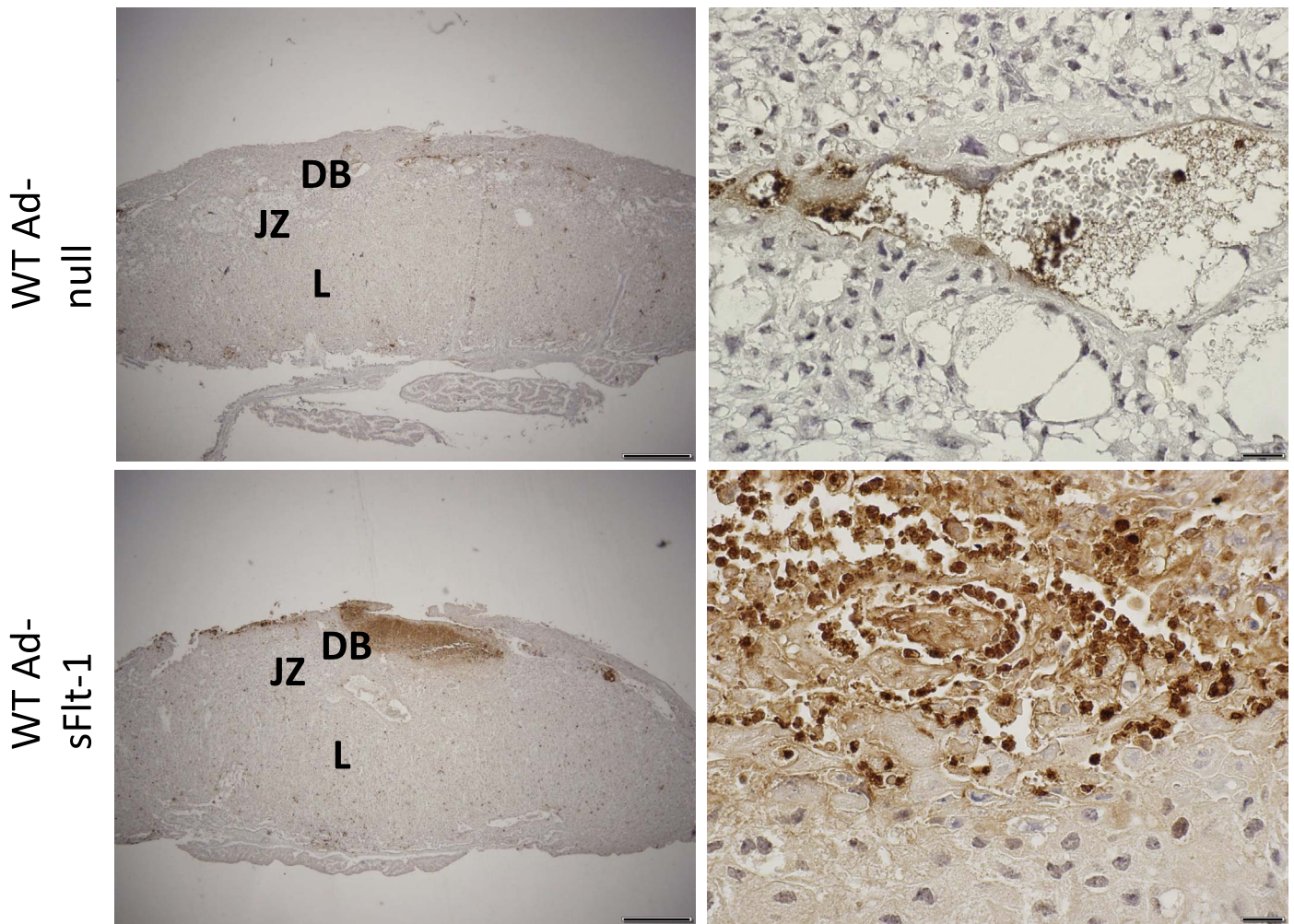
FIG. 2. Presence of neutrophils was verified by staining against MPO. Immunohistochemical staining against MPO (brown) was performed to verify the presence of neutrophils and NETs in placentas of sFlt-1-treated WT mice (second row). While WT sFlt-1 placentas show large MPO-positive areas, placentas from control Ad-null mice (first row) show little or no positivity for MPO. DB, decidua basalis; JZ, junctional zone; L, labyrinth. Scale bars = 500 μm (left column) and 20 μm (right column), respectively.

to $Padi4^{-/-}$ mice, which had miscarriages in 43% of all pregnancies. The mice injected with Ad-null did not have miscarriages in either genotype. Using an additional approach to determine susceptibility to miscarriage resulting from sFlt-1 overexpression, we plotted the cumulative percentage of mice that had lost their pregnancies at a particular sFlt-1 concentration in the x-axis (Fig. 5B). The steeper slope of the curve for the WT mice showed that these mice were more prone to pregnancy loss than $Padi4^{-/-}$ mice at comparable sFlt-1 concentrations. For both genotypes, there was a concentration of sFlt-1 above which the mice lost all their litters: For the WT mice, this "threshold" was reached at concentrations >1000 ng/ml in contrast to $Padi4^{-/-}$ mice, in which sFlt-1 concentrations >3000 ng/ml always resulted in miscarriage.

Placentas from Preeclamptic Women Show Increased Neutrophil Invasion and Formation of NETs in the Decidua Compared to Nonhypertensive Controls

Because the finding of large amounts of NETs in the placentas of Ad-sFlt-1–treated mice was striking, we sought to evaluate the importance of this finding in humans with preeclampsia who are often characterized by aberrant expression of sFlt-1 [38]. We therefore examined placentas from 5 women with preeclampsia and performed immunohistochemical staining for H3cit as well as for neutrophils in the decidua basalis and villous tissue (Fig. 6). Prior studies had reported that NETs could be found in the intervillous space of preeclamptic placentas [14], but the decidua basalis had not been analyzed for NETs. We noted large accumulations of NET-ting neutrophils in the decidua of the preeclamptic placentas, very similar to those lesions seen in the WT Ad-sFlt-1 mice. In addition, we also noted H3cit/NETs or in the intervillous tissue similar to prior studies. We evaluated nonhypertensive control pregnancy placentas and found very low levels of neutrophil accumulation without signs of H3cit/NETs. Thus, it is likely that the proinflammatory and procoagulant effects of NETs resulting from sFlt-1 elevation also contribute to preeclampsia in humans.

DISCUSSION

In this study, we report an important role for NETs in a mouse model of pregnancy loss resulting from overexpression of sFlt-1, an endogenous antiangiogenic protein that has been implicated in human placentation disorders [39]. Recent publications suggest an involvement of neutrophils and NETs in human placentation disorders such as preeclampsia and
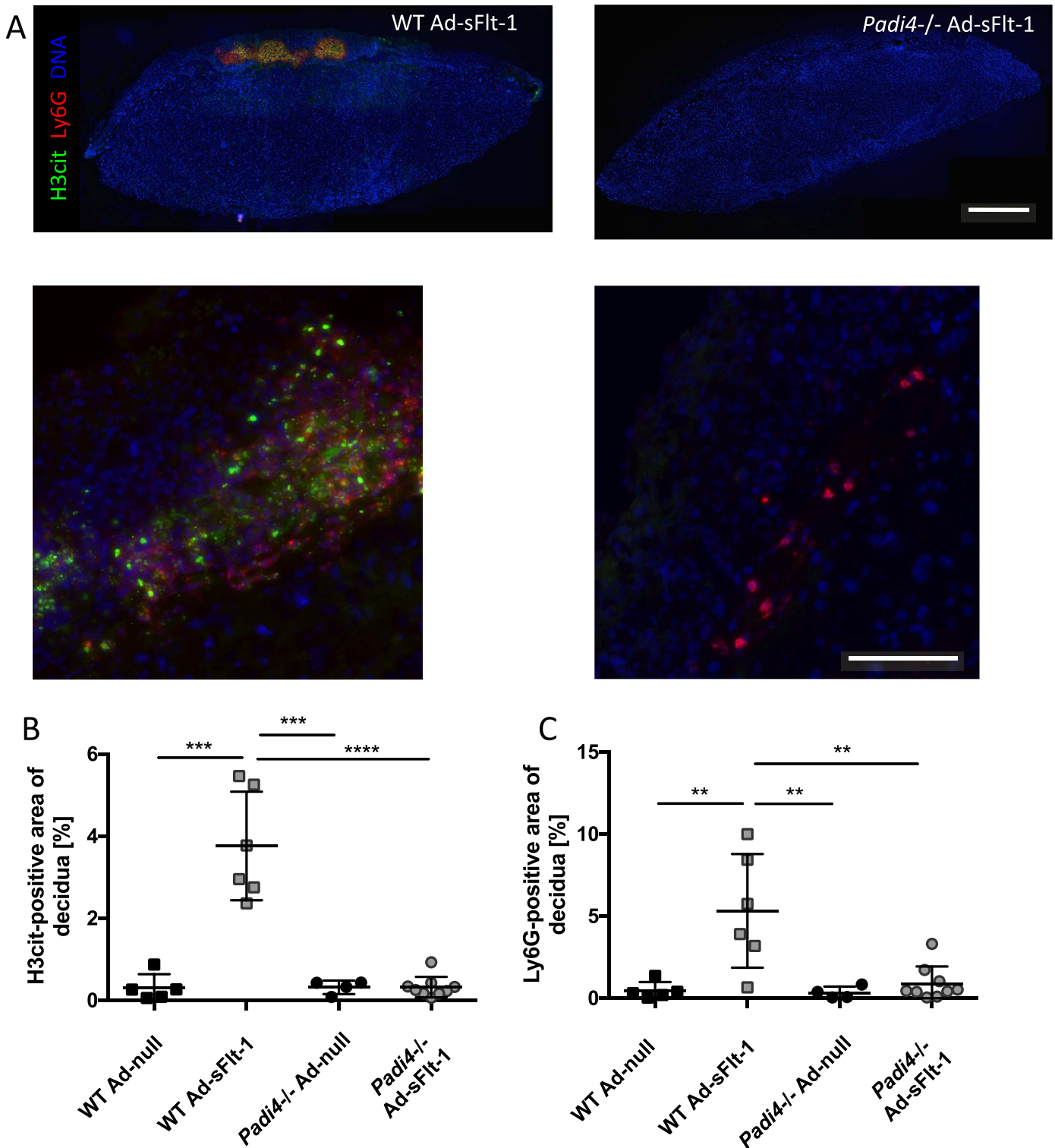
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FIG. 3. Neutrophil invasion and NETosis are reduced in the placentas of Ad-sFlt-1-treated $Padi4^{-/-}$ mice compared to those in WT mice. **A**) Placentas of either WT mice or $Padi4^{-/-}$ mice that had been injected with Ad-sFlt-1 virus at Day 8.5 of pregnancy were harvested at Day 17.5 of pregnancy, and immunofluorescence staining for the neutrophil marker Ly6G (red), H3cit (green), and DNA (blue) was performed. While WT mice showed large accumulations of neutrophils and NETs in the decidua basalis of the placentas, $Padi4^{-/-}$ mice showed no NETs and were largely protected from neutrophil invasion. Bar upper row = 1 mm; lower row = 100 μm. **B**) Immunofluorescent images were used to quantify the H3cit-positive and Ly6G-positive areas of the deciduas of WT and $Padi4^{-/-}$ mice that had been injected with Ad-sFlt-1 or Ad-null virus. Only WT mice injected with Ad-sFlt-1 showed a significant neutrophil infiltration and NETosis (n = 4-9). $**P < 0.01$; $***P < 0.001$.
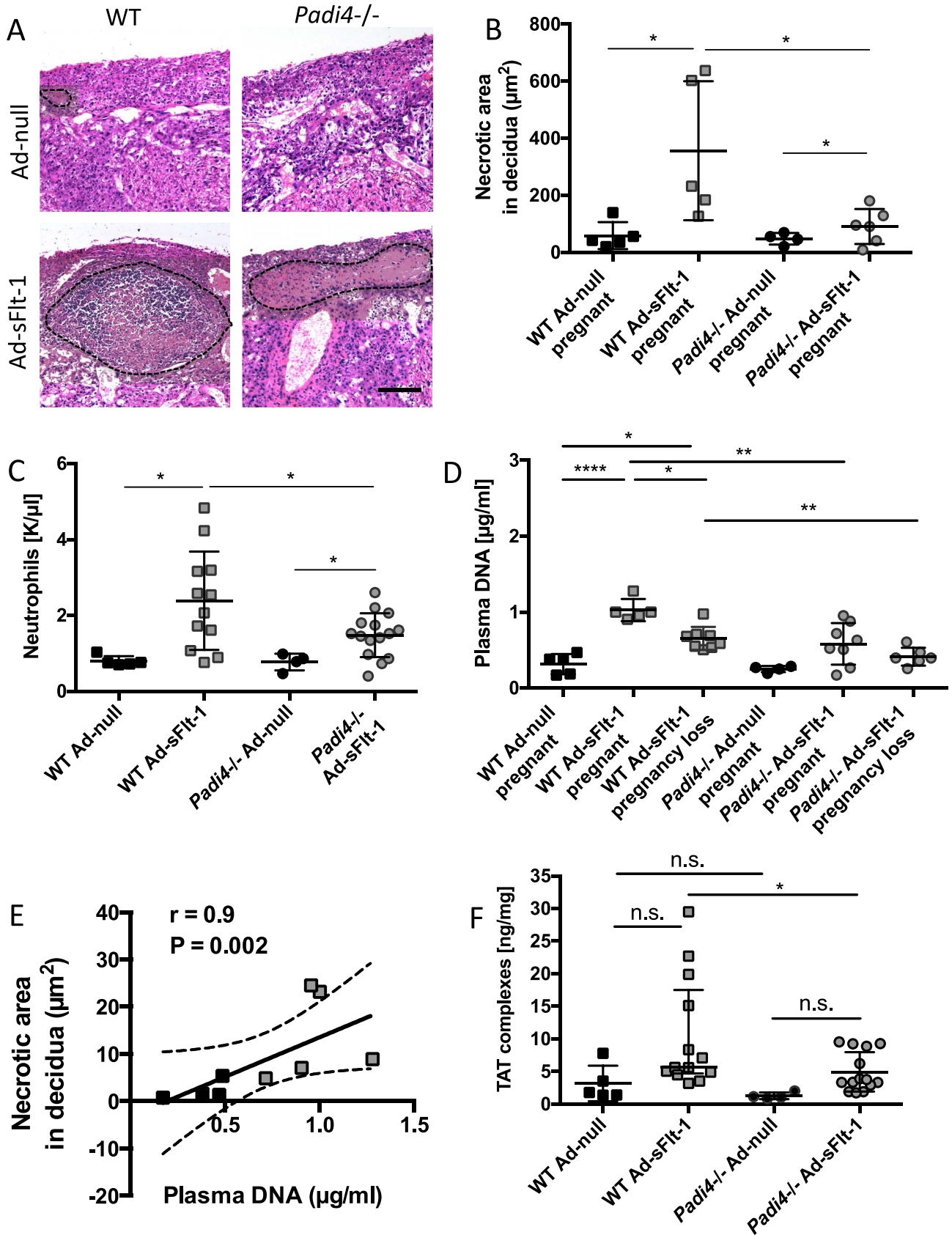
FIG. 4. PAD4 deficiency limits placental necrosis and ameliorates systemic inflammation and thrombosis. **A**) Placentas of either WT mice or $Padi4^{-/-}$ that had been injected with Ad-sFlt-1 or Ad-null virus at Day 8.5 of pregnancy were harvested at Day 17.5 of pregnancy. Bar = 100 μm. **B**) Necrotic areas per placenta were quantified using H&E-stained micrographs. Mice injected with sFlt-1 showed placental necrosis compared to Ad-null mice in either WT or $Padi4^{-/-}$ mice. However, necrotic areas were significantly larger in WT mice than in $Padi4^{-/-}$ Ad-sFlt-1 mice. n = 4 to 6. **C**) Injections of Ad-sFlt-1 induced systemic neutrophilia in WT and $Padi4^{-/-}$ mice compared with mice treated with Ad-null virus. Pregnant mice were injected at Day 8.5 of
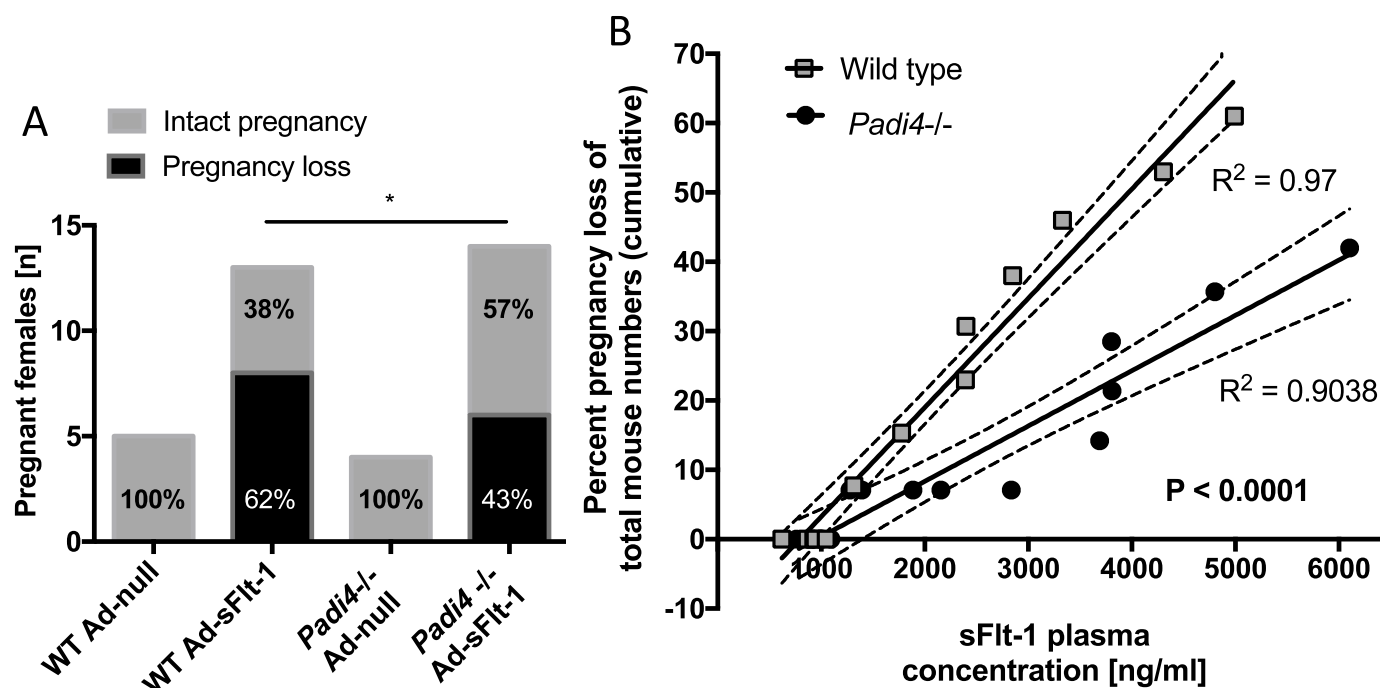
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FIG. 5. PAD4 deficiency ameliorates sFlt-1–induced pregnancy loss. **A**) Pregnant WT or *Padi4*$^{-/-}$ mice were injected with either Ad-sFlt-1 virus or Ad-null virus on Day 8.5 of pregnancy, and the rate of pregnancy loss was recorded for all three groups. None of the mice injected with Ad-null virus had abortion of the pregnancy, whereas in the WT mice injected with Ad-sFlt-1 virus, more than half of the mice lost their litters. *Padi4*$^{-/-}$ Ad-sFlt-1 mice were partially protected from pregnancy loss. **B**) The cumulative percentage of mice that had sFlt-1–induced abortion is displayed as a function of the sFlt-1 plasma concentration. The curve of the WT mice is significantly steeper than that of the *Padi4*$^{-/-}$ mice, indicating a higher susceptibility to sFlt-1-induced pregnancy loss even at lower plasma concentrations of sFlt-1. *$P < 0.05$

spontaneous pregnancy losses [10, 14, 40]. Increased neutrophil activation, including a stimulation of neutrophil-endothelial cell interactions, as well as infiltration of the systemic vasculature of patients, has long been known to be a pathophysiological feature of preeclampsia [12, 41]. The key finding of this paper is that NETs also play a role in pregnancy loss in so far as *Padi4*$^{-/-}$ mice are partially protected from it. Taken together, our data suggest that inhibiting NETosis may be a novel strategy to prevent or treat placentation disorders.

Our findings have many implications for our understanding of the pathogenesis of preeclampsia. Placentally derived inflammatory factors such as syncytiotrophoblast microparticles (STBM) and IL-8 can stimulate the production of NETs in vitro and women suffering from preeclampsia have elevated levels of cell-free DNA in their plasma, which may be an indicator of increased NETosis in these women [15]. Furthermore, placental factors can activate neutrophils to generate more ROS both in normal pregnancies and in preeclampsia. Reactive oxygen species [42], in turn, can induce NETosis [26, 43, 44]. It has been proposed that the reduction in placental perfusion during preeclampsia, originating from inadequate invasion and modification of maternal spiral arterioles, may result in ischemia-reperfusion injuries

within the placenta [40], which would increase oxidative stress and increase NETosis [6]. The aim of our study was to determine the role of NETs in a model of sFlt-1 overexpression during pregnancy. To that end, placental histology and miscarriages were studied in a mouse model of sFlt-1 overexpression in WT mice or in *Padi4*$^{-/-}$ mice, the latter being unable to form NETs as the enzyme PAD4 has been shown to be crucial for NETs formation [26]. However, a major limitation of our study is our inability to characterize preeclampsia phenotypes such as hypertension or fetal growth restriction in this model as the C57BL/6 strain was very sensitive to sFlt-1 overexpression, with pregnancy losses occurring at a high percentage. Future studies using lower doses of sFlt-1 at a later time point in pregnancy may be needed to evaluate phenotypes such as fetal growth restriction and hypertension. Alternatively, we could study the role of NETs in other mouse strains that are less sensitive to the toxic effects of sFlt-1, which would, however, require backcrossing the PAD4 deficiency to a different mouse strain.

Padi4$^{-/-}$ mice not only showed a lack of NETs but also significantly less neutrophil invasion with reduced necrotic areas within the decidua basalis compared with WT Ad-sFlt-1 mice (Figs. 3 and 4, A and B). Under baseline conditions,

pregnancy. Peripheral blood counts were measured 8 days later in mice that had retained their pregnancies and those that had experienced pregnancy loss. Neutrophilia was significantly more pronounced in WT mice than in *Padi4*$^{-/-}$ mice (n = 4–15). **D**) Plasma DNA levels were strongly elevated in pregnant mice injected with Ad-sFlt-1 and moderately elevated in WT mice. In *Padi4*$^{-/-}$ mice, Ad-sFlt-1 injections did not lead to a significant elevation of plasma DNA. n = 4 to 8. **E**) Analysis of correlation between necrotic area within the placenta and plasma DNA levels was performed for WT placentas that had received either Ad-null (black squares) virus or Ad-sFlt-1 virus (light gray squares). The positive correlation between both parameters suggests a possible influence of NETs on the extent of placental necrosis. n = 9. **F**) TAT complexes, indicative of ongoing thrombin generation, were determined in WT and *Padi4*$^{-/-}$ mice injected with Ad-sFlt-1 or Ad-null virus. Only WT showed elevated TAT levels in a number of mice; *Padi4*$^{-/-}$ mice were protected from preeclampsia-related thrombosis. n = 4 to 14. *$P < 0.05$; **$P < 0.01$, ****$P < 0.0001$. n.s., not significant.
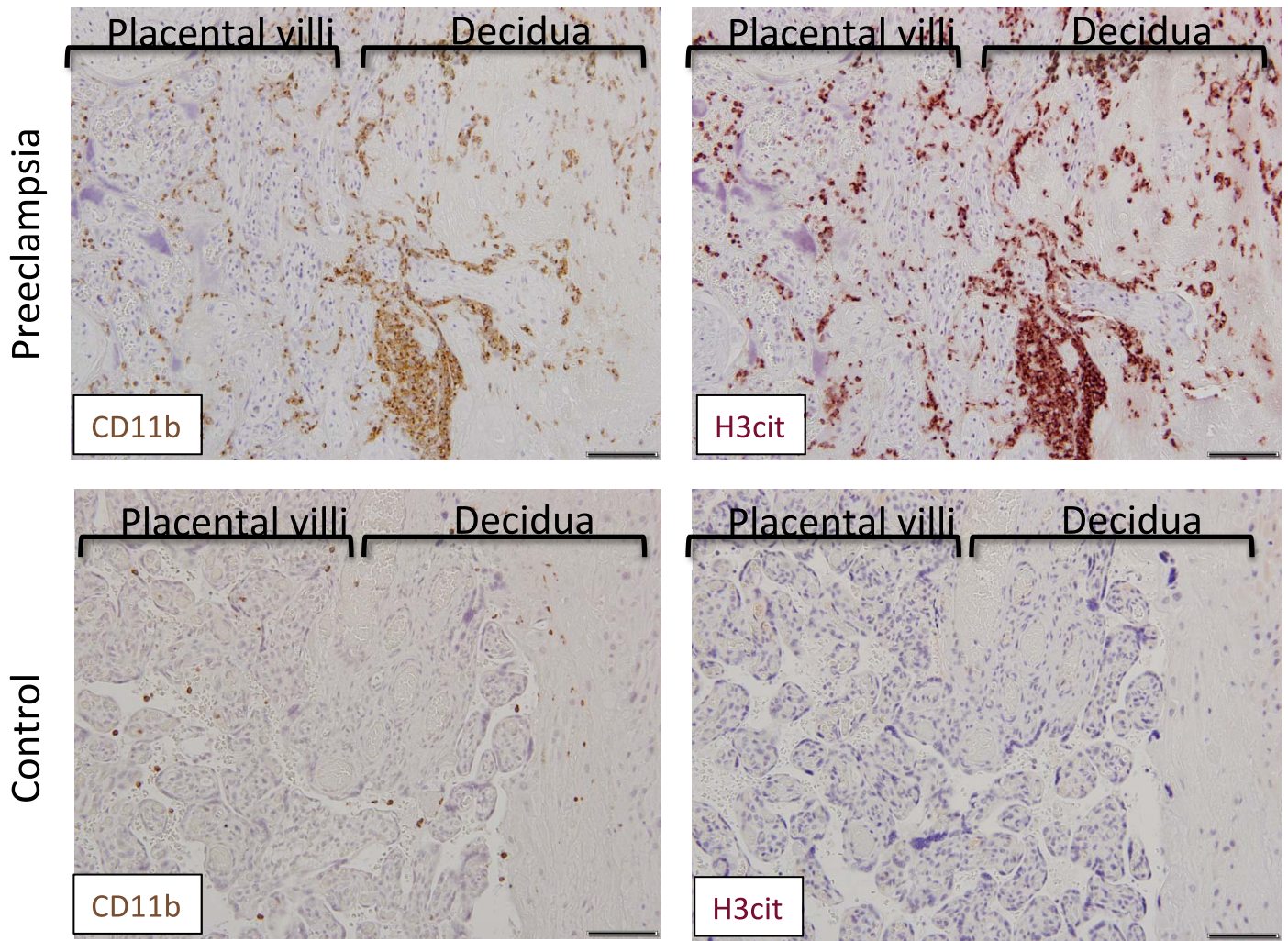
FIG. 6. Human placentas from preeclamptic women show neutrophil infiltration and NETs accumulation in the decidua. Immunohistochemistry of representative consecutive sections of human preeclamptic placentas shows CD11b-positive neutrophils (brown staining) and H3cit-positive areas (indicating NETs; red staining) in the same pattern in the decidua and to a lesser extent in the villous tissue (first row). Nonhypertensive pregnancy placentas (Control, second row) have occasional neutrophil infiltration, but no NETs were observed. Bar = 100 μm.

$Padi4^{-/-}$ mice have numbers of circulating neutrophils similar to those in WT mice [26], and leukocyte rolling along activated endothelium is not impaired in $Padi4^{-/-}$ mice [45]. In addition, $Padi4^{-/-}$ neutrophils are capable of producing ROS, have no deficiency in degranulation and are recruited normally in a model of thioglycolate-induced peritonitis [46]. Therefore, reduced neutrophil infiltration in the placentas of the $Padi4^{-/-}$ mice is unlikely to derive from a defect in neutrophil migration and invasion, although an involvement of other PAD4-regulated epigenetic gene regulations cannot be fully excluded [47]. Extracellular chromatin and histones are strongly cytotoxic to endothelium and at very high concentrations are lethal in mice [36]. They also impair the clearance of apoptotic neutrophils by macrophages, promote platelet aggregation [48], may cause the release of proinflammatory cytokines [49], and could increase and maintain an ongoing inflammatory response [50]. Thus, while sFlt-1 overexpression induced necrosis in the decidua basalis of both WT and $Padi4^{-/-}$ mice, generation of extracellular chromatin during NETosis may have worsened tissue injury secondary to sFlt-1 overexpression and triggered the recruitment of additional neutrophils in this model, similar to our observations in a model of myocardial infarction [6].

WT Ad-sFlt-1 mice also showed significantly higher levels of plasma DNA after sFlt-1 overexpression than the corresponding $Padi4^{-/-}$ groups. The DNA levels of pregnant WT mice treated with Ad-sFlt-1 were higher than those of WT mice that had lost their litters, which was most likely due to the fact that NETs were primarily released from the placentas. Therefore, the loss of the source of extracellular DNA/NETs successively led to the reduction of extracellular DNA levels in the circulation of those mice that lost their litters [51].

It is difficult to determine the origin of the elevated levels of plasma DNA, as these could stem from ongoing NETosis as well as from necrotic placental tissue or even other sFlt-1-induced endothelial damage within the vasculature. The fact that $Padi4^{-/-}$ mice also showed an increase in plasma DNA despite their inability to form NETs, indicates that the DNA must, in part, have come from other sources than neutrophils undergoing NETosis. However, in the WT mice, there was a strong correlation between plasma DNA and necrotic area while in the $Padi4^{-/-}$ mice, which also show placental necrosis, no such correlation could be found (Fig. 4E). This finding suggests that above a certain DNA level, NETosis and not the necrotic area of the placenta is the principal determinant of plasma DNA concentration and that the level of NETosis in

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turn influences the amount of tissue damage. Additionally, the WT Ad-sFlt-1 mice also showed more systemic neutrophilia than $Padi4^{-/-}$ Ad-sFlt-1 mice as well as increased coagulation in several mice (Fig. 4, C and F). This indicates that extra-placental inflammatory effects of sFlt-1 overexpression, most likely mediated through sFlt-1–induced endothelial damage and thrombosis, are also exacerbated through the production of NETs.

Inhibition of NETosis may not eliminate the underlying causes of abnormal placentation such as the disequilibrium between angiogenic and antiangiogenic factors or the failure of the physiological conversion of the maternal spiral arteries. Indeed, PAD4 deficiency did not provide complete protection from pregnancy loss after Ad-sFlt-1 challenge but reduced its incidence by 19%, which indicates that other factors may contribute to pregnancy outcome in this model. However, inhibition of NETosis appears to prevent the exacerbation of the disease caused by the cytotoxic, pro-inflammatory and pro-thrombotic effects of NETs. Therefore, targeting NETs in preeclampsia and related disorders such as fetal growth restriction may be a worthwhile therapeutic approach. Interestingly, while NETs were originally described as an immune defense mechanism, they appear to be less critical for immunoprotection than originally anticipated. In a murine model of polymicrobial sepsis PAD4 deficiency did not affect bacteremia but ameliorated endotoxemic shock [46] and patients suffering from Papillon-Lefèvre syndrome, which includes the inability to form NETs, are prone to severe juvenile periodontal disease but are otherwise healthy [52]. Given that preeclampsia is a noninfectious inflammatory condition, it appears safe to assume that PAD4 inhibition would not lead to a strong risk of infection in these patients. Analysis of human preeclamptic placentas showed neutrophil accumulations and NETs in both the decidua, similarly to our observations in mice, and in the intervillous space, as had been previously reported [14]. Therefore, PAD4 inhibition or NET dissolution may be a new therapeutic target in women suffering from preeclampsia or related placentation disorders.

Selective PAD4 inhibitors have not been studied in vivo so far [28], although this is the subject of ongoing research, and whether PAD4 inhibition affects embryonic development is unclear at the moment, although PAD4-deficient mice have normal litters [26]. General PAD inhibitors such as chlorami-dine have been shown to strongly impact embryonic development and would therefore not be suitable for use in the context of pregnancy [53]. On the other hand, DNase I is already being used as an inhalation agent for patients with cystic fibrosis [54] and may be an interesting therapeutic agent for the clearance of extracellular chromatin, especially in light of the fact that our group has shown anti-inflammatory effects of DNase I treatment in myocardial ischemia/reperfusion injury [35]. Targeting NETs might be especially worthwhile in those women suffering the hemolysis, elevated liver enzymes, low platelets), which occurs in approximately 20% of women with severe preeclampsia and is characterized by a disorder of the coagulation system. In these patients, excessive NETosis could be induced by heme, and procoagulant properties of NETs/histones might have especially deleterious effects [55]. In these patients, dissolution of NETs or histone-inhibition could be particularly beneficial [56].

In conclusion, PAD4 deficiency in this model of sFlt-1 overexpression decreases local inflammation in the placenta and systemic inflammation and thus appears to protect against sFlt-1–induced pregnancy loss. Further studies need to be done in other mouse models of impaired placentation to evaluate further a pathogenic role for NETs in pregnancy complications.

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