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ORIGINAL PAPER

CXCR4$^+$ Dendritic cells promote angiogenesis during embryo implantation in mice

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Abstract Early pregnancy is characterized by decidual adaption to the developing embryo involving angiogenesis and vascular growth. Failure of decidual ~~vasculature~~ A1 expansion is linked to diseases of pregnancy. Dendritic cells (DC) have been associated with vascular growth during early gestation, though it is unknown whether their capacity to modulate angiogenesis is ubiquitous to all DC subsets. Here, we show that DC normally found associated with the decidual vasculature co-express the C–X–C chemokine receptor type 4 (CXCR4). In addition, we demonstrate that impaired homing of CXCR4$^+$DC during early gestation provoked a disorganized decidual vasculature with impaired spiral artery remodeling later in gestation. In contrast, adoptive transfer experiments provided evidence that CXCR4$^+$DC are able to rescue early pregnancy by normalizing decidual vascular growth and delivery of pro-angiogenic factors, which results in adequate remodeling of the spiral arteries during placental development. Taken together, our results indicate an important role of CXCR4$^+$DC in the regulation of decidual ~~vascular expansion~~ A1 and highlight the importance of the CXCL12/CXCR4 pathway during this process, suggesting that this may represent a key pathway to evaluate during pregnancy pathologies associated with impaired vascular expansion.

Keywords Dendritic cells · CXCR4 · Angiogenesis · Early gestation

Introduction

Dendritic cells (DC), professional antigen presenting cells that induce and regulate immune responses, have been also implicated in vascular growth and angiogenesis. Hence, DC have shown the potential to modulate the angiogenic response through their ability to produce a wide variety of vasoactive mediators [1] in different physiopathological settings. For instance, lymph node vascular growth in mice has been shown to depend on VEGF production by DC [2]. In human ovarian carcinoma, tumor-infiltrating DC induce angiogenesis through production of TNF-α and IL-8 [3]. Moreover, expression of β-defensin in mice has been shown to enhance recruitment of DC precursors to tumors and promote neovascularization through increased production of VEGF [4]. Dendritic cells were also found to support angiogenesis and promote development of ectopic implants in a murine model of endometriosis [5]. Likewise, lesion growth in an experimental model of choroidal neovascularization has been reported in association with increased infiltration of DC bearing the VEGFR2 [6]. All these findings exemplify the ability of DC in promoting angiogenesis in addition to the recognised role played by these cells in the orchestation of the immune response.

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During early pregnancy, endothelial cells (EC) in close proximity to decidual cells proliferate to form a new dense vascular network in the pregnant uterus [7]. It is thought that the newly formed decidual vasculature serves as the first exchange apparatus for the developing embryo until the placenta becomes functionally competent [8]. Increased vascular permeability and angiogenesis are critical to successful implantation, decidualization and placentation [9]. Indeed, treatment with the angiogenesis inhibitor AGM-1470 in gravid mice resulted in complete failure of embryonic growth [10]. Disturbances in uterine blood flow have been associated with higher perinatal morbidity and mortality due to preterm delivery, pre-eclampsia and pregnancy disorders such as intrauterine growth restriction (IUGR). Thus, vascular development through the process of angiogenesis is pivotal in determining the success of pregnancy [9]. Interestingly, beside their role in maternal immune surveillance during normal pregnancy [11–14], DC appear to be critical for angiogenesis during decidualization. Indeed, Plaks et al. [15] have shown that DC depletion using the CD11c-DTR transgenic mouse strain, which allows conditional ablation of DC in vivo, interferes with the normal dynamics of vascular remodeling by affecting vessel permeability and blood flow to the implantation site, leading to early pregnancy loss. Moreover, normal expansion of the decidual vascular network fails to occur in the absence of DC, as evidenced by the decreased expression of the endothelial markers platelet endothelial cell adhesion molecule (PECAM-1, also known as CD31) and endoglin observed in DC depleted implantation sites [16]. Importantly, defective expansion of the decidual vascular network during implantation could in turn lead to alterations in placental development and function [17]. Indeed, DC depletion during implantation was also associated with impaired differentiation of the trophoblast [16], most notably of invasive proliferin-expressing giant cells which are likely to promote decidual vascular growth during placentation [18, 19]. Thus, unraveling the contribution of DC to the angiogenesis process associated with implantation is important to gain insight in the role of these cells during early pregnancy.

In this study, we investigated the mechanisms involved in the modulation of decidual angiogenesis by DC during early mouse pregnancy. Experiments in Fms-related tyrosine kinase 3 ligand (Flt-3L)-treated gravid mice, which display an expanded DC pool, demonstrated an up-regulation of the chemokine (C–X–C motif) ligand 12 (CXCL12) in the decidua together with increased perivascular infiltration of DC co-expressing the C–X–C chemokine receptor type 4 (CXCR4). Blockade of the CXCR4 pathway during early pregnancy provoked a disorganization of the expanding maternal vasculature. We additionally highlight the pro-angiogenic role played by $CXCR4^{+}$DC in avoiding pregnancy failure upon conditional depletion of DC. Thus, our results attribute a major role to $CXCR4^{+}$DC in the regulation of decidual vascular expansion during early pregnancy and highlight the importance of the CXCL12/CXCR4 pathway during this process.

Methods

Animals

Five-to six-week C57BL/6 CD11c.DTR mice female mice, which express a diphteria toxin receptor (DTR) under the control of the CD11c promoter [15], were purchased from Jaxmice® and maintained in our animal facility with a 12L/12D cycle. The presence of a vaginal plug after cohabitation of CD11c.DTR females ($H2^{b}$) with Balb/c males ($H2^{d}$) was denoted as day 0.5 of pregnancy. Procedures that involved mice were approved by the state authority for Animal Use in Research and Education (LaGeSo) and were conducted in strict accordance with guidelines for the care and use of laboratory research animals promulgated by the Medicine University of Berlin.

Depletion of DC

CD11c.DTR females with vaginal plugs were separated from males and were injected i.p. on gestation day (gd) 4.5 with DT (2 ng/g BW in PBS) for depletion of DC. Control CD11c.DTR females received an i.p. injection of PBS. On gd 7.5, mice (n = 5–7) were narcotized and blood obtained by retro-orbital puncture was collected in heparin-free tubes for serum sampling. The mice were then sacrificed and the total implantation sites were processed for histological sectioning and RNA isolation according to standard procedures.

Expansion of DC

In order to expand uterine DC during early pregnancy, some CD11c.DTR and C57BL/6 females mated to Balb/c mice (n = 5–7) and with vaginal plugs were treated with one daily i.p. injection of human recombinant Flt-3L (500 ng/g BW in PBS, BioxCell) for seven consecutive days.

Adoptive transfer experiment

Uterine cells from DC expanded C57BL/6 female mice mated to Balb/c males were isolated on gd 5.5 as described before [20]. Uterine cells were stained with anti-CD11c FITC and CXCR4-PE and selected using a FACS Aria cell sorter (BD Biosciences). More than 95 % pure $CXCR4^{+}CD11c^{+}$ cells were obtained. Before injection, cells were counted by trypan blue exclusion. CD11c.DTR pregnant mice were used as

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recipients and 10^5 $CXCR4^+CD11c^+$ cells were intravenously injected 24 h after DC depletion (DT 2 ng/g BW). Recipient pregnant female mice were sacrificed on gd 7.5 and 13.5 (n = 5/days analyzed).

Treatment with CXCR4 antagonist

AMD3100 was administered to Flt-3L treated CD11c.DTR pregnant mice via i.p. injection on gd 5.5 (Sigma, 10 mg/ml in PBS). Gravid female mice were sacrificed on gd 7.5 (n = 5) and 13.5 (n = 5).

In vivo fluorescence labeling of uterine blood vessels

On gd 5.5, some control and expanded DC female mice (n = 5) were injected i.v. with TRITC-conjugated BS-I lectin (4 μg/g BW; from Bandeiraea simplicifolia, Sigma) as previously described [5]. After 30 min mice were euthanized, and implantation site tissues were processed for histological sectioning according to our standard protocols. Nuclei were counterstained by incubating 5 min in DAPI solution, followed by washing and mounting in Shandon Immu-Mount TM (Thermo Scientific). Photo documentation was performed using the Zeiss LSM confocal system. Perivascular DC (CD11c-~~GFP~~) were enumerated in the central and lateral regions of the mesometrial decidua (MD) on three o more sections/implantation site from control and expanded DC mice. The quantification of perivascular DC was performed on images after they were captured at 200×.

Histology

For histological analysis, implantation sites on gd 7.5 and 13.5 were fixed with 10 % buffered formalin, dehydrated in ethanol, embedded with paraffin, and stained following Masson trichrome protocol. Tissue sections were examined using a light microscope (Axiophot) and photographs taken with Axio Cam HRc. Photo documentation was performed using the digital image analysis system Spot advanced software, version 8.6 (Visitron Systems).

DBA lectin/PAS dual staining

After deparaffinization and rehydratation, the paraffin-embedded serial sections were stained using the protocol previously described by Zhang and coworkers [21].

Immunofluorescence staining

Serial uterine tissue sections from gd 7.5 were stained following our standard protocol [20]. Briefly, slides were washed 3 times in TBS for 5 min, blocked with 2 % normal serum for 20 min and incubated overnight at 4 °C with the following primary Abs: anti- CD31 (1:200, Santa Cruz Biotechnology), anti-Endoglin (1:100, Santa Cruz Biotechnology) and anti- α-SMA (1:100, Sigma Aldrich). Negative controls were established by replacing the primary Ab with irrelevant IgG. After washing, CD31, Endoglin and α-SMA stained sections were incubated 1 h at room temperature with TRITC-conjugated secondary antibodies (Jackson ImmunoResearch). Nuclei in all sections were counterstained by incubating 5 min in DAPI solution, followed by washing and mounting in Immumount medium (Shandon). Sections were analyzed using a confocal laser scanning microscope (cLSM 510, Carl Zeiss).

Assessment of serum VEGF and sFtl-1 concentrations

Serum samples were tested in competitive ELISA using kits obtained from R&D Systems to quantify VEGF (Duoset mouse VEGF) according to the manufacturer's recommendations. The quantification of Flt-1 serum levels was performed using the Mouse Flt-1 Quantikine Immunoassay (R&D Systems) following the manufacturer's recommendations.

RNA isolation and quantitative PCR analysis

Total RNA was extracted from implantation site tissues on gd 7.5 and from placental tissues on gd 13.5 using the Nucleospin RNA/protein isolation kit (Macherey–Nagel). After DNase digestion (Invitrogen), cDNA was generated using random primers (Invitrogen). Real-time qPCR was performed on the TaqMan 7500 System (Aplied Biosystems). For each reaction, 1 μL cDNA, synthesized from 1 μg RNA in 25 μL, was used in a total volume of 11 μL containing 6.25 μL of Power SYBR Green PCR mastermix (Applied Biosystems), 3.75 μL DEPC water and 450 nM of the appropriate forward and reverse primers. The following primers were used: CXCR4 (forward primer: 5′- AGCATGACGGACAAGTACC-3′, reverse primer: 5′-GATGATATGGACAGCCTTACAC-3′); CXCL12 (forward primer: 5′-GAGAGCCACATCGCCAG AG -3′, reverse primer: 5′-TTTCGGGTCAATGCACACT TG -3′); VEGF (forward primer: 5′-ATCTTCAAGCCGTCC TGTGT-3′, reverse primer: 5′-GCATTCACATCTGCTGTG CT-3′); HIF-2α (forward primer: 5′-TGAGTTGGCTCAT-GAGTTGC-3′, reverse primer: 5′-TATGTGTCCGAAGGA AGCTG-3′) and PlGF (forward primer: 5′-CCACGCTCCTG TGAAACTAGA-3′, reverse primer: 5′-GACCAAACCTC AAAGCATGG-3′). The PCR profile was as follows: 2 min 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Subsequently, a melting curve analysis was performed which consisted of 70 cycles of 10 s with a temperature increment of 0.5 °C/cycle starting at 60 °C. The relative expression was calculated according to the equation Rel. Exp (RE) = $2^{-\Delta\Delta Ct}$.

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Western blot

Protein extracts from gd 7.5 uterine tissues were obtained using a Total Protein Extraction kit (Chemicon International) according to the manufacturer's instructions. Protein concentrations were determined by Bradford Assay (BioRad). Twenty micrograms of total protein were then separated by SDS-PAGE under reducing conditions and transferred onto PVDF membranes (Amersham Biosciences). After blocking for 1 h in PBSTM (5 % non-fat dry milk 0.1 % Tween 20 PBS), the blots were incubated overnight at 4 °C with VEGF (1:2,000; R&D Systems), VEGFR1 (1:500; R&D Systems) and β-actin (1/8,000; Sigma) Abs diluted in 3 % BSA PBS as previously described [22]. This was followed by washing in PBSTM and incubation with HRP conjugated goat anti-rabbit IgG (1:5,000; Jackson ImmunoResearch, Germany) in 3 % BSA-PBS. The chemiluminescence reagent kit (GE Health care Europe) was used, according to the manufacturer's instructions. Autoradiographs were scanned by an imaging densitometer.

Statistics

Results were analyzed with GraphPad Prism 5.0 (GraphPad Software Inc). Data are presented as mean ± SD from two or three replicate experiments. All comparisons, except the one in Fig. 3 for which we used analysis of variance and Tukey's test, were performed using the nonparametric Mann–Whitney *U* test. A P value of less than 0.05 was taken as statically significant.

Results

CXCR4$^+$ DC are localized in close proximity to the decidual vasculature

In order to define better their contribution to the angiogenesis process during implantation, we first evaluated the localization of DC within the pregnant uterus using the *CD11c.DTR* (~~diphtheria toxin~~) transgenic mice, which harbour a gene encoding a DTR/GFP fusion protein [23]. As we have previously published [24], DC were normally found closely associated with the decidual vasculature at the MD and also with the myometrium on gd 5.5 (Fig. 1a). Next we characterized the distribution of DC within implantation sites in which the DC pool has been expanded by the administration of Ftl-3L. As shown in Fig. 1a (right panel), we observed a greater number of perivascular DC at the MD compared to non-expanded DC mice. Increased infiltration of perivascular DC in these mice was associated with a significant down-regulation of systemic sFlt-1 levels on gd 7.5 (Fig. 1b). Since VEGF stimulates the synthesis of CXCL12, which is involved in recruitment of proangiogenic myeloid cells expressing CXCR4 [25, 26], we next focused our analysis on the CXCL12/CXCR4 pathway in the context of DC expansion. When analysed on gd 5.5, expanded DC mice exhibited a higher mRNA expression of CXCL12 and CXCR4 compared to control mice (Fig. 1c). Further analysis showed that many of the DC located in the vascular zone of expanded DC implantations co-expressed CXCR4 (Fig. 1d). Importantly, flow cytometric analysis of uterine cell suspensions obtained during gd 5.5 showed that the absolute percentage of CXCR4$^+$CD11c$^+$ cells was not significantly modified upon treatment with Flt-3L (Fig. 1e).

Blockade of CXCR4 reduces DC recruitment and impairs decidual vascular expansion early in gestation

To provide further evidence that the CXCL12/CXCR4 pathway is important for the recruitment of DC during early gestation, we inhibited CXCL12 signaling with an extensively used CXCR4-specific inhibitor (AMD3100) [27, 28]. Gravid eDC female mice were treated with AMD3100 on gd 5.5 as described in Fig. 2a. Although CXCR4 antagonist ~~eDC~~ treated mice progressed to gd 13.5, the fetal survival registered in this group was significantly decreased compared to eDC mice (Fig. 2a). While evaluation of the implantation sites on gd 7.5 confirmed that administration of CXCR4 antagonist (AMD3100) reduced the number of DC recruited into the MD (Fig. S1A), no statistically significant changes were observed in the NK cells and trophoblast cells (cytokeratin$^+$, CK) between both groups (Fig. S1B-C). To better define the status of the decidual vasculature on the two groups that progress to gd 7.5, we analysed the expression of CD31, which is constitutively expressed by vascular endothelium [29]. Interestingly, AMD3100 eDC gravid females also exhibited a restricted and disorganized CD31 and endoglin staining pattern when compared to eDC female mice (Fig. 2b). Analysis of α-smooth muscle actin (α-SMA) staining to evaluate vascular maturation/plasticity (Fig. 2c) revealed a low expression within the decidual vascular zone (vz) of AMD3100-eDC female mice on gd 7.5, indicating that most vessels in this region are immature. On the contrary, eDC females showed increased numbers of α-SMA positive vessels, which denotes the maturation and stabilization of the newly formed vascular network (Fig. 2c). Interestingly, Western blot experiments showed that the decidual VEGF to sFlt-1 expression ratio was significantly decreased in AMD3100 treated eDC deciduas (Fig. 2d). Later on gd 13.5, analysis of the trichrome staining showed that whereas eDC implantation sites had dilated, thin-walled arteries in the central, proximal region of the decidua basalis (Fig. 2e), the vessels in this region of AMD3100-eDC mice

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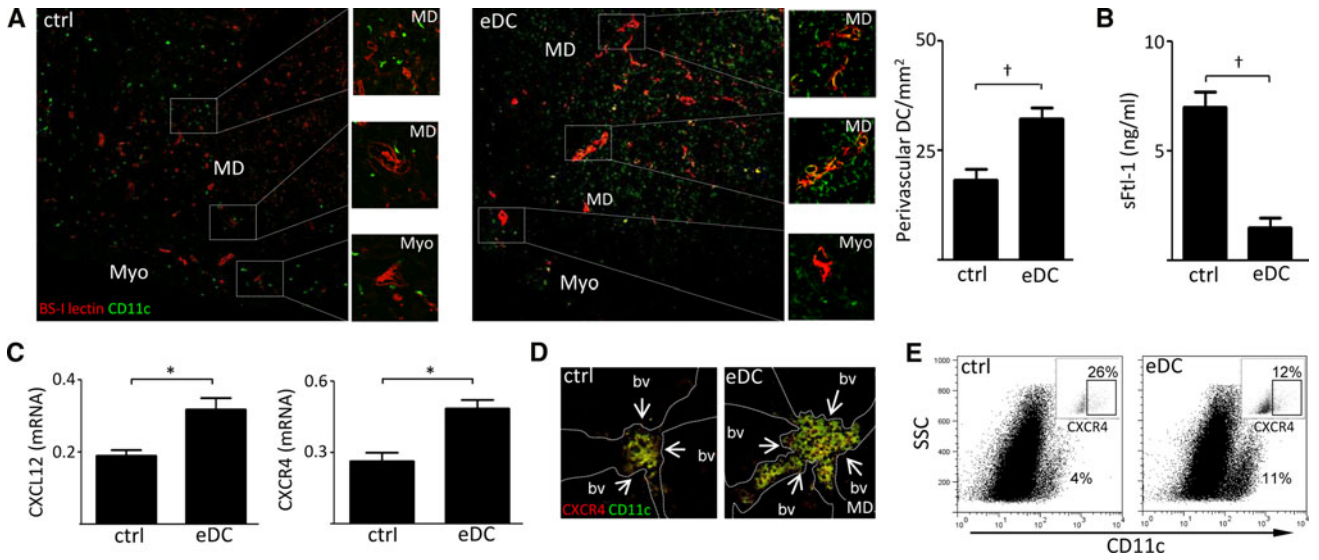

Fig. 1 CXCR4$^+$DC associate preferentially with vascular zone in the pregnant uterus. **a** Topographical relationship between DC and the mesometrial vascular bed in control (ctrl) and expanded DC (eDC) implantations. Immunofluorescence analysis of uterine sections obtained during gd 5.5 shows that CD11c$^+$ cells (*green*) preferentially associate with BS-1 lectin (*red*) decidual blood vessels. The densities of perivascular DC cells were scored, as described in "Methods" section. **b** sFtl-1 levels, as measured by ELISA on gd 7.5. Expansion of DC significantly decreased serum Ftl-1 respect to control mice. **c** Decidual CXCR4 and CXCL12 expression during gd 5.5, as analysed by qPCR. Expansion of DC was associated with increased levels of both CXCR4 (*left panel*) and CXCL12 mRNA (*right panel*) compared to controls. **d** Analysis of CXCR4 expression in decidual DC isolated from control and expanded DC mice on gd 5.5. The merge images show intense co-expression of CD11c (*green*) and CXCR4 (*red*) within the vascular zone in the mesometrial pole (MD). *Arrows* point at CXCR4$^+$DC within blood vessels (bv). **e** Representative flow cytometric analysis of uterine cell suspensions obtained on gd 5.5 to determine the CXCR4 expression on CD11c$^+$ cells. Insert dot plots indicated the CXCR4 expression of uterine DC from control and eDC implantations. In all panels, results correspond to at least two independent experiments using five to seven animals/group. In all figures, significant differences are noted as $^*p < 0.05$ and $^\dagger p < 0.01$ as analysed by the nonparametric Mann–Whitney U test

had narrower lumens and cuffed appearance, indicative of a thickened arterial wall (Fig. 2e). In addition, we observed that fetuses carried by AMD3100-eDC females appeared to be smaller compared to eDC female mice on gd 13.5 (Fig. 2f).

CXCR4$^+$DC exhibit pro-angiogenic function in vivo and rescue gestation

To provide definite evidence that the CXCR4$^+$DC are important in the modulation of angiogenesis during early gestation, we isolated uterine CXCR4$^+$DC from non-transgenic B6 mice on gd 5.5 and transferred them into DC depleted recipient pregnant females (CD11c.DTR) after DT injection (Fig. 3a). Of note, we chose the CD11c.DTR model since DC depletion leads to an impaired vascular expansion and attenuated maturation causing embryo resorption on gd 7.5 [15]. Compared to DC depleted (dDC) mice, which showed no sign of viable embryos, adoptive transfer of CXCR4$^+$DC early in gestation improved fetal survival on gd 7.5 (Fig. 3b). Figure 3c shows that adoptive transfer of CXCR4$^+$DC into dDC mice restored the pattern of CD31 expression observed in control mice. Interestingly, the analysis of endoglin, an accessory receptor for TGF-β that is predominantly expressed on angiogenic blood vessels in vivo [30], confirmed these results by showing a similar expression of this marker on the MD of the CXCR4-dDC and control implantations (Fig. 3c). DC depletion also led to a significant increase in serum concentrations of sFlt-1 (Fig. 3d). In contrast, this effect was abrogated upon adoptive transfer of CXCR4$^+$DC, confirming the pro-angiogenic potential of this cell subset during early gestation. Interestingly, CXCR4-dDC females showed an increased decidual VEGF expression on gd 7.5 compared to control and dDC implantations (Fig. 3e).

Changes in the angiogenesis process associated with implantation have been mostly linked with the modulation of placental physiology during early and mid pregnancy [17, 31]. Thus, our next aim was to compare the progression of gestation upon adoptive transfer of CXCR4$^+$DC focusing on the placentation period. Figure 4a shows that dDC gravid females adoptively transferred with CXCR4$^+$DC progressed to gd 13.5 and the abortion rate registered in this group was similar to the control mice. During mid gestation, the maternal spiral arteries expand in order to increase blood flow to the placental bed [32]. Trichrome staining showed that control and CXCR4$^+$DC adoptively transferred dDC mice had dilated, thin-walled arteries in the central, proximal region of the decidua basalis at gd 13.5 (Fig. 4b). Analysis of the presence of PAS$^+$DBA$^+$ granulated NK cells, which

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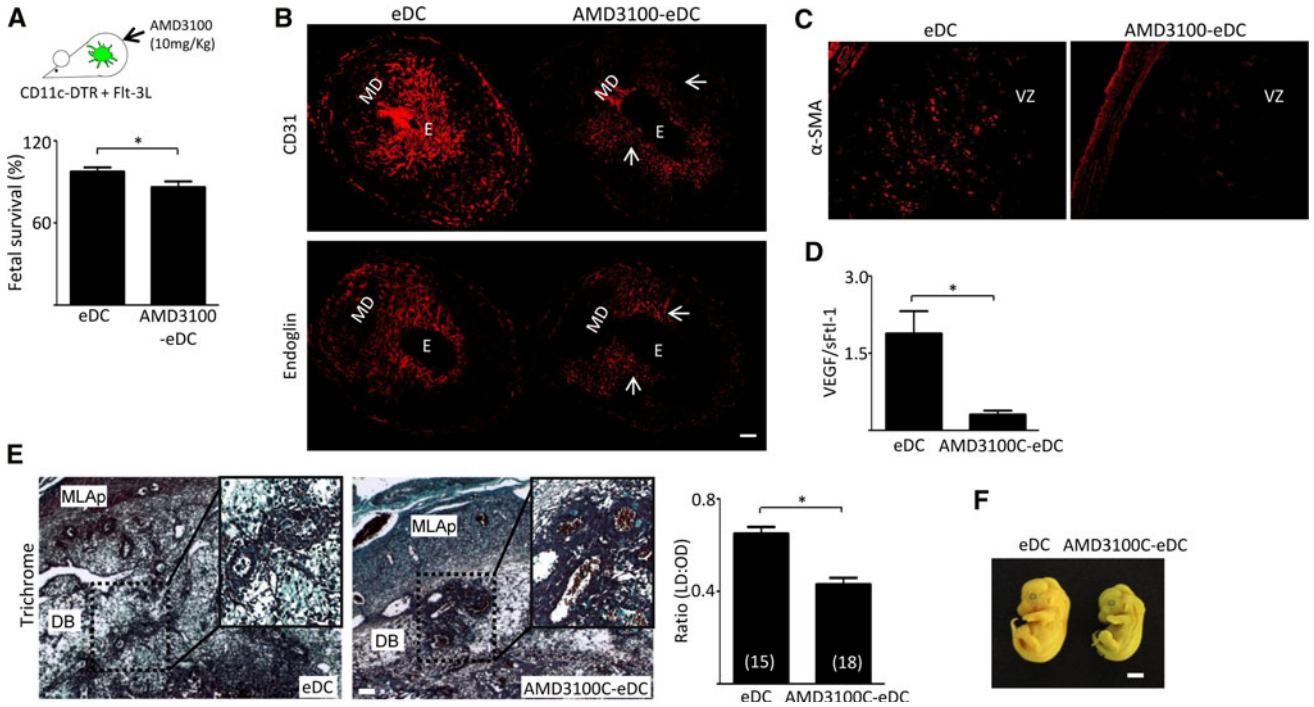

Fig. 2 CXCL12 blockade inhibits DC recruitment and decidual vascular expansion. **a** Protocol for CXCL12 blockade into expanded DC (eDC, CD11cDTR) female mice. CD11c.DTR females were treated with AMD3100 on gd 5.5 as described in "Methods" section. Bars depict the mean percentage of fetal survival rates ± SD. The percentage of fetal survival was calculated as follows: % fetal survival = V/(R + V) × 100, whereby R represents the number of fetal rejection and V stands for viable implantation sites. **b** Analysis of CD31 and endoglin expression on uterine sections obtained during gd 7.5. *Arrows* denoted a disorganized vascular expansion in AMD3100-eDC implantations. *Bar* 200 µm. **c** α-SMA analysis of uterine sections from expanded DC (eDC) and AMD3100 treated eDC gravid females on gd 7.5. **d** Decidual VEGF/sFtl-1 ratios as analysed by Western blot on gd 7.5. **e** Trichrome-stained decidua sections of eDC and AMD3100-eDC mice on gd 13.5 show the maternal vessels in the decidua basalis. Quantification of the inner lumen to-outer diameter ratio (LD/OD) of the spiral arterial walls obtained on gd 13.5. *Bar* 200µm. Data are expressed as mean ± SD and the numbers of vessels analysed are shown on each summary bar. **f** Fetuses (gd 13.5) obtained from eDC to AMD3100-eDC female mice. *Bar* 0.25 cm. In all panels, results correspond to at least two independent experiments using six to seven animals/group. In all figures, significant differences are noted as $*p < 0.05$ as analysed by the nonparametric Mann–Whitney U test. Abbreviations: *E* embryo, *MD* mesometrial decidual, *VZ* vascular zone, *MLAp* mesometrial lymphoid aggregate of pregnancy, *DB* decidua basalis

facilitate dilation of spiral arteries [31], showed similar abundances and distribution pattern (vascular-associated, va) in the decidua basalis in both groups (Fig. 4c). We finally characterized the placental hypoxia-inducible transcription factor (HIF)-2α expression, a reliable indicator of hypoxic distress of the developing embryo [33]. Figure 4d shows similar expression of HIF-2 α and placental growth factor (PlGF; which enhances vessel permeability) between control and CXCR4$^+$DC adoptively transferred dDC mice, suggesting that placental physiology is similar between both groups.

Discussion

Increased endometrial vascular permeability is one of the prerequisites for the success of embryo implantation, followed by progressive endothelial cell growth and angiogenesis at the implantation site. In this study, we have shown that the CXCL12/CXCR4 axis is important for the promotion of angiogenesis associated with early gestation, which could influence the placentation process. Pharmacologic blockage of the CXCR4 pathway reduces the recruitment of DC into the decidua, provoking attenuated vascular expansion with consequences later in gestation. Thus, CXCR4$^+$DC exhibit a proangiogenic role which is required for the progression of healthy pregnancy.

Though the CXCL12/CXCR4 pathway has been studied in the context of reproduction, the understanding of its function is only emerging. First demonstrated in humans, trophoblast expression of CXCL12 contributes to two important processes during early pregnancy: (1) as an autocrine modulator of proliferation and invasion [34, 35], and (2) by promoting the recruitment and homing of CD56^{bright} CD16$^-$ NK cells [36, 37], which participate in the remodelling of spiral arteries during placentation. A similar function modulating immune cell trafficking has recently been demonstrated in mice, where the CXCL12/CXCR4 axis appears to be important for recruitment of FoxP3$^+$ NK cells and regulatory T cells that promote maternal tolerance

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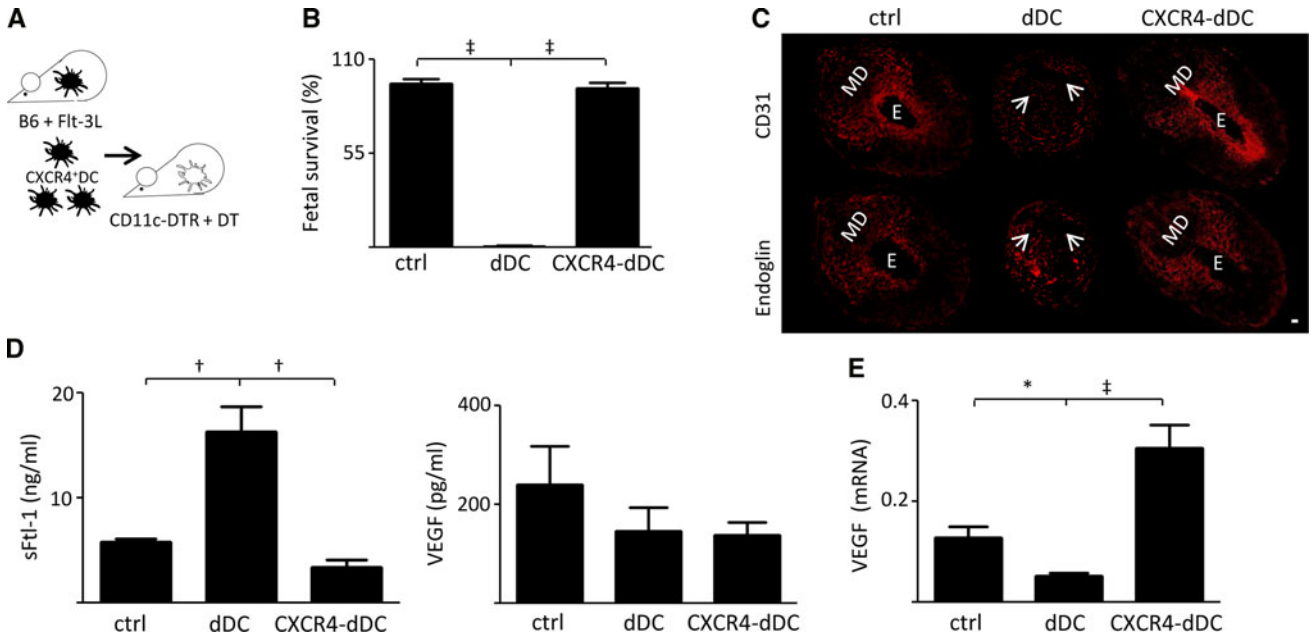

Fig. 3 CXCR4$^+$DC prevent pregnancy failure upon DC depletion. **a** Protocol for adoptive transfer of syngeneic CXCR4$^+$DC into DC depleted (dDC, CO11c.DTR) female mice. B6 females were treated with Flt-3L followed by selection of CXCR4$^+$CD11c$^+$ uterine cells, as described in "Methods" section. CXCR4$^+$DC were then adoptively transferred to CD11c-DTR mice upon DT treatment on gd 4.5. **b** Percentage of fetal survival registered on gd 7.5 in control and DC depleted (dDC) mice following transfer of CXCR4$^+$DC. Improved fetal survival rates were observed upon adoptive transfer in dDC mice. **c** Analysis of CD31 and endoglin expression on uterine sections obtained during gd 7.5. *Bar* 100 μm. *Arrows* denote lack of the maternal vasculature zone. **d** sFtl-1 and VEGF levels, as measured by ELISA on gd 7.5. Adoptive transfer of CXCR4$^+$DC significantly decreased serum Ftl-1 respect to dDC, implying an increased bioavailability of VEGF. **e** Decidual VEGF expression during gd 7.5, as analysed by qPCR. Adoptive transfer of CXCR4$^+$DC significantly increased local VEGF levels respect to control and dDC mice. In all panels, results correspond to at least two independent experiments using five to six animals/group. In all figures, significant differences are noted as $^*p < 0.05$, $^\dagger p < 0.01$ and $^\ddagger p < 0.001$ as analysed by the Tukey's test

[38, 39]. Ours is however the first study to highlight the involvement of the CXCL12/CXCR4 pathway in decidual angiogenesis during early pregnancy, as AMD3100 treated eDC females displayed several defects including a decreased vascular density in the mesometrial decidua, impaired vessel maturation and later on, defective remodelling of the spiral arteries on the decidua basalis. This is in agreement with the function ascribed to this chemokine as a major pro-angiogenic factor promoting endothelial cell migration, capillary sprouting and branching morphogenesis both in vitro and in vivo [40]. It was further demonstrated that angiogenesis is amplified due to a positive feedback loop in which the CXCR4/CXCL12 interaction induces VEGF release from endothelial cells [41] and conversely, VEGF and other classic angiogenic factors enhance CXCL12/CXCR4 expression on the endothelium [42]. Though the precise mechanism remains to be determined, it is tempting to speculate that decreased VEGF production by CXCR4$^+$ cell populations may contribute to the significant reduction of the VEGF to sFlt-1 ratio observed on AMD3100 treated decidual tissue. In mice and humans, VEGF becomes detectable at very early stages of pregnancy [43], and mostly derives from up-regulated expression on trophoblast cells in the context of a relatively hypoxic environment during placentation [44]. Such environmental conditions are also most likely to enhance CXCL12/CXCR4 pro-angiogenic functions, as suggested by studies showing an induction of CXCL12 expression by HIF-1α in ischemic tissues and increased expression of CXCR4 on the surface of different cell types including EC, monocytes, macrophages and tumor cells [45, 46]. Besides trophoblast cells, VEGF production by decidual stromal cells and NK cells could also contribute to the modulation of angiogenesis in the pregnant uterus [43, 47]. However, our results suggest that the decreased decidual VEGF to sFlt-1 ratio upon blockade of the CXCR4/CXCL12 pathway may be due to impaired functions of subsets other than uNK cells, since AMD3100 treated females displayed a similar frequency of vascular associated DBA$^+$ cells. Regarding the importance ascribed to the CXCL 12/CXCR4 pathway in promoting NK cell recruitment to the uterus [38], the latter finding may be related to the earlier time frame analysed in the present study, as recent studies have confirmed that perivascular and intramural uNK cells are found at very low tissue densities during the gd 6.5–7.5 period [48].

Our results showed that AMD3100 greatly interfered with the accumulation of CD11c$^+$ cells in the mesometrial

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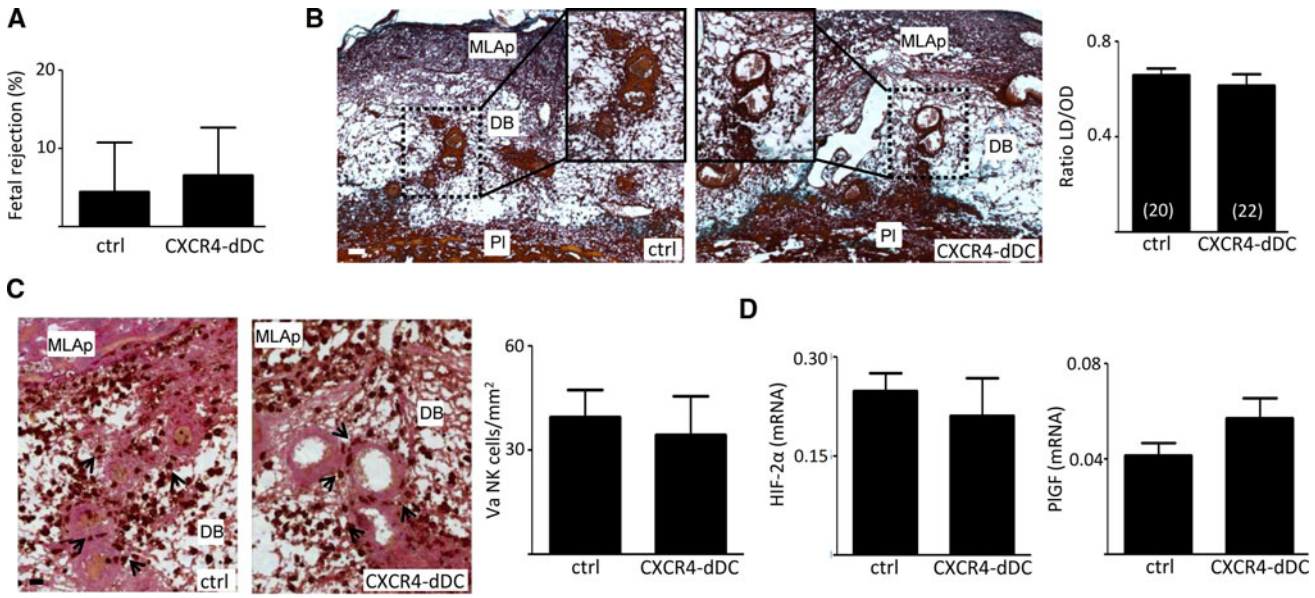

Fig. 4 DC depleted gravid females proceed to gd 13.5 upon adoptive transfer of CXCR4^{+}DC. **a** *Bars* depict the mean percentage of fetal rejection rates ± SD. The percentage of fetal rejection was calculated as follows: % fetal rejections = R/(R + V) × 100, whereby R represents the number of hemorrhagic implantations = fetal rejections and V stands for viable fetal-placental units, **b** Representative images of Masson Trichrome-stained decidua sections of control and CXCR4-dDC mice on gd 13.5 show the maternal vessels in the decidua basalis with open lumens and thin arterial walls. *Scale bar* 200 µm. Quantification of the inner lumen-to-outer diameter ratio (LD/OD) of the spiral artery walls obtained from control and CXCR4-dDC females on gd 13.5. Data are expressed as mean ± SD and the numbers of vessels analysed are shown on each *summary bar*. **c** Photomicrographs of dual DBA-PAS stained sections of implantation sites from gd 13.5 are presented. The number of uterine NK cells associated with spiral arteries were scored. When NK cells were intramural, they were scored as vascular associated (Va*; arrows*). Eight to ten sections/implantation site were scored. *Bar* 100 µm. Abbreviations: *MLAp* mesometrial lymphoid aggregate of pregnancy, *DB* decidua basalis, *PL* placenta, **d** Placental hypoxia-inducible factor (HIF)-2 alpha and Placenta growth factor (PIGF) expression on gd 13.5, as analysed by qPCR. In all panels, results correspond to at least two independent experiments using five to six animals/group

decidua of Flt-3L treated mice, suggesting that the CXCL 12/CXCR4 pathway could be involved in the recruitment of specific DC subsets to the pregnant uterus. Indeed, CXCR4 expression appears to be a normal feature of the DC subset recruited to the decidual vascular zone during early pregnancy, as the absolute percentage of uterine CXCR4^{+} CD11c^{+} cells was not modified upon Flt-3L treatment. In this context, the vascular defects observed upon treatment with AMD3100 may result, at least partially, from impaired DC functions in the promotion of decidual angiogenesis. While not previously addressed in the context of pregnancy, recent reports have highlighted the involvement of CXCR4^{+} expressing monocytes/bone marrow derived cells in angiogenic and neovascularization responses in different physiopathological settings [28, 49]. Thus, our results showing that adoptive transfer of CXC R4^{+} DC was able to rescue normal pregnancy progression by improving decidual vascular development provide definite evidence that this cell subset is an important physiological mediator of the pregnancy-associated angiogenic response. Which would be the mechanisms involved in the modulation of decidual angiogenesis by CXCR4^{+} DC? First, adoptive transfer of CXCR4^{+} DC significantly decreased serum concentrations of sFlt-1, in agreement with previous results suggesting that DC fine-tune angiogenesis during decidual development by modulating VEGF bioavailability [15]. An increased serum VEGF bioavailability would in turn have consequences for the mobilization of DC and homing to the uterus, as it has been demonstrated that exposure to VEGF modulates the survival and differentiation of monocyte-derived DC and most importantly, up-regulates CXCR4 expression [50]. Additionally, local VEGF expression was significantly increased upon adoptive transfer of CXCR4^{+} DC both respect to DC depleted and control mice, suggesting that the positive feedback loop between VEGF and the CXCL12/CXCR4 axis is an important mechanism controlling decidual angiogenesis during early pregnancy. Our hypothesis is that during the early stages of normal pregnancy, the hypoxic decidual environment induces local VEGF and CXCL12 expression, which in turn cooperatively promote the recruitment of CXCR4^{+} DC to amplify the angiogenic response. The angiogenic expansion of the decidual vascular bed during early stages of pregnancy would be a critical prerequisite for uNK cell mediated remodeling of the spiral arteries ensuring normal placental development and function. In this regard, it must be noted that DC depleted implantation

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sites are characterized by impaired recruitment and differentiation of NK cells [16], which fail to produce normal levels of IFN-γ necessary for spiral artery remodeling [51].

Importantly, our results further highlight the notion that DC are pivotal for decidual vascular development during early stages of pregnancy. The finding that adoptive transfer of $CXCR4^+$ DC was able to restore normal pregnancy rates indeed shows that impaired angiogenesis is the primary defect in DC depleted female mice, and that defective stromal cell proliferation and decidua formation results from an inability to sustain tissue growth in the context of inadequate vascularization. This may provide an explanation as to why, despite being a critical mediator controlling the decidualization response, treatment with progesterone failed to rescue pregnancy progression in DC depleted female mice [15]. Finally, the findings reported in the present study may have important implications for the understanding of the mechanisms involved in the pathogenesis of pregnancy disorders arising from defective vascularization such as preeclampsia and intrauterine growth restriction. Indeed, recent studies have shown an association between preeclampsia and increased decidual densities of DC [52], and up-regulated expression of CXCL12 in preeclamptic placental tissue showing an abnormal localization to syncitiotrophoblast cells [53]. Thus, these results encourage an extensive examination of the contribution of DC to the etiology of preeclampsia and other disorders related to immunological and angiogenic imbalances during early gestation.

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