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Histone methyltransferase Nsd2 ensures maternal–fetal immune tolerance by promoting regulatory T-cell recruitment

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Regulatory T cells (Tregs) are fundamentally important for maintaining systemic immune homeostasis and are also required for immune tolerance at the maternal–fetal interface during pregnancy. Recent studies have suggested that epigenetic regulation is critically involved in Treg development and function. However, the role of H3K36me has not yet been investigated. Here, we found that the H3K36me2 methyltransferase Nsd2 was highly expressed in Tregs. Although loss of Nsd2 did not impair systemic Treg development or function, the level of Tregs at the maternal–fetal interface was significantly decreased in pregnant Nsd2 conditional knockout mice. Consequently, maternal–fetal immune tolerance was disrupted in the absence of Nsd2 in Tregs, and the pregnant mice showed severe fetal loss. Mechanistically, Nsd2 was found to upregulate CXCR4 expression via H3K36me2 modification to promote Treg cell recruitment into the decidua and suppress the anti-fetal immune response. Overall, our data identified Nsd2 as a critical epigenetic regulator of Treg recruitment for maternal–fetal tolerance.

Keywords: Regulatory T cell; Cell migration; Immune tolerance

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INTRODUCTION

As a major suppressive subset of CD4⁺ T cells, Foxp3⁺ regulatory T cells (Tregs) play an important role in the maintenance of immune tolerance [1–4]. Accumulating evidence has revealed that epigenetic mechanisms critically regulate the differentiation and function of T-cell subsets, including Tregs. For instance, Ezh2, which is the methyltransferase that catalyzes histone H3K27 trimethylation (H3K27me3), is required for the stabilization of Treg signature genes, especially when cells are in an activated status. Mice with Ezh2-deficient Tregs spontaneously develop autoimmune diseases [5]. Another well-studied epigenetic regulator in Tregs is MLL4, which catalyzes histone H3K4 monomethylation (H3K4me1) at the locus encoding Foxp3 to promote Treg cell differentiation [6]. Recently, H3K36 methylation was found to be an abundant modification in the genome, to be associated with actively transcribed genes and to play important roles in biological processes such as cell differentiation and tumorigenesis [7, 8]. Our previous study showed that the H3K36 dimethyltransferase Nsd2 is critical in the germinal center B-cell response, acting by regulating both B-cell-follicular dendritic cell interactions and follicular helper T-cell differentiation [9, 10]. However, the roles of H3K36 modification in Treg development and function have not yet been defined.

In addition to preventing autoimmunity, Tregs play a role in pregnancy and are suggested to be involved in establishing

maternal–fetal immune tolerance and thus preventing immune rejection reactions against fetal alloantigens [11–21]. Upon blastocyst implantation, dramatic morphological and biochemical remodeling occurs in uterine endometrial stromal cells, which differentiate into decidual stromal cells [12]. This process is called decidualization and generates the maternal–fetal interface between the uterine mucosa and the extraembryonic tissues of the conceptus to support the development of the embryo. During this process, multiple maternal immune cells, including natural killer (NK) cells and Tregs, also populate the decidua to support fetal growth and suppress the anti-fetal immune response [13]. Decidual Tregs dramatically increase during pregnancy, and the frequency of Tregs in the decidua is higher than that in peripheral lymphoid organs [12, 19]. Clinical studies have revealed a strong association between a reduced Treg frequency and pregnancy-related diseases, such as recurrent spontaneous pregnancy loss and preeclampsia [22–28]. Studies performed with a mouse model have demonstrated that both thymic natural Tregs and peripheral induced Tregs are required for successful pregnancy [26, 29–31]. Moreover, Treg cells undergo systemic expansion during pregnancy [32]. Importantly, to suppress the anti-fetal immune response, Tregs must be recruited to the local maternal–fetal interface, which is the decidua. A previous study reported that the chemokine receptor CCR5 is involved in the accumulation of Tregs, especially activated Tregs, in the gravid uterus [33].

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Adoptive transfer experiments showed that CCR5-deficient Tregs migrated into the uterus less efficiently than their wild-type (WT) counterparts. However, under physiological conditions, CCR5 loss did not result in a marked reduction in Tregs in the uterus, and no fetal loss was observed [33], suggesting that there are other redundant chemokines involved in this process. Subsequent studies indicated that the CCR7-CCL19 and CXCR4-CXCL12 axes might facilitate Treg recruitment [34, 35], but genetic evidence is lacking. Therefore, further investigations are needed to understand the mechanisms underlying Treg recruitment to the maternal–fetal interface.

Here, we found that the H3K36 dimethyltransferase *Nsd2* was highly expressed in Tregs. *Nsd2* expression was dispensable for Treg development and function. However, we found that during pregnancy, *Nsd2* deficiency in Tregs resulted in a low frequency of Tregs at the maternal–fetal interface and fetal loss. This reduction in Tregs observed in the absence of *Nsd2* was largely due to CXCR4 downregulation. Our study revealed a critical *Nsd2*-dependent epigenetic regulatory mechanism for Treg recruitment to the maternal–fetal interface and provided genetic evidence for the role of CXCR4-mediated Treg migration into the decidua during pregnancy.

RESULTS

The H3K36 dimethyltransferase *Nsd2* is dispensable for Treg differentiation and function

To explore the potential role of H3K36 modification in Tregs, we initially analyzed the expression of all 8 known H3K36-related methyltransferases and found that *Nsd2* was the only enzyme that was upregulated in Tregs compared to conventional T cells (Supplementary Fig. 1a, b). Therefore, Treg-specific *Nsd2* conditional knockout (KO) mice were generated by crossing mice with *loxP*-flanked *Nsd2* alleles with *FoxP3*^{YFP-Cre} mice (*Nsd2*^{fl/fl} *FoxP3*^{YFP-Cre} mice were designated *Nsd2*^{Treg KO} mice, and *Nsd2*^{+/+} *FoxP3*^{YFP-Cre} mice were designated *Nsd2*^{Treg WT} mice). In adult mice, the Treg frequency and cell number in peripheral lymphoid tissues were not altered (Fig. 1a). The T-cell activation status assessed by CD44 and CD62L staining was also normal in the absence of *Nsd2* (Fig. 1b). We then aged the mice to 4 months old and found no abnormal infiltration of immune cells into various organs in *Nsd2*^{Treg KO} mice (Fig. 1c). An *in vitro* suppression experiment was then performed to assess Treg function. As shown in Fig. 1d, *Nsd2* KO Tregs displayed a suppressive capacity similar to that of WT Tregs, suggesting that *Nsd2* is dispensable for the suppressive function of Tregs. These data suggested that Treg-expressed *Nsd2* is not required for Treg development or function or for the maintenance of systemic immune homeostasis.

To further investigate whether *Nsd2* is required for Treg function in inflammatory settings, *Nsd2*^{Treg KO} and control mice were treated to establish experimental autoimmune encephalomyelitis (EAE), an inducible mouse model of multiple sclerosis. Flow cytometric analysis of immune cells from the central nervous system showed that the *Nsd2*^{Treg KO} mice harbored Tregs at a frequency comparable to that of the control *Nsd2*^{Treg WT} mice (Fig. 1e). The effector T-cell subsets identified by staining for the cytokines IL-17A and IFN γ were also not altered by *Nsd2* loss (Fig. 1f). Accordingly, *Nsd2*^{Treg KO} mice exhibited disease scores similar to those of *Nsd2*^{Treg WT} mice (Fig. 1g). Overall, although highly expressed in Tregs, *Nsd2* is dispensable for Treg development and immunosuppressive function under both homeostatic conditions and inflammatory conditions.

Nsd2 deficiency results in reduced Tregs at the maternal–fetal interface and in fetal resorption

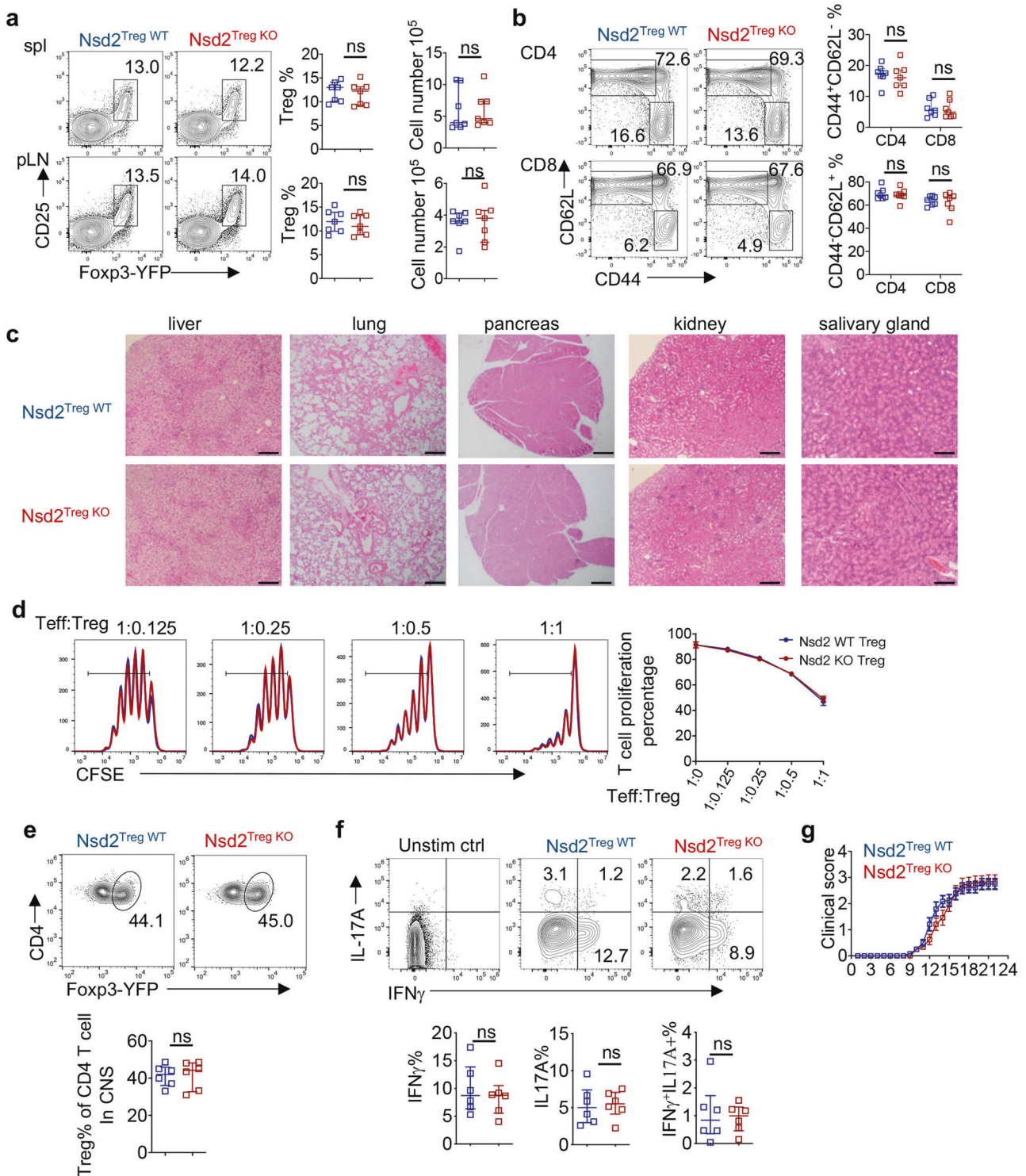
By suppressing the anti-fetal immune response in the decidua, Tregs play a vital role in immune tolerance during pregnancy. We therefore explored whether Treg-expressed *Nsd2* is involved in

maternal–fetal tolerance. Consistent with previous reports, systemic Tregs (splenic Tregs) significantly expanded during pregnancy (Supplementary Fig. 2a). *Nsd2* WT and KO Tregs expanded to a similar level, suggesting that *Nsd2* was dispensable for systemic Treg expansion during pregnancy (Supplementary Fig. 2a). However, to our surprise, following mating with male mice, pregnant *Nsd2*^{Treg KO} mice displayed significantly less accumulation of Tregs in the decidua than pregnant female *Nsd2*^{Treg WT} mice (Fig. 2a). The Treg frequencies in peripheral LNs, uterus-draining LNs and blood were unaffected by *Nsd2* deficiency (Supplementary Fig. 2b). The decidua Treg subset composition was not affected by *Nsd2* deficiency, suggesting that all Treg subsets were similarly reduced in the *Nsd2*^{Treg KO} decidua (Fig. 2b). Accordingly, more IFN γ -expressing T helper 1 (Th1) cells were detected in the *Nsd2*^{Treg KO} decidua, indicating immune tolerance failure (Fig. 2c). As a consequence, we observed increased fetal resorption in female *Nsd2*^{Treg KO} mice compared with WT littermate controls (Fig. 2d, e). The resorption rate per pregnant female and the incidence of fetal resorption were also significantly increased (Fig. 2f, g). Notably, although the fetal resorption rate was also significantly increased in *Nsd2*^{Treg KO} mice, 90% of the fetuses remained alive in the absence of *Nsd2*, suggesting that the remaining Treg cells and other decidual cells still contributed to immune tolerance. The extent of fetal loss in *Nsd2*^{Treg KO} mice was similar to that observed in previously reported settings in which peripheral Tregs were selectively ablated [30]. Histological examination of placental tissues clearly revealed a more congested and widened spongiotrophoblast, necrotic labyrinth, hemorrhage and necrosis or thrombosis in decidual vessels in female *Nsd2*^{Treg KO} mice (Fig. 2h). Immunohistochemical staining revealed dramatically increased neutrophil infiltration in the *Nsd2*^{Treg KO} decidua (Supplementary Fig. 3). A previous study suggested that fetal abortion and local inflammation affect the hemodynamics of the uterine artery [36]. Therefore, we performed ultrasound biomicroscopy analysis of pregnant mice and found that both the uterine artery resistance index and the pulsatility index were significantly increased in *Nsd2*^{Treg KO} mice (Fig. 2i). Overall, *Nsd2* deficiency selectively reduced the Treg frequency in the decidua and resulted in fetal resorption without impacting systemic Treg development or function.

Nsd2 promotes Treg recruitment into the decidua by regulating CXCR4 expression

We then investigated the mechanism underlying the reduction in the decidua upon *Nsd2* deletion. Given that systemic Treg development and function were not affected, we asked whether local Treg maintenance in the decidua was impaired by *Nsd2* deficiency. Therefore, Treg expansion and apoptosis were examined by flow cytometry. Surprisingly, neither the expansion nor apoptosis of Tregs in the decidua was affected by *Nsd2* deficiency (Fig. 3a, b). We also assessed the expression of Treg function-associated cell-surface markers and found that most of these markers were expressed at normal levels on decidual Tregs; however, PD-1 displayed a slight increase in the absence of *Nsd2* (Fig. 3c). Thus, these data suggest that the local fitness of Tregs in the decidua was not affected by *Nsd2* loss.

To further explore how *Nsd2* deficiency reduced Treg abundance specifically in the decidua, we performed mRNA sequencing (RNA-seq) analysis of *Nsd2*-deficient and WT Treg cells. Consistent with the fact that overall Treg development and function were normal, only a limited number of differentially expressed genes were detected between *Nsd2* KO and WT Treg cells, specifically, 48 upregulated genes and 39 downregulated genes (Fig. 4a). Most of the functionally relevant Treg genes exhibited normal levels of transcription. Notably, the mRNA level of *Cxcr4*, the gene encoding the chemokine receptor CXCR4, was significantly decreased in *Nsd2* KO Tregs (Fig. 4a). Flow cytometry staining verified the reduced expression of CXCR4 at the protein



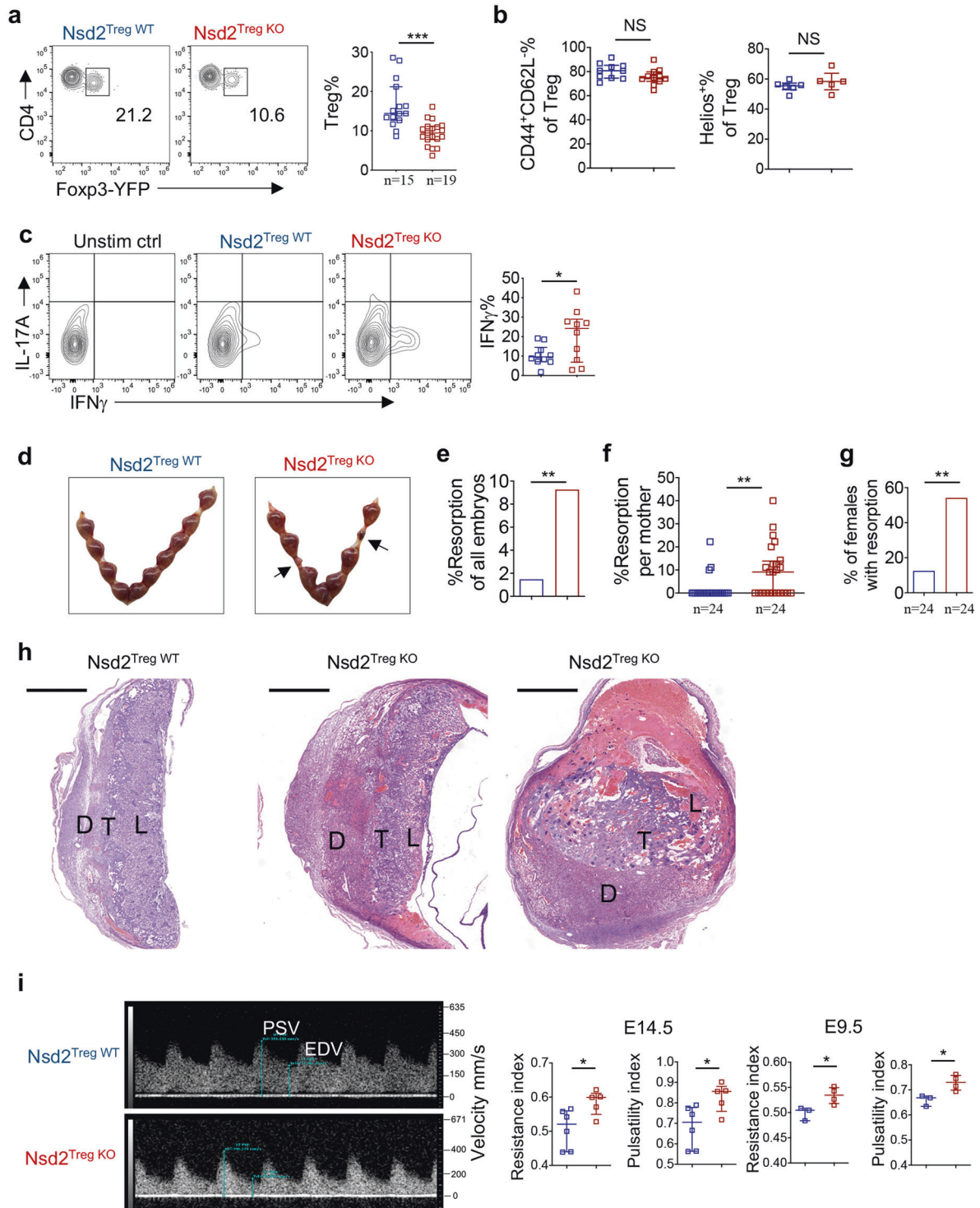


Fig. 2 *Nsd2* deficiency results in reduced Tregs at the maternal–fetal interface and in fetal resorption. **a** Representative flow cytometric analysis of YFP⁺ Treg cells in the decidua of *Nsd2*^{Treg WT} and *Nsd2*^{Treg KO} mice previously mated with male BALB/c mice on Days E13.5–E14.5. **b** Percentage of CD44⁺CD62L^{low} and Helios⁺ Tregs determined by flow cytometry in the decidua of *Nsd2*^{Treg WT} and *Nsd2*^{Treg KO} mice previously mated with male BALB/c mice on Days E13.5–E14.5. **c** Flow cytometric analysis of IFN γ and IL-17A expression in decidual CD4⁺ T cells. *n* = 10. **d** Representative image of resorption of allogeneic embryos in the uterus of *Nsd2*^{Treg WT} and *Nsd2*^{Treg KO} mice on Day E14.5. Arrows indicate resorptions. **e** Percentage of resorbed embryos in all *Nsd2*^{Treg WT} and *Nsd2*^{Treg KO} pregnancies resulting from matings with male BALB/c mice. Two-sided Fisher's exact test was used to assess the significance. **f** Percentage of resorption observed in individual mothers with the indicated genotype. **g** Incidence of pregnancies with at least one resorption. Two-sided Fisher's exact test was used to assess the significance. **h** Histopathological evaluation of placentas from female *Nsd2*^{Treg WT} (left) and *Nsd2*^{Treg KO} (right, two examples) mice mated with male BALB/c mice; low-magnification survey of representative H&E-stained sections of placenta. D decidua, T trophoblast, L labyrinths. Scale bars, 1000 μ m. **i** Representative Doppler image of the uterine artery (left, E14.5), which was used to determine the uterine artery resistance and pulsatility indices (right) of *Nsd2*^{Treg WT} and *Nsd2*^{Treg KO} female mice on Days E14.5 and E9.5. **P* \leq 0.05; ***P* \leq 0.01; ****P* \leq 0.001

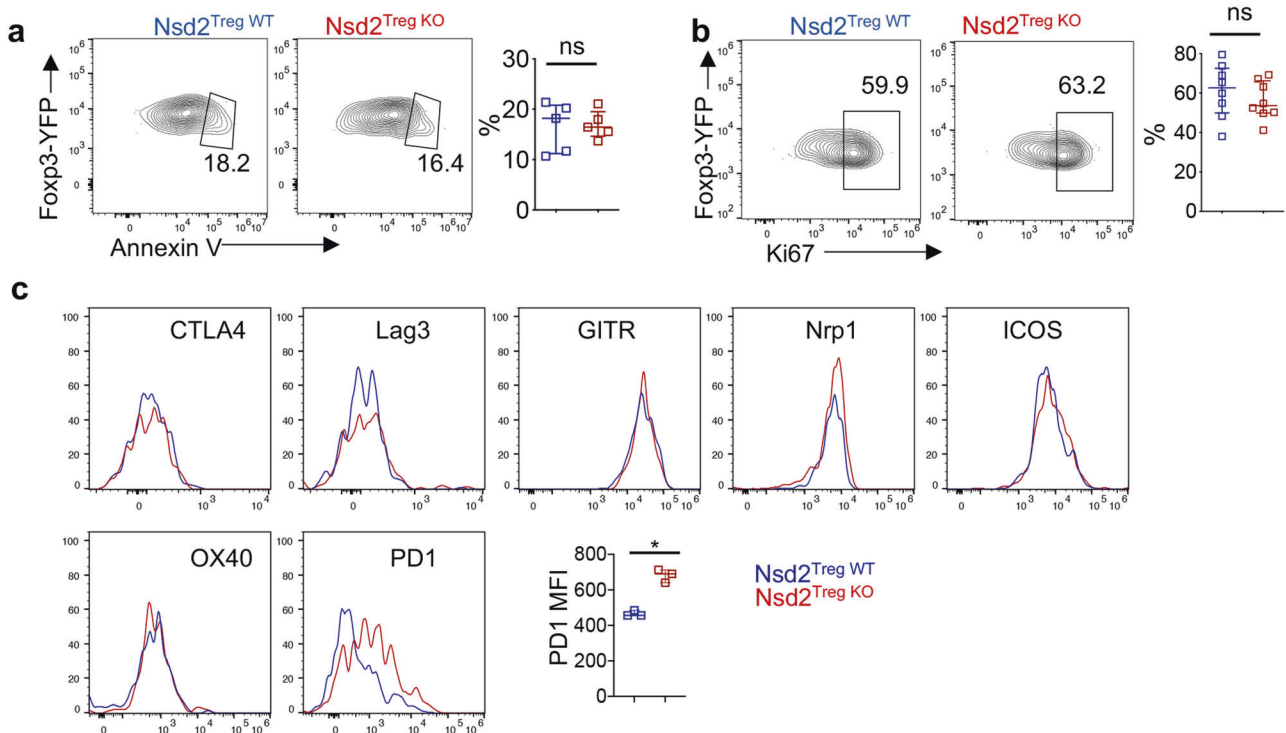


Fig. 3 Nsd2 deficiency did not affect the apoptosis or proliferation of decidual Tregs. **a** Cell-surface staining for Annexin V on decidual Tregs isolated from Nsd2^{Treg WT} and Nsd2^{Treg KO} mice. $n = 5$. **b** Flow cytometric analysis of Ki67 expression in decidual Tregs from Nsd2^{Treg WT} and Nsd2^{Treg KO} mice. $n = 8$. **c** Histogram overlay of CTLA-4, LAG-3, GITR, Nrp1, ICOS, OX40 and PD-1 expression and plots of the PD-1 ($n = 3$) mean fluorescence intensity (MFI) of decidual Treg cells from Nsd2^{Treg WT} and Nsd2^{Treg KO} mice. *** $P \leq 0.001$; ns not significant

level (Fig. 4b). Nsd2 loss did not affect the expression levels of CCR4 and CCR5, which have been previously reported to be involved in Treg recruitment into the decidua (Fig. 4b). Nsd2 catalyzes the dimethylation of histone H3 at lysine 36, which is a permissive mark and thus positively influences gene transcription outcomes [7, 8]. Therefore, we performed chromatin immunoprecipitation qPCR (ChIP-qPCR) to analyze H3K36me2 modification of the *Cxcr4* locus. We observed significantly reduced H3K36me2 levels in Nsd2-deficient Tregs, suggesting that Nsd2 might directly regulate CXCR4 expression by catalyzing H3K36me2 (Fig. 4c). To assess whether the reduction in CXCR4 in Nsd2 Tregs had functional consequences, an in vitro Transwell migration assay was performed, and we found that Nsd2-deficient Tregs migrated toward CXCL12, the ligand for CXCR4, less efficiently than their WT counterparts (Fig. 4d). A previous study showed that the CXCL12-CXCR4 axis is critical for NK-cell recruitment into the decidua and that trophoblast cells express high levels of CXCL12 [37–39]. It has also been reported that the CXCL12-CXCR4 axis may regulate Treg accumulation at the maternal–fetal interface, although genetic evidence is still lacking [34]. Therefore, ex vivo adoptive transfer experiments were performed to directly examine whether Nsd2 deficiency impairs Treg recruitment into the decidua. We transferred Nsd2 KO or WT Treg cells into congenically distinct pregnant recipient mice and analyzed the distributions of these donor-derived Tregs in lymphoid tissues and the decidua. As shown in Fig. 4e, Nsd2 KO and WT Tregs were distributed at similar frequencies in the uterus-draining lymphoid nodes. In contrast, there was a dramatic reduction in the number of Nsd2 KO Tregs in the decidua, suggesting a severe defect in Nsd2 KO Treg recruitment to the maternal–fetal interface (Fig. 4e). Collectively, these data demonstrated that Nsd2 is required for Treg migration into the decidua to control maternal–fetal immune tolerance during pregnancy, potentially by regulating CXCR4 expression.

It has been reported that CXCR4 is critical for Treg migration into the bone marrow [40]. To corroborate our finding that Nsd2 regulates CXCR4 expression, we also analyzed Tregs in the bone marrow and found that the frequency of Tregs was significantly decreased in the bone marrow of Nsd2^{Treg KO} mice compared to that of WT mice, which further supported our finding (Fig. 4f).

Treg-specific CXCR4 conditional KO mice displayed decidual Treg reduction and fetal abortion phenotypes similar to those of Nsd2^{Treg KO} mice

Although a previous study indicated that CXCR4 is potentially important for Treg migration into the decidua, supportive genetic evidence is lacking [34]. Therefore, we constructed Treg-specific CXCR4 conditional KO mice by crossing *Cxcr4*^{fl/fl} mice with Fopx3-Cre-YFP mice (designated CXCR4^{Treg KO} mice). Following mating with male BALB/c mice, the Treg frequency was significantly reduced in the decidua of pregnant female CXCR4^{Treg KO} mice compared to that of pregnant female WT mice, whereas the splenic Treg frequency was not altered at all (Fig. 5a, b). Correspondingly, pregnant CXCR4^{Treg KO} mice displayed increased fetal loss compared with that of pregnant WT control mice (Fig. 5c–e). Therefore, CXCR4^{Treg KO} mice phenocopied Nsd2^{Treg KO} mice in terms of decidual Treg frequency reduction and fetal loss, further suggesting that Nsd2 regulates CXCR4 expression to promote Treg recruitment to the maternal–fetal interface.

Nsd2 promotes Treg migration into the decidua primarily by regulating CXCR4 expression

The above data suggest that Nsd2 can enhance Treg migration into the decidua by regulating CXCR4 expression. However, whether CXCR4 regulation is the predominant function of Nsd2 in Tregs remains to be further investigated. To address this question, we crossed Nsd2 conditional KO mice (*Nsd2*^{fl/fl}*Fopx3*^{YFP-Cre}) with

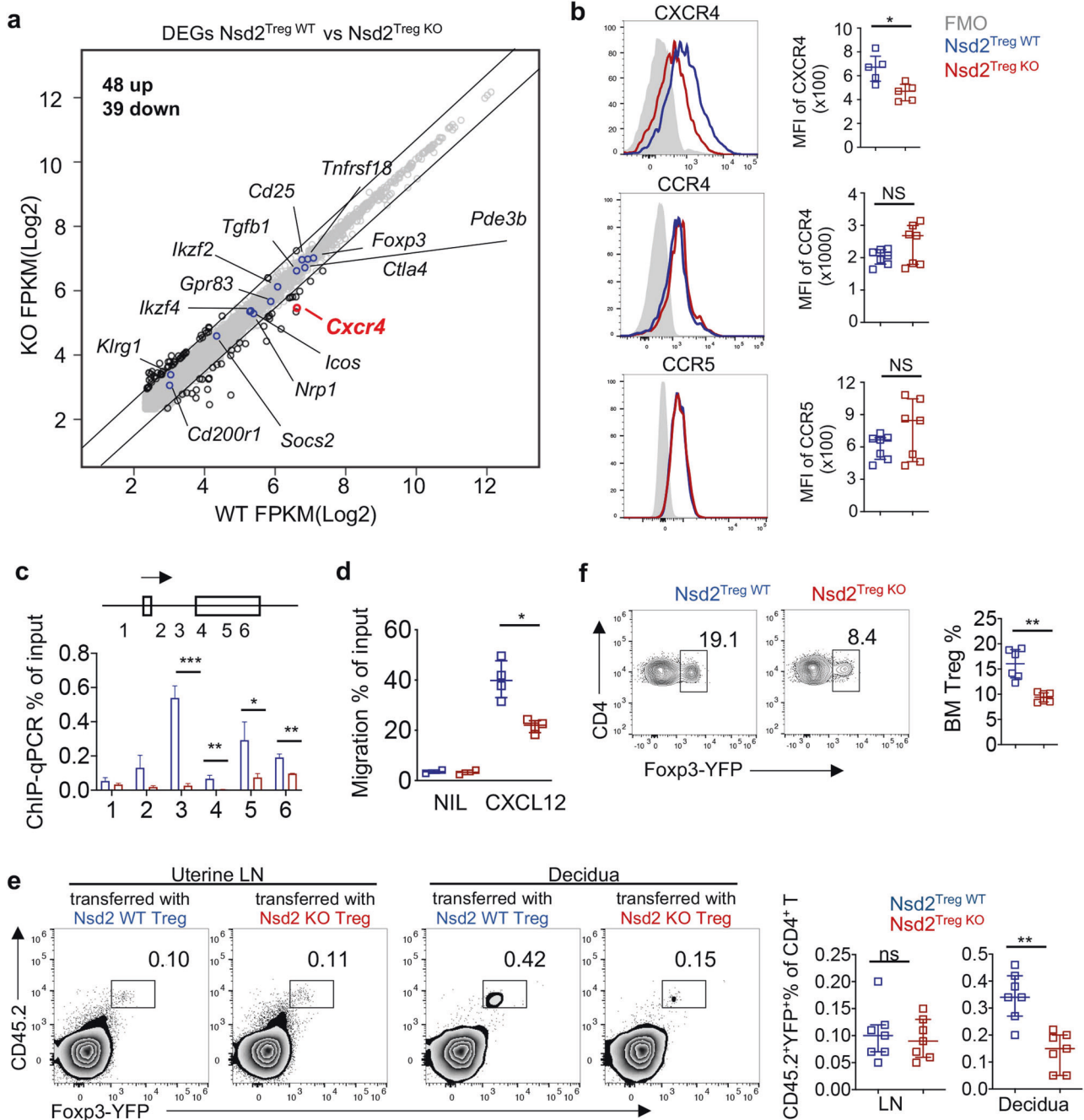


Fig. 4 *Nsd2* promotes Treg recruitment into the decidua by regulating CXCR4 expression. **a** RNA-seq analysis of $CD25^+ YFP^+$ Treg cells from $Nsd2^{Treg\ WT}$ and $Nsd2^{Treg\ KO}$ mice. The examples of functionally relevant Treg genes without significant differential expression are labeled with blue dots. **b** Flow cytometric analysis of CXCR4, CCR4, and CCR5 expression in splenic Tregs. $n = 5-7$. **c** ChIP-qPCR for H3K36me2 modifications on the CXCR4 locus. Primers specific for the six indicated regions were used for the analysis. **d** Transwell migration assay performed with Tregs from $Nsd2^{Treg\ WT}$ and $Nsd2^{Treg\ KO}$ mice of the indicated genotype in response to medium alone (nil) and 1 $\mu\text{g/ml}$ SDF, shown as the percentage of input cells. **e** A total of 2×10^6 $CD25^+ YFP^+$ Tregs from $Nsd2^{Treg\ WT}$ or $Nsd2^{Treg\ KO}$ mice were adoptively transferred into pregnant $CD45.1^+$ BoyJ mice on Day E12.5. The cell percentages of transferred Tregs in the uterine-draining lymph nodes and decidua were examined by flow cytometry 48 h after injection. $n = 7$. **f** Flow cytometric analysis of the Treg ($CD4^+ YFP^+$) percentage in the bone marrow (BM) of 8-week-old $Nsd2^{Treg\ WT}$ and $Nsd2^{Treg\ KO}$ mice. $n = 7$. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns not significant

Cxcr4^{fl/fl} mice to obtain *Nsd2/CXCR4* double KO mice (designated *Nsd2/CXCR4^{Treg dko}* mice). We speculated that if CXCR4 regulation is the primary function of *Nsd2* in Tregs, *Nsd2* deficiency would not cause further fetal loss in the setting of CXCR4 loss. Indeed, following mating with male BALB/c mice, *Nsd2/CXCR4* double KO mice did not display more severe fetal loss than *Cxcr4* single-gene KO mice but rather a similar fetal absorption phenotype (Fig. 6a, b).

This was also the case for the reduced Treg level phenotype in the decidua—*Nsd2/CXCR4* double deficiency resulted in a similar reduction in Tregs in the decidua compared to CXCR4 deficiency alone (Fig. 6c). Therefore, these data suggest that *Nsd2* promotes Treg migration into the decidua primarily by regulating CXCR4 expression, although we cannot completely exclude the involvement of other minor mechanisms.

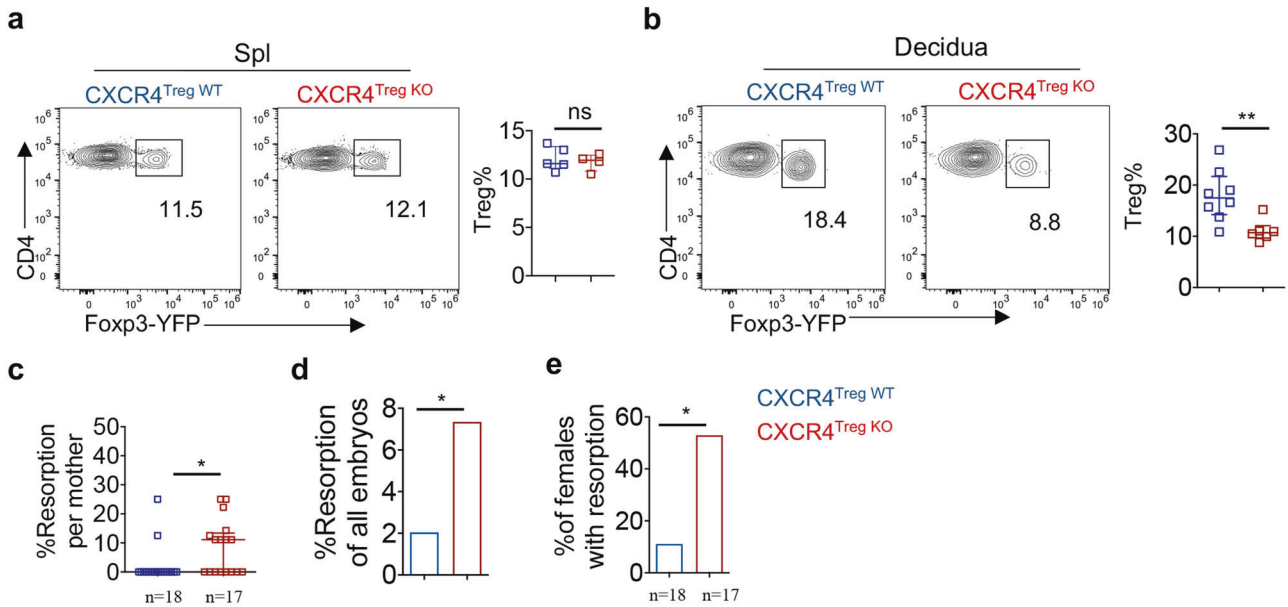


Fig. 5 Treg-specific CXCR4 conditional KO mice displayed decidal Treg reduction and fetal abortion phenotypes similar to those of *Nsd2*^{Treg KO} mice. **a** Flow cytometric analysis of the Treg (CD4⁺YFP⁺) percentage and number in the spleen (Spl) of 8-week-old CXCR4^{Treg WT} (*n* = 5) and CXCR4^{Treg KO} mice (*n* = 4). **b** Representative flow cytometric analysis of YFP⁺ Treg cells in the decidua of CXCR4^{Treg WT} and CXCR4^{Treg KO} mice previously mated with BALB/c males on Days E13.5–E14.5. *n* = 6–8. **c** Percentage of resorption observed in individual mothers with the indicated genotype. **d** Percentage of resorbed embryos in all CXCR4^{Treg WT} and CXCR4^{Treg KO} pregnancies resulting from mating with male BALB/c mice. Two-sided Fisher's exact test was used to assess the significance. **e** Incidence of pregnancies with at least one resorption. Two-sided Fisher's exact test was used to assess the significance. **P* ≤ 0.05; ***P* ≤ 0.01; ns not significant

DISCUSSION

The epigenetic regulation of Treg development and function remains incompletely understood. Here, we investigated the role of *Nsd2*-mediated H3K36me2 modification in Tregs. Although upregulated in Tregs compared to conventional T cells, *Nsd2* was not required for Treg development or function in lymphoid organs and was thus dispensable for systemic immune homeostasis. However, following mating with allogeneic male mice, *Nsd2*-deficient Tregs failed to be efficiently recruited to the maternal–fetal interface in pregnant mice, and thus, local immune tolerance was disrupted. Mechanistically, we found that *Nsd2* promoted the expression of the chemokine receptor CXCR4 to facilitate Treg migration into the decidua, where the CXCR4 ligand CXCL12 is abundantly expressed, as reported. Using conditional KO mice, we provided genetic evidence for the role of CXCR4 in Treg recruitment to the maternal–fetal interface and its epigenetic regulatory mechanism involving the H3K36me2 methyltransferase *Nsd2*. Clinically, the CXCR4 expression level is negatively associated with miscarriage, and women with spontaneous miscarriage have lower CXCR4 expression at the maternal–fetal interface [41]. Our data are consistent with this observation and provide mechanistic insight. It will be important to assess whether the CXCR4 reduction in spontaneous miscarriage patients leads to a local Treg decrease.

Because of the fundamental importance of immune suppression, Tregs have been proposed to be used in immunotherapies for immune-related diseases. For example, a number of clinical trials evaluating Treg adoptive transfer in kidney and liver transplantation have been initiated, with the ultimate goal of achieving a drug-free tolerance status [42, 43]. Treg therapy was also reported to be effective in clinical studies of type 1 diabetes mellitus [44]. In terms of pregnancy-related diseases, such as recurrent spontaneous pregnancy loss, although no clinical studies have been performed yet, much work has been done with mouse pregnancy models. Adoptive transfer of Tregs efficiently prevented fetal rejection in an abortion-prone mouse model [34, 45, 46]. Even for pathogen infection-induced

pregnancy disorders, Treg transfer exhibited a strong beneficial effect [47]. These mouse studies provide a strong rationale for pursuing clinical applications in pregnancy diseases in the future. However, as a cell-based therapy, the clinical translation of Treg adoptive transfer is complicated and challenging. For example, to suppress the anti-fetal immune response, infused Tregs need to be efficiently recruited locally to the maternal–fetal interface. Our finding that the H3K36me2 methyltransferase *Nsd2* regulates Treg migration provides a potential strategy to facilitate Treg accumulation at the maternal–fetal interface. As an epigenetic enzyme, *Nsd2* can easily be therapeutically targeted with small molecules. It is therefore feasible to design and obtain *Nsd2*-specific agonists and use them in combination with Treg adoptive transfer therapy for pregnancy-associated disease therapy.

MATERIALS AND METHODS

Mice

Nsd2^{fl/fl} mice were previously described [10]. *Cxcr4*^{fl/fl} mice, *Foxp3*^{cre-YFP} transgenic mice and B6-CD45.1 (Ptprca Pepcb/BoyJ) mice were obtained from the Jackson Laboratory. BALB/c male mice were obtained from the Animal Core Facility of Nanjing Medical University. Mice were maintained in a specific pathogen-free environment, and animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

Timed mating

Two female mice were set up in the afternoon with individual BALB/c males. Females were checked daily for the presence of a vaginal plug in the morning, and plugged females were separated from males; the day of plug detection was considered Day E0.5. Plugged females were analyzed for resorbed fetuses and flow cytometry at E14.5.

Cell preparations

Thymocyte, splenocyte, and LN cell suspensions were prepared by mashing the organs through 70- μ m cell strainers. Decidual cells were isolated as described previously, with modifications [48, 49]. In brief, fresh decidual samples were washed extensively in PBS with 100 IU/mL

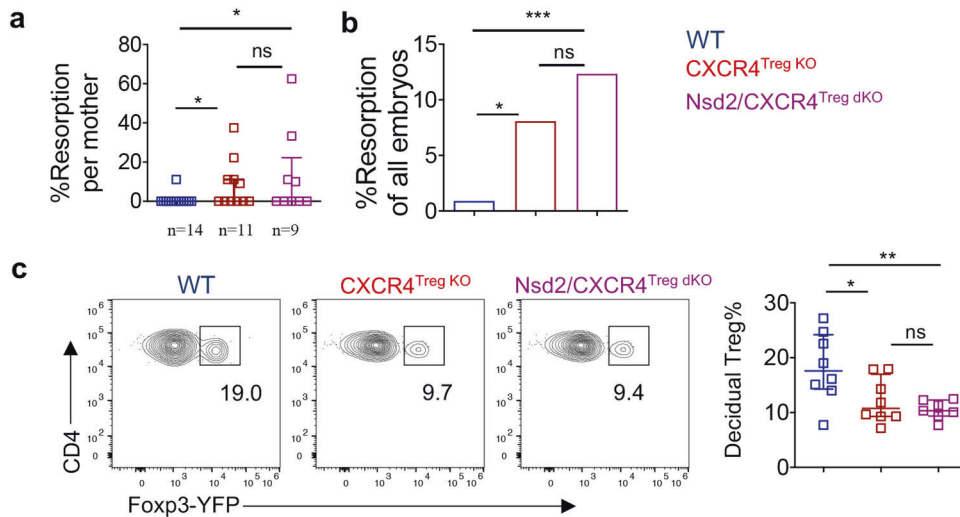


Fig. 6 Nsd2 promotes Treg migration into the decidua primarily by regulating CXCR4 expression. **a** Percentage of resorption observed in individual WT, CXCR4^{Treg KO}, and Nsd2/CXCR4^{Treg dKO} pregnancies resulting from mating with male BALB/c mice. **b** Percentage of resorbed embryos in all WT, CXCR4^{Treg KO}, and Nsd2/CXCR4^{Treg dKO} pregnancies resulting from mating with male BALB/c mice. Two-sided Fisher's exact test was used to assess the significance. **c** Representative flow cytometric analysis of YFP⁺ Tregs in the decidua and analysis of the decidua of female WT, CXCR4^{Treg KO} and Nsd2/CXCR4^{Treg dKO} mice mated with male BALB/c mice and analyzed on Days E13.5–E14.5. *n* = 8. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001; ns not significant

penicillin/streptomycin and then minced into small pieces. Decidual lymphocytes were digested at 37°C in 0.5 mg/mL collagenase type IV (Worthington Biochemical Corporation) and 0.1 mg/mL DNase I (Sigma-Aldrich) in RPMI 1640 medium for 40 min. The suspensions were strained through nylon mesh and separated on a 40–80% Percoll density gradient (GE Healthcare) after centrifugation at 1200×*g* for 25 min at room temperature.

Flow cytometry and cell sorting

For surface staining, cells were incubated with the indicated antibodies on ice for 30 min and washed with staining buffer (2% FBS in PBS with 0.1% NaN₃ and 50 mM EDTA). The following antibodies were used for flow cytometry: anti-CD4 (GK1.5), anti-IL-17A (TC11-18H10), anti-CD44 (IM7), anti-CD45.2 (104), anti-CD8a (53-6.7), anti-CTLA4 (UC10-4B9), anti-CXCR4 (L276F12), anti-CCR4 (2G12), anti-CCR5 (4B12), anti-CCR7 (HM-CCR5), anti-Foxp3 (FJK-16s), anti-GITR (DTA-1), anti-ICOS (7E.17G9), anti-IFN-γ (XMG1.2), anti-Ki67 (16A8), anti-LAG-3 (C9B7W), anti-OX-40 (OX-86), anti-PD-1 (RMP1-30), anti-CD25 (PC61.5), anti-CD62L (MEL-14), and anti-Neuropilin-1 (3DS304M). For cytokine-producing T-cell analysis, cells were stimulated for 4 h with phorbol myristate acetate (PMA; 50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences). Cells were stained for surface markers and then fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) before intracellular staining. Data were acquired on a Beckman Cytoflex and analyzed with FlowJo 10 software. Cell sorting was performed with a BD Aria Fusion. The sorting efficiency was tested, and only cells with high purity (>98%) were used for the experiments.

Migration assays

Migration assays were performed as previously described [50]. Briefly, dLN lymphocytes were incubated in RPMI 1640 with 0.5% fatty acid-free BSA (Sigma-Aldrich), 1% penicillin/streptomycin, and 10 mM HEPES for 1 h at 37°C to promote the resensitization of chemokine receptors. Then, the cells were plated onto transwell inserts placed in 24-well tissue culture plates (Corning) containing the indicated concentrations of stromal cell-derived factor 1 (SDF/CXCL12) in migration medium. Controls without SDF (null) or without the Transwell insert (input) were also studied. Plates were then incubated at 37°C in 5% CO₂ for 3 h, followed by enumeration of migrated cells by flow cytometry.

Western blot analysis

For western blot analysis, 1 × 10⁶ sorted CD4⁺ naïve T cells or Treg cells were lysed in 100 μl of SDS loading buffer. Nonspecific binding to

membranes was blocked by 5% milk in PBST (PBS with 0.05% Tween 20) for 1 h at room temperature, and then membranes were incubated with primary antibodies diluted in 5% BSA in PBS overnight at 4°C with constant rotation. Then, the membranes were incubated with secondary antibodies diluted in PBST for 1 h at room temperature. Chemiluminescence was detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

In vitro suppression assay

A total of 5 × 10⁴ CFSE-labeled naïve CD4⁺ T cells were cocultured with different numbers of CD4⁺ CD25⁺ YFP⁺ Treg cells in the presence of 1 × 10⁵ mitomycin-treated splenocytes and 2 μg/ml anti-CD3 in round-bottom 96-well plates for 3 days. The proliferation of labeled naïve T cells was determined by flow cytometry.

Adoptive Treg cell transfer

CD4⁺CD25⁺YFP⁺ Treg cells were purified using a BD Aria Fusion cell sorter. Sorted Treg cells were activated for 24 h with anti-CD3 (5 μg/ml; 145-2C11; Bio-X-Cell) plus anti-CD28 (5 μg/ml; 37.51; Bio-X-Cell) at a concentration of 10⁶ cells/ml in RPMI supplemented with 10% FBS, nonessential amino acids, sodium pyruvate, L-glutamine, HEPES, and β-mercaptoethanol. A total of 1 × 10⁶ activated cells were injected i.v. into pregnant BoyJ mice at E12.5. Forty-eight hours later, the dams were euthanized, and the decidua and LNs were collected. Leukocyte isolation and immunophenotyping of Tregs were performed to determine the proportion of adoptively transferred Tregs present in each tissue.

EAE model

The EAE mouse model was established as previously described. Eight-week-old mice were immunized subcutaneously with 200 μl of emulsified CFA and 200 mg of MOG35–55 peptide (MEVGVYRSPFSRVVHLYRNGK) and received intravenous injections of 200 ng of pertussis toxin at the time of immunization, which was repeated 48 h later. Mice were assigned scores daily on a scale of 0–5 in a double-blinded manner with the following criteria: 0, no disease; 1, tail paralysis; 2, wobbly gait; 3, hindlimb paralysis; 4, forelimb paralysis; and 5, moribund or dead. Gradations of 0.5 were assigned to mice exhibiting signs that fell between two of the scores listed above.

RNA-seq

Total RNA from 10⁶ splenic regulatory T cells was sorted for RNA extraction and library construction at RiboBio Co., Ltd. on an Illumina HiSeq 2500

platform and a 50-bp single-end module. Each sample combined a mixture of RNA from 3 mice of each genotype. Sequence reads were mapped to the mm10 reference genome using HISAT2 with default settings. Differential gene expression analysis was performed using the Bioconductor DESeq2 package with a threshold of RPKM of 5 and fold change of 1.5. Genes with reads per kilobase of transcript per million mapped reads were included for analysis. Raw data files and processed files were deposited in the Gene Expression Omnibus public database (GSE179489).

ChIP-qPCR assay

Briefly, sorted CD4⁺CD25⁺YFP⁺ Treg cells were cross-linked in 1% formaldehyde for 5 min at room temperature, quenched with 0.125 M glycine for 5 min at room temperature and lysed in 0.2% SDS with a protease inhibitor tablet for 10 min on ice. Genomic DNA was then fragmented by sonicating. For immunoprecipitation, anti-dimethyl histone H3 (07-274; Millipore) was used. DNA purification was performed using the ChIP DNA Clean & Concentrator (ZYMO Research) according to the manufacturer's instructions.

The sequences of the CXCR4 chip-qPCR primer sets were as follows:

Forward 15'-CATCTTAGTGTCTGCGTG-3'
Reverse 15'-ATTTTCATAAGCACATAGGG-3'
Forward 25'-TAGGAGTGC GGTTCTGA-3'
Reverse 25'-GCTGCTCCATCCAAA-3'
Forward 35'-TCGCTTAGGGAGGGT-3'
Reverse 35'-CGACCAACACACAGCG-3'
Forward 45'-CCATCTACTTCATCATCT-3'
Reverse 45'-AAAGAGGAGGTACGCCAC-3'
Forward 55'-GGCAGTCTATGTGGCGGT-3'
Reverse 55'-AACACCACCATCCACA-3'
Forward 65'-TAATGGTGGGTCTCGTC-3'
Reverse 65'-CAGCAGGCAAAGAAAGC-3'

Statistical analyses

Unless otherwise noted, statistical analysis was performed using the Mann–Whitney *U* test for individual biological replicates in Prism 9 (GraphPad). Fisher's exact test was used to assess the significance of the ratios of healthy and resorbed fetuses. The data in the figures are displayed as the median with interquartile range (IQR). *P* values are denoted in figures by **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

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AUTHOR CONTRIBUTIONS

LZ, YC, and XW conceptualized the project, designed the experimental approaches, analyzed the data, and prepared the paper. LZ, XL, YY, YW, and JW performed most of the experiments.

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COMPETING INTERESTS

The authors declare no competing interests.

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