IFN-γ induces aberrant CD49b⁺ NK cell recruitment through regulating CX3CL1: a novel mechanism by which IFN-γ provokes pregnancy failure

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Interferon-γ (IFN-γ), a pleiotropic lymphokine, has important regulatory effects on many cell types. Although IFN-γ is essential for the initiation of uterine vascular modifications and maintenance of decidual integrity, IFN-γ administration can also cause pregnancy failure in many species. However, little is known about the effector mechanisms involved. In this study, using an IFN-γ-induced abortion mouse model, we reported that no Dolichos biflorus agglutinin lectin-positive uterine natural killer (uNK) cells were observed in the uteri from IFN-γ-induced abortion mice. By contrast, the percentage of CD3⁺CD49b⁺ NK cells in the uterus and blood from a foetal resorption group was significantly higher than that of the control group. Similarly, significantly upregulated expression of CD49b (a pan-NK cell marker), CX3CL1 and CX3CR1 (CX3CL1 receptor) was detected in the uteri of IFN-γ-induced abortion mice. Using isolated uterine stromal cells, we showed that upregulated expression of CX3CL1 by IFN-γ was dependent on a Janus family kinase 2-signal transducers and activators of transcription 1 (JAK2-STAT1) pathway. We further demonstrated the chemotactic activity of CX3CL1 in uterine stromal cell conditioned medium on primary splenic NK cells. Finally, we observed increased recruitment of CD49b⁺ NK cells into the endometrium after exogenous CX3CL1 administration. Collectively, our findings indicate that IFN-γ can significantly increase uterine CX3CL1 expression via activation of the JAK2-STAT1 pathway, thus inducing CD49b⁺ NK cell uterine homing, and eventually provoke foetal loss. Thus, we provide a new line of evidence correlating the deleterious effects of IFN-γ on pregnancy with the aberrant regulation of CX3CL1 and CD49b⁺ NK cells.

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Abbreviations: Ab, antibody; CM, conditioned medium; Ct, cycle threshold; CX3CR1, CX3CL1 receptor; DAB, diaminobenzidine; DBA, Dolichos biflorus agglutinin; ECs, endothelial cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GD, gestational day; H&E, haematoxylin and eosin; IFN-γ, interferon gamma; JAK, Janus family kinase; KLRG1, killer lectin-like receptor G1; MHC, major histocompatibility complex; MLAp, mesometrial lymphoid aggregate of pregnancy; NK, natural killer; PFA, paraformaldehyde; pSTAT1, phosphorylated STAT1; PTX, pertussis toxin; RPL, recurrent pregnancy loss; STAT, signal transducers and activators of transcription; TGF/β, transforming growth factor β1; uNK, uterine NK

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The purpose of the present study was to investigate whether IFN-γ-induced pregnancy failure was associated with the uterine production of CX3CL1 and NK cell uterine homing. We demonstrated herein that IFN-γ induced a highly increased proportion of CD49b+ NK cells in the uterus and peripheral blood and it induced a significantly upregulated uterine expression of CX3CL1. Additionally, our data showed that uterine CX3CL1 facilitated CD49b+ NK cell recruitment into the uterus. To our knowledge, this is the first evidence showing that IFN-γ, via stimulating uterine CX3CL1 production, induces CD49b+ NK cell uterine homing and thus pregnancy failure in mice.

Results

IFN-γ administration resulted in foetal resorption. To evaluate the adverse effects of IFN-γ on pregnancy, syngeneically mated BALB/c females received an injection of 5000 U IFN-γ intraperitoneally on gestational day 6 (GD6), and the incidence of foetal resorption was assessed 2 days after treatment. A dose of 5000 U IFN-γ was used after preliminary comparisons of different doses (data not shown). We observed that IFN-γ administration significantly increased the resorption rate (Figure 1b). The control mice exhibited gross morphologically normal implantation sites (Figure 1ai). By contrast, IFN-γ at a dose of 5000 U resulted in embryo loss. Resorbing uterine contents were characterized by the degeneration of the decidua accompanied with thrombosis and haemorrhage. The remnants of decidual tissue had already passed into the uterine lumen with the embryos (Figure 1aii). Further histological examination of the control mice revealed a representative view of a GD8 embryo with well-developed deciduas and embryonic capsule (Figure 1a(iii)). By contrast, implantation sites from IFN-γ-treated mice displayed poorly developed deciduas and the absence of embryos (Figure 1a(iv)). However, the ovaries of IFN-γ-treated mice contained normal corpus luteum and exhibited no overt histological abnormalities compared with the control group (Figures 1av and vi). Interestingly, when the splenic cells of IFN-γ-induced abortion mice after erythrocyte lysis were transferred into syngeneically mated BALB/c on GD6, we also observed a 62.5% resorption rate 2 days posttransfer (Figure 1c), suggesting that IFN-γ-induced resorption was due to leucocytes. Thus, further experiments were designed to explore the causes underlying IFN-γ-induced resorption.

IFN-γ treatment enhanced the accumulation of the CD49b+ NK cell subset. Because uNK cells have critical functions in pregnancy,21 we examined whether IFN-γ treatment would alter the uNK cells. By performing immunostaining analysis, we found that the DBA lectin-positive cells were restricted to decidua basalis and mesometrial lymphoid aggregates of pregnancy (MLAp) of implantation sites in solvent control mice (Figures 2ai and iii). By contrast, no DBA-positive reaction was observed in the sections of uterus from IFN-γ-induced abortion mice (Figures 2aii and iv). Unexpectedly, CD49b was expressed at a significantly higher level in IFN-γ-treated mice (Figure 2b). Similar results were observed when CD49b expression was analysed on GD7 (Supplementary Figure 1A). Further, uteri were harvested to assess the percentage of CD3 CD49b+ NK cells in CD45+ leucocytes (see Supplementary Figure 1B for the gating strategy). The percentage of CD3 CD49b+ NK cells (lower-right quadrant) in the uterus from the IFN-γ-treated group was significantly higher than that from the control group (Figure 2c), as expected. Interestingly, a similarly significant increase was found in the peripheral blood (see Supplementary Figure 1C for the gating strategy) of IFN-γ-treated mice when compared with the control mice (Figure 2d). Overall, our findings suggested that IFN-γ induced a marked increase of CD49b+ NK cells in the uterus and peripheral blood.

IFN-γ significantly increased uterine CX3CL1 expression via activation of the JAK2-STAT1 pathway. To analyse whether the changes of NK cells were due to chemokines, quantitative PCR was performed to detect the expression of various chemokines in the uteri. The expression of CX3CL1 mRNA (Figure 3a, top panel) was markedly upregulated by IFN-γ treatment on GD8. The significantly enhanced expression of CX3CL1 was also confirmed by western blotting (Figure 3a, bottom panel). Furthermore, histological analysis revealed stronger staining in the luminal epithelium and glandular epithelium of the uterus from IFN-γ-treated mice compared with control mice (Figure 3b and Supplementary Figure 2A). Similarly, the expression of CX3CL1 protein was also upregulated in the uterus of IFN-γ-treated mice when analysed on GD7 (Supplementary Figure 2B). Interestingly, CX3CR1 expression was also significantly upregulated in the uterus from IFN-γ-treated mice (Figure 3c).

To further reveal how IFN-γ upregulated uterine expression of CX3CL1, we performed uterine stromal cell culture experiments. When uterine stromal cells were treated with IFN-γ at doses of 10, 100, 250 or 500 U/ml for 12 h, CX3CL1 protein expression was markedly induced in response to IFN-γ at a dose of 250 or 500 U/ml compared with the control group (Supplementary Figure 2C). Immunocytochemical staining displayed similar results (Figure 4a). When IFN-γ was administered at a dose of 250 U/ml, CX3CL1 expression levels varied in a time-dependent manner. The CX3CL1 expression level increased within 1 h, peaked at 6 h, continued for at least 12 h and then declined later (Supplementary Figure 2D). Thus, the treatment of IFN-γ at a dose of 250 U/ml for 12 h was applied in the following study.

Because the Janus family kinase-signal transducers and activators of transcription (JAK-STAT) pathway was widely investigated in IFN-γ-mediated signal transduction and transcriptional regulation signalling,22 we tested whether the regulation of CX3CL1 expression by IFN-γ occurred via the JAK-STAT pathway. When uterine stromal cells were pre-treated with AG490, a specific JAK2 inhibitor,23 there was a dose-dependent inhibition of CX3CL1 upregulation by IFN-γ (data not shown). At 10 μM, AG490 completely abrogated IFN-γ-mediated responses in uterine stromal cells (Figure 4b), suggesting that JAK2 mediated the IFN-γ-stimulated CX3CL1 expression. However, incubation with AG490 alone had no effect on the CX3CL1 basal level (Figure 4b). In addition, IFN-γ treatment strikingly increased phosphorylation of STAT1,
and AG490 pretreatment decreased phosphorylation of STAT1 by 55%, as expected (Figure 4c). To further confirm whether STAT1 phosphorylation affected CX3CL1 expression, fludarabine, a selective STAT1 inhibitor, was used.24 As shown in Supplementary Figure 2E, while fludarabine exhibited no influence on the amount of STAT1 protein, it inhibited STAT1 phosphorylation in a dose-dependent manner. Uterine stromal cells, after exposure to fludarabine at 100 μM for 2 h, showed a loss of 60% and 50% of CX3CL1 and pSTAT1 (phosphorylated STAT1), respectively (Figure 4d), suggesting a close correlation between STAT1 activation and the production of CX3CL1. Collectively, these data strongly suggested that IFN-γ upregulated CX3CL1 expression through a JAK2-STAT1 pathway. We then next explored whether and how IFN-γ-driven CX3CL1 regulated NK cell migration.

CX3CL1 facilitated peripheral NK cell migration. To explore whether CX3CL1 would induce the migration of NK cells, we first verified that peripheral NK cells expressed CX3CR1 at their surface. We performed immunostaining on NK cells, and observed a bright staining when cells were incubated with CX3CR1 Ab (Figure 5a). Then, the ability of peripheral NK cells to respond to CX3CL1 was assessed. Increasing the dose of CX3CL1 triggered a significant increase in cell migration; doses began at 100 ng/ml, and this effect reached a plateau at 500 ng/ml (Supplementary Figure 3A). Furthermore, an overnight preincubation of NK cells with pertussis toxin (PTX) at 500 ng/ml massively blocked the stimulatory effects of CX3CL1 (Figure 5b), indicating a CX3CL1-induced chemotaxis rather than chemokinesis.25

To mimic the uterine local environment, uterine stromal cells were isolated, cultured and used to prepare conditioned medium (CM). The chemotactic activity of the stromal cell CM (termed control CM) on NK cells was assessed. Chemotaxis to control CM increased robustly to 9.58-fold over that of the control group (Supplementary Figure 3B). Similarly, PTX partially abolished CM-mediated migration (data not shown).

Figure 1  IFN-γ administration resulted in foetal resorption. Syngeneically mated BALB/c female mice were injected with solvent or IFN-γ intraperitoneally on GD6 and killed on GD8. (a) Representative macroscopic views of a healthy uterine horn from a solvent-injected mouse (i) and an aborted uterine horn from an IFN-γ-injected mouse (ii) are shown. H&E staining of uterine (iii, iv) and ovarian (v, vi) paraffin sections from solvent-injected mice and IFN-γ-injected mice are shown. Arrows indicate the corpus luteum. Photomicrographs are representative of at least three mice in each group. Scale bar: 500 μm. (b) Ratio of foetal abortions induced by IFN-γ is shown. The numbers above the bars indicate the number of mice with abortion/normal pregnancy. The ratio of foetal abortions was calculated from the following formula: (no. of abortion/no. of abortion plus no. of normal pregnancy) × 100%. ***P < 0.001 by χ2. (c) Splenic cells of placebo-treated or IFN-γ-induced abortion mice after erythrocyte lysis were transferred into syngeneically mated BALB/c mice on GD6, and mice were killed 2 days posttransfer. The ratio of foetal abortions is shown, and the numbers above the bars indicate the number of mice with abortion/normal pregnancy. The ratio of foetal abortions was calculated as above. CL, corpus luteum; DB, decidua basalis; E, embryo; no., number.
IFN-γ treatment enhanced the accumulation of the CD49b⁺ NK cell subset. Syngeneically mated BALB/c female mice were injected with solvent or IFN-γ intraperitoneally on GD6 and killed on GD8. (a) Analysis of DBA lectin-stained uNK cells in the uteri by immunohistochemistry. Arrows indicate DBA lectin-positive cells. Photomicrographs are representative of five mice in each group. Panels iii and iv are higher magnifications of areas marked by the black rectangles in panels i and ii, respectively. Scale bar: 500 μm (i and ii) and 25 μm (iii and iv). (b) CD49b expression in uteri was analysed by quantitative PCR (top panel) and western blotting (bottom panel). Data show the mean ± S.E.M. of four independent experiments and are obtained from four mice of each group, respectively. *P < 0.05 by independent samples T-test. Flow cytometric analysis of cell suspensions from uteri (c) and peripheral blood (d). See Supplementary Figures 1B and C for the gating strategy and the percentages of CD3⁻CD49b⁺ NK cells (lower-right quadrant). Data show the mean ± S.E.M. of four (uteri) or five (blood) independent experiments and are obtained from four (uteri) or five (blood) mice of each group, respectively. **P < 0.01 by independent samples T-test. DB, decidua basalis; E, embryo.
Figure 3  IFN-γ significantly increased uterine CX3CL1 and CX3CR1 expression. Syngeneically mated BALB/c female mice were injected with solvent or IFN-γ intraperitoneally on GD6 and killed on GD8. (a) CX3CL1 expression was analysed by quantitative PCR (top panel) and western blotting (bottom panel) in uteri. Data show the mean ± S.E.M. of four independent experiments and are obtained from four mice of each group, respectively. *P < 0.05 by independent samples T-test. (b) CX3CL1 expression was analysed by immunohistochemistry in uteri. Arrows indicate that stronger staining is observed in the LE and GE of the uteri from IFN-γ-injected mice. Photomicrographs are representative of three mice in each group. Panels ii, iii and v, vi are higher magnifications of different areas marked by the black rectangles in panels i and iv, respectively. Scale bar: 500 µm (i and iv) and 50 µm (ii, iii, v and vi). (c) CX3CR1 expression was analysed by quantitative PCR (top panel) and western blotting (bottom panel) in uteri. Data show the mean ± S.E.M. of five (quantitative PCR) or three (western blotting) independent experiments and are obtained from five (quantitative PCR) or three (western blotting) mice of each group, respectively. *P < 0.05 and **P < 0.01 by independent samples T-test. E, embryo; GE, glandular epithelium; LE, luminal epithelium.

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More importantly, we further found that chemotaxis to IFN-γ-treated stromal cell CM (termed IFN-γ CM) was significantly increased compared with control CM, and this enhancement of chemotaxis by IFN-γ CM can be significantly reversed by preincubation of stromal cells with AG490 (Figure 5c). To clarify the effect of CX3CL1 in IFN-γ CM, a neutralizing anti-CX3CL1 monoclonal antibody (mAb) was used. Blocking of CX3CL1 with neutralizing mAb could partially inhibit the migration of NK cells toward IFN-γ CM, compared with the addition of an isotypic IgG control (Figure 5d).

Although CX3CL1 induced robust migration of peripheral NK cells in vitro, this may not reflect the action of this drug in vivo. To verify the effect of CX3CL1 in vivo, recombinant mouse CX3CL1 was administered intraperitoneally to BALB/c females. Compared with mice treated with placebo, mice treated with CX3CL1 showed a higher percentage of CD3⁺ CD49b⁺ NK cells in the uterus (Figure 6a). Similarly, a significant increase in CD3⁺ CD49b⁺ NK cells was found in the peripheral blood of CX3CL1-treated mice (Figure 6b). Therefore, these data showed that CX3CL1 was associated with increased proportion of CD49b⁺ NK cells in vivo. Collectively, our above results strengthened the idea that upregulated uterine expression of CX3CL1 by IFN-γ was conducive for the NK cell uterine homing from the periphery.
IFN-γ has been widely evaluated as a potential mediator of pregnancy failure in humans. We describe here that IFN-γ can significantly increase uterine CX3CL1 expression via activation of the JAK2-STAT1 pathway, thus inducing CD49b+ NK cell uterine homing, and eventually provoke foetal loss in syngeneically mated BALB/c mice. To our knowledge, this is the first comprehensive study to correlate the deleterious effects of IFN-γ during pregnancy with the aberrant regulation of CX3CL1 and NK cells.

IFN-γ concentration per implantation site was prominent during early pregnancy in the mice, and uNK cells were the main source of IFN-γ.10,26 As reported by Ashkar and Croyns,10 IFN-γ concentration was ~4 U per implantation site on GD6 and peaked on GD10, with ~10 U per implantation site. Implantation sites of IFN-γ- and IFN-γRα-null mice did not undergo normal gestation-induced spiral artery modification and contained elevated numbers of incompletely differentiated uNK cells and widespread necrotic decidua, suggesting that IFN-γ contributed to the initiation of uterine vascular modifications, maturation of uNK cells and maintenance of decidual integrity.27 In this report, when each female mouse received an injection of 5000 U IFN-γ intraperitoneally on GD6, we observed a higher resorption rate on GD8. Thus, although IFN-γ had critical roles in successful pregnancy, a supraphysiological dose of IFN-γ was harmful to conceptus. However, the ovaries of IFN-γ-treated mice exhibited no overt histological abnormalities, suggesting that IFN-γ did not exert its effects on ovaries in this model.

**Discussion**

IFN-γ has been widely evaluated as a potential mediator of pregnancy failure in humans. We describe here that IFN-γ can significantly increase uterine CX3CL1 expression via activation of the JAK2-STAT1 pathway, thus inducing CD49b+ NK cell uterine homing, and eventually provoke foetal loss in syngeneically mated BALB/c mice. To our knowledge, this is the first comprehensive study to correlate the deleterious effects of IFN-γ during pregnancy with the aberrant regulation of CX3CL1 and NK cells.

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The best studied mouse model of spontaneous foetal loss was the mating of CBA/J females with DBA/2 males.3 The abnormal resorption rate in the CBA/J × DBA/2 mating combination was thought to be because of activated NK cells.
and mononuclear cells expressing Mac-1 (CD11b) and F4/80. Additionally, human RPL (recurrent pregnancy loss) is associated with NK cells. Our data indicated that the percentage of CD3−CD49b+ NK cells in the blood and uterus from the foetal resorption group was significantly increased. Thus, our results suggested that CD49b+ NK cells were incompatible with successful pregnancy, which seemed to be consistent with a previous report that the cytotoxicity of CD49b+ NK cells was higher than that of CD49b− NK cells. Surprisingly, we observed no DBA-positive uNK cells in IFN-γ-induced abortion mice. Increasing evidence supports the idea that uNK cells provide major contributions to decidual and vascular remodelling. Thus, necrotic decidua and poor angiogenesis were found within the implantation sites of aborted mice that displayed the absence of uNK cells. 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CD49b+ NK cells were recruited from peripheral NK cells. Nonetheless, our data seemed to support the idea that upregulated uterine expression of CX3CL1 was associated with an increased proportion of CD49b+ NK cells in the uterus after IFN-γ administration in vivo. Moreover, IFN-γ-driven expression of CX3CL1 in uterine stromal cells was closely correlated with the increased migration of NK cells. In addition, the NK cell migration was significantly decreased when CX3CL1-neutralizing mAb was added to the CM, which clearly demonstrated that the chemotaxis of the uterine stromal cells CM was partially because of the presence of CX3CL1. Finally, we observed a greater increase in the recruitment of NK cells into the endometrium after exogenous CX3CL1 administration. Fraticelli et al. reported that functional CX3CR1 was expressed strongly in NK cells. In mice, CX3CR1 was identified at a late stage of NK cell development and regulated NK cell activity in vivo via promoting NK cell trafficking. We verified that NK cells expressed CX3CR1 at their surface and, interestingly, that CX3CR1 expression was significantly upregulated in the uterus after IFN-γ treatment. The leucocytes may account for the increased CX3CR1 expression because we could not detect CX3CR1 in uterine stromal cells (data not shown). The unavailability of CX3CR1 Ab for flow cytometry restricted us from detecting CX3CR1 expression on NK cells directly in vivo. Nonetheless, our data seemed to support the idea that CD49b+ NK cells were recruited from peripheral NK cells, and a bias towards CD49b+ NK cells may lead to pregnancy failure. It was reported that CX3CR1- or CX3CL1-deficient mice did not exhibit any overt histological abnormalities and behavioural abnormalities. It would be fascinating to investigate whether the responses of CX3CL1−/− or CX3CR1−/− mice to IFN-γ stimulus would be indistinguishable from those of wild-type mice.

In summary, our data indicate that exogenous IFN-γ administration leads to the aberrant modulation of CD49b+ NK cells in the uterus via the upregulated expression of CX3CL1. We have now added chemokines, regulators of leucocyte trafficking, to the list of factors that cause IFN-γ-induced pregnancy failure. This may be a novel mechanism by which IFN-γ causes pregnancy failure and may provide a theoretic basis for human embryo abortion therapy.

**Materials and Methods**

**Mice.** Eight- to ten-week-old bred BALB/c mice were purchased from Vital River Laboratories (VRL, Beijing, China). Mice, housed in a temperature- and humidity-controlled room with a constant photoperiod (12 L : 12 D), were fed ad libitum and had free access to tap water. Studies involving mouse usage were approved by the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). Pregnancy was achieved by caging female mice with a fertile male at a 2 : 1 ratio, and the day when a copulatory plug was observed was termed GD1.

**Treatment of mice with IFN-γ or CX3CL1 and histology.** For IFN-γ treatment, homogeneously mated BALB/c females were injected intraperitoneally with 5000 U IFN-γ (Peprotech, London, UK) or placebo (sodium phosphate containing 0.1% BSA) on GD6. For CX3CL1 treatment, homogeneously mated BALB/c females were injected intraperitoneally with 1 μg of CX3CL1 (R&D Systems, Minneapolis, MN, USA) or placebo (PBS containing 0.1% BSA) on GD6. Mice were killed by cervical dislocation on GD8, and foetal resorption was assessed by observing the contents of uterus. Mice without gross implantation sites or tissue debris in the uterus were considered not pregnant and excluded from the experiment.

For histological analysis, uteri and ovaries were removed and fixed in 4% parafomaldehyde (PFA) overnight at 4 °C. After fixation, tissues were treated with ethanol and xylene and embedded in paraffin. Sections of 5 μm in thickness were prepared and stained using haematoxylin and eosin (H&E).

**Splenocyte transplantation.** Splenocytes from placebo-treated or IFN-γ-induced abortion donor mice were gently homogenized in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 1% FBS (HyClone), penicillin/streptomycin (100 U/ml) and then shifted through a 37 μm cell strainer. Donor cells were depleted of RBCs using an ammonium chloride lysing solution (0.14 M NH₄Cl, 10 mM KHCO₃, and 1 mM EDTA). Cells were then washed with RPMI-1640 medium and resuspended in RPMI-1640 medium. Homogeneously mated BALB/c females on GD6 were used as recipients and were intravenously injected with 1 × 10⁷ donor cells in 100 μl RPMI-1640. Two days later, recipients were killed, and the uteri were examined for the ratio of foetal abortions.

**Total RNA isolation and quantitative PCR.** Total RNAs were extracted with a kit (BioTeke, Beijing, China) and then used as templates for reverse transcription (Promega, Madison, WI, USA). cDNA was amplified using SYBR Green MasterMix (ComWin Biotech Co. Ltd, Beijing, China) according to the manufacturer’s instructions. Quantitative PCR was performed with a LightCycler 480 (Roche, Indianapolis, IN, USA). The primers used are summarized in Supplementary Tables 1 and 2, and the target gene mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The fold change was calculated as 2−ΔΔCt (cycle threshold).

**Western blotting.** The following primary Abs were used: anti-CD49b, anti-STAT-1, anti-pSTAT-1 (Cell Signalling Technology Inc., Danvers, MA, USA), anti-CX3CL1 (eBioscience, San Diego, CA, USA), anti-CX3CR1 (eBioscience), anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GAPDH (Hangzhou Goodhere Biotechnology Co. Ltd, Hangzhou, China). Proteins were extracted by nondenaturing lysis buffer (Applygen, Beijing, China), and the concentration was determined by a bichoronic acid Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins were separated by SDS–PAGE and transferred onto a nitrocellulose membrane (Pall, New York, NY, USA). The membranes were blocked in 5% skimmed dry milk in TBST at 37 °C for 1 h and then incubated with primary Abs at 4 °C overnight, followed by incubation with secondary Abs conjugated to HRP at 37 °C for 1 h (KPL, Gaithersburg, MD, USA). Chemiluminescence reactions were performed with an ECL Detection Kit (Pierce), and images were acquired using a...
Kodak X-OMat film (Carestream, Xiamen, China). Bands were analysed using Bio-Rad Quantity One software (Bio-Rad, Hercules, CA, USA), and expression was calculated as the ratio of the signal for the specific protein to the signal for actin or GAPDH.

**Immunocytochemical staining.** Anti-CX3CL1 primary Ab and biotinylated-DBA lectin (Sigma-Aldrich, St. Louis, MO, USA) were used. Cryosections (8 μm) of uteri were fixed in 4% PFA for 15 min. After being washed in PBS, the sections were blocked with 3% hydrogen peroxide for 5 min and sequential 10% horse normal serum (2%BSG-BIO, Beijing, China) at 37 °C for 1 h. Then, the cryosections were incubated with anti-CX3CL1 primary Ab or biotinylated-DBA lectin at 4 °C overnight, followed by incubation with secondary Ab conjugated to HRP or streptavidin-HRP (ZSGB-BIO) at 37 °C for 1 h.

The isolated uterine stromal cells were cultured for 12 h and fixed in 4% PFA for 15 min. After being washed in PBS, the cells were blocked with PBS containing 1% BSA at 37 °C for 1 h and then incubated with anti-CX3CL1 primary Ab at 4 °C overnight, followed by incubation with a secondary Ab conjugated to HRP at 37 °C for 1 h. Cell and tissue slides were stained with dianinobenzidine (ZSGB-BIO) and counterstained with haematoxylin. Images were taken using a Nikon ECLIPSE Ni-U microscope and the NIS software (Nikon, Tokyo, Japan).

**Cell death and disease.**

**Isolation and primary culture of uterine stromal cells.** Uterine stromal cells were isolated from non-pregnant BALB/c mice, according to the protocol previously described, with minor modifications.46 In brief, uteri were dissected longitudinally and minced into small fragments. Uterine pieces were then placed in 1% trypsin (Sigma-Aldrich) and incubated in a sequence of PBS, for 1 h at 4 °C and 1.5 h at room temperature with pipetting up and down every 10 min. The tissues remaining after the digestion were washed two times with PBS and incubated for subsequent digestion in 0.1% collagenase (Sigma-Aldrich) at 37 °C for 1 h with pipetting up and down every 10 min. At the end of the digestion, tissues were immediately diluted in DMEM/F12 at a 1:1 ratio (HyClone) with 10% FBS and mixed thoroughly. Then, the digested cells (primarily stromal cells) were sifted through 76 and 37 μm cell strainers in a sequence and centrifuged. The pellet was washed two times with PBS. Cells were seeded at a density of 10^6 cells per 35 cm^2 in a dish containing DMEM/F12 (1:1) supplemented with 10% FBS. After uterine stromal cells adhered to the culture dishes, cells were transferred to serum-free DMEM/F12 (1:1) and starved for 24 h before treatment with various concentrations of IFN-γ. For inhibitor pretreatment, cells were incubated with various concentrations of AG490 (Sigma-Aldrich) or fludarabine (Selleck Chemicals, Houston, TX, USA) for 2 h before IFN-γ stimulation. After 12 h in culture, stromal cell CM was removed, centrifuged at 12,000 r.p.m. for 7 min and stored at −20 °C before use. The purity of isolated uterine stromal cells was above 90% (data not shown).

**Isolation of splenic NK cells.** NK cells were aseptically isolated by mechanical dispersion of the whole GD8 BALB/c spleen in RPMI-1640 medium supplemented with 1% FBS and penicillin/streptomycin (100 U/ml). Cell suspensions were subsequently passed through a 37 μm nylon mesh. After centrifugation, cells adhering to the culture dishes were transferred to serum-free DMEM/F12 (1:1) and starved for 24 h before treatment with various concentrations of IFN-γ. For inhibitor pretreatment, cells were incubated with various concentrations of AG490 (Sigma-Aldrich) or fludarabine (Selleck Chemicals, Houston, TX, USA) for 2 h before IFN-γ stimulation. After 12 h in culture, stromal cell CM was removed, centrifuged at 12,000 r.p.m. for 7 min and stored at −20 °C before use. The purity of isolated uterine stromal cells was above 90% (data not shown).

**Chemotaxis assay.** Quantitative NK cell transmigration assays were evaluated in 5 μm pore Transwell inserts (Corning, Corning, NY, USA), as described previously.32 Splenic CD3+CD49b+ NK cells in 100 μl were loaded in the upper well, and 600 μl of medium supplemented with various concentrations of CX3CL1 or stromal cells CM was added to the lower compartment. Cells were allowed to migrate for 2.5 h at 37 °C, with 5% CO₂, and then cells in the bottom chamber were collected and counted for 150 s using a FACS caliber (BD Biosciences, Franklin Lakes, NJ, USA). For treatment with PTX, cells were pretreated with PTX at 500 ng/ml overnight before the assay. Where indicated, blocking of CX3CL1 was performed by adding 5 μg/ml anti-CX3CL1-neutralizing mAb (R&D Systems) or control rat IgG.
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