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Genetic deletion of Cxcl14 in mice alters uterine NK cells

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ABSTRACT

The uterine natural killer cells (uNK cells) are the major immune cells in pregnant uterus and the number of uNK cells is dramatically increased during placentation and embryo development. The uNK cells are necessary for the immune tolerance, cytokine secretion and angiogenesis of placenta. Former studies indicated that the population expansion of uNK cells was accomplished through recruitment of NK cell precursors from the spleen and bone marrow, but not proliferation of NK cells. However, the necessary molecules within this process were little understood. Here in our study, we found the co-localized expression of Cxcl14 protein with uNK cells in E13.5 pregnant uterus. Moreover, we used Cxcl14 knockout mice to examine uNK cells in mesometrial lymphoid aggregate of pregnancy (MLAp) and decidua basalis (DB) of E13.5 pregnant uterus and found significantly decreased uNK cells in Cxcl14$^{-/-}$ pregnant uteri compared with Cxcl14$^{+/-}$ pregnant uteri. To further explorer the molecular change in MLAp and DB after Cxcl14 knockout, we isolated the MLAp and DB from Cxcl14$^{+/+}$ and Cxcl14$^{-/-}$ pregnant uteri and performed microarray analysis. We found many genes were up and down regulated after Cxcl14 knockout. In conclusion, our results suggested the important function of Cxcl14 in uNK cells and the proper level of Cxcl14 protein were required to recruit NK cells to pregnant uterus.

1. Introduction

During placentation, specialized immune cells subset, including uNK cells, macrophage, monocytes, dendritic cells and neutrophils were recruited and activated. Their number increased dramatically at this stage, providing many immune cytokines for placentation [3,13]. These cells have been found in both human and rodents. The NK cell lineage, named with uNK cells [11] was one of these important cells. Around E4.5, a few, small, granular lymphocytes appear in the mesometrial region of implantation site [4]. As gestation advances, these cells proliferate, differentiate, enlarge and generate abundant cytoplasmic granules [9]. These uNK cells accumulate in mesometrial lymphoid aggregate of the pregnancy—MLAp also called metrial gland [4]. The uNK cells are transiently existed, because there is only one tenth of peak population remains near term and their population will decrease in the uterus after parturition [5]. The uNK cells are critical for the immune tolerance, cytokine secretion and angiogenesis during placentation [13]. Previous studies suggested that the expansion of uNK cells was accomplished through recruitment of NK cell precursors from the spleen and bone marrow [3,13]. However, the necessary molecules within this process were not clear.

Cxcl14, a chemokine of CXC family was cloned in 1999 [6], but the function of Cxcl14 *in vivo* is still elusive. Previous studies indicated that Cxcl14 was involved in chemo attraction, angiogenesis and cancer [2,12,16]. The expression of Cxcl14 was detected from E8.5 to E17.5, both in the embryo and placenta, indicating the effect of Cxcl14 in embryogenesis and placentation [20,22]. Similarly to mice, Cxcl14 mRNA was found highly expressed in human mid-gestation placenta [22]. Here, we investigated the expression and function of Cxcl14 in murine placentation. We found the expression of Cxcl14 was co-localized with uNK cells at E13.5. Moreover, we observed impaired population of uNK cells in mesometrial lymphoid aggregate of pregnancy (MLAp) and decidua basalis (DB) on E13.5 Cxcl14$^{-/-}$ pregnant uterus. Through microarray analysis, we found the expression of many genes was up and down regulated in MLAp and DB after Cxcl14 knockout. Our study first investigated the function of Cxcl14 on recruitment of uNK cells in MLAp and decidua of pregnant uterus and it will help us to understand the function of Cxcl14 *in vivo*.

2. Materials and methods

2.1. Generation of Cxcl14$^{-/-}$ mice and genotyping

Cxcl14 gene conditional knockout mice were produced as described previously by using Cre/loxP system ([23]). Cxcl14$^{+/-}$ mice

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Table 1
Primers for genotyping and RT-PCR.

	Primer		Size (bp)
Cxcl14(flox/flox)	1	5-GGTCCAAGTGTAAGTGTTCC-3	995 (primer1, 2) wild
	2	5-GTGAAGCTCATGACTCGGGTC-3	800 (primer1, 3) flox
	3	5-GCAATCCATCTTGTTCAATGGCC-3	
CAG-Cre(Tg/+)	1	5-CCTACAGCTCCTGGGCAACGTGC-3	1087 Tg
	2	5-CTAATCGCCATCTTCCAGCAGG-3	
	3	5-CTAGGCCACAGAATTGAAAGATCT3-	324 Internal control (IL-2)
	4	5-GTAGGTGGAAATTCTAGCATCATCC-3	
Cxcl14(genomic)		5-GCCCTCTGTCGCGGTCCTGC-3	325 wild 200 mutant
		5-GTCTTGTCAAAGGCAGAGAG-3	
Actin		5-CCCTAGGCACCAGGGTGT-3	272
		5-TTGAAGGTCTCAAACATGATCTG-3	
Cxcl14(mRNA)		5-GGGTCCAAGTGTAAGTGTTC-3	617
		5-GTAGTGCTGTGAACGGTCTC-3	
Gcm1		5-GCTGTCATCCCTTGTGGTCC-3	459
		5-CTGGCACGGTGACTTTCTATTC-3	
Tpbpα		5-GCCACTGTGCCATTGTCTAAG-3	340
		5-CCAGAACACTGCCTTTTGAATA-3	

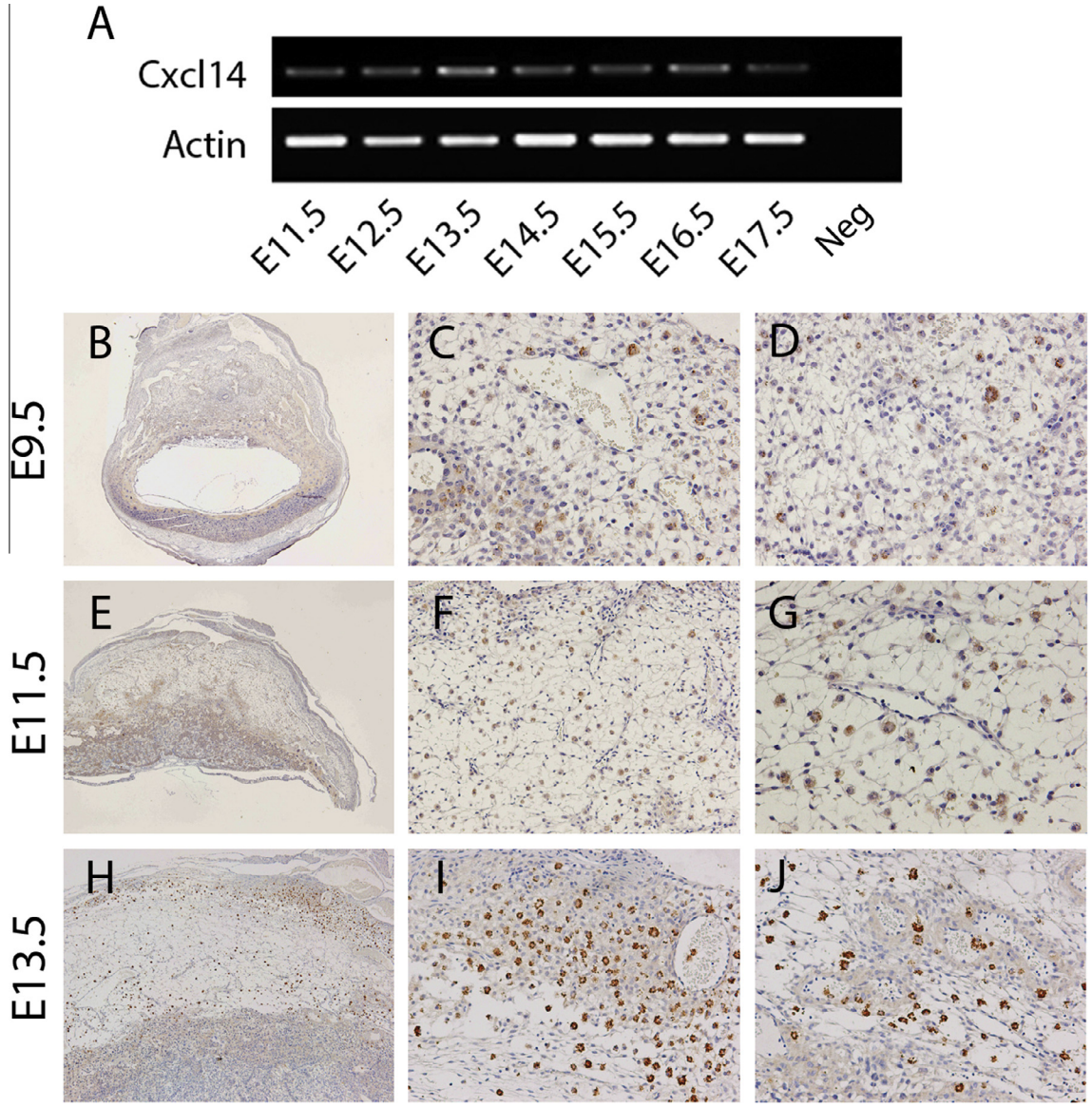

Fig. 1. Cxcl14 protein continuous expressed in mesometrial lymphoid aggregate of pregnancy (MLAp) and decidua basalis (DB) in wild-type implantation site and pregnant uterus. (A) Cxcl14 showed continually expression in placenta during mid and late gestation stage. "Neg" represents negative control. (B–D) Cxcl14 is prominently localized at decidua with a weak signal at E9.5 (X16, X200 and X200). (E–G) Cxcl14 expressed in MLAp and decidua basalis (F, G) of E11.5 pregnant uterus (X16, X100 and X200). (H–J) Cxcl14 showed a stronger expression in MLAp (I) and decidua (J) of E13.5 pregnant uterus (X20, X100, and X100).

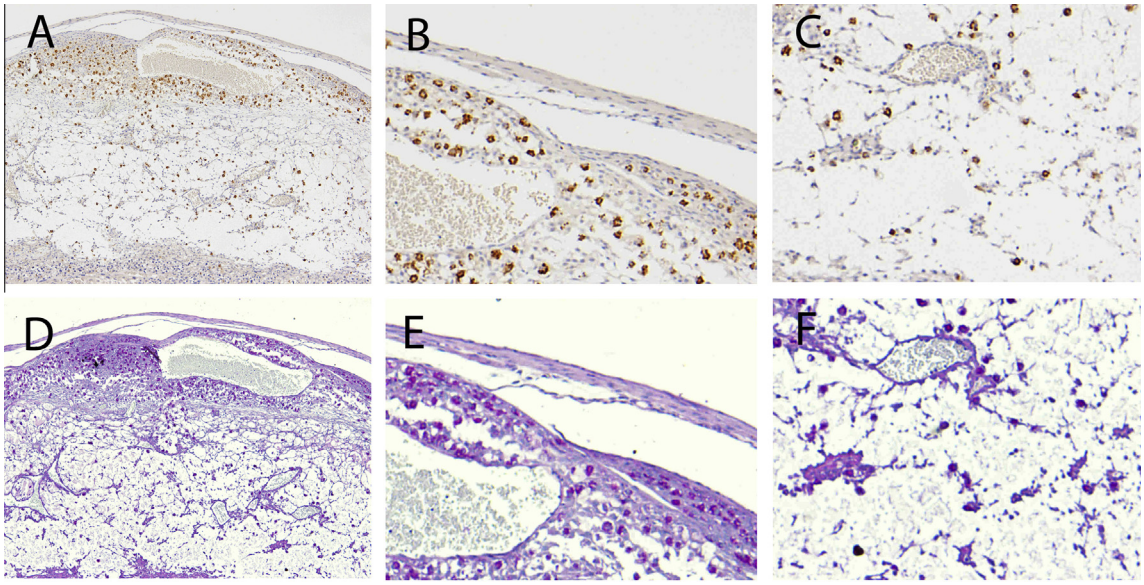

Fig. 2. Cxcl14 in MLAp and decidua basalis of E13.5 Cxcl14$^{+/+}$ pregnant uterus is expressed in uterine nature killer cells (uNK cells). (A–C) Immunohistochemistry of E13.5 Cxcl14$^{+/+}$ sagittal placental section with anti-Cxcl14 antibody. Note that Cxcl14 protein prominently localized in MLAp (B) and decidua basalis (C) (X40, X100 and X100). (D, E) PAS staining of E13.5 Cxcl14$^{+/+}$ serial placental section. Note that Cxcl14 in MLAp (E) and decidua basalis (F) is mainly expressed in uNK cells (X40, X100 and X100).

were obtained by cross Cxcl14$^{(flox/flox)}$ mice with CAG-Cre$^{(Tg/+)}$ mice in C57BL/6 background. Then intercross Cxcl14$^{+/-}$ mice to generate Cxcl14$^{-/-}$. Tail DNA was collected to performed genomic genotyping. For mRNA genotyping, embryos of littermates were collected. Primers for genotyping were shown in Table 1. The noon of the day when the vaginal plug observed was defined as E0.5. Animals were housed under normal light conditions (12 h light/12 h dark) with free access to food and water. Cxcl14$^{+/+}$ mice were used for Cxcl14 expression and PAS staining studies.

2.2. Immunohistochemistry

Freshly collected placentas were fixed overnight in 10% neutral buffered formalin at room temperature, dehydrated in increased concentrations of ethanol, cleared with xylene, and embedded in paraffin. Sections (5 μm) of the paraffinized blocks were made close to the placental midline (we stained every 10th slide with hematoxylin/eosin (H&E). Slides were examined in order to find the midpoint of the placenta-site of umbilical attachment, which was used as the major reference point for comparisons between mutants and wild-type littermates). For immunohistochemistry, tissue sections were deparaffinized and rehydrated. After washed three times in PBS for 5 min each, the endogenous peroxidases were inactivated using 1% H_2O_2 in PBS at room temperature for 10 min and followed by three further 5-min washes in PBS. By blocking with 5% BSA in PBS for 1 h at room temperature, the sections were stained with anti-Cxcl14 (GeneTex) overnight at 4 °C. The following day, slides were washed in PBS three times for 5 min each and then incubated with HRP-labeled secondary antibody (ZSGB-BIO) for 1 h at 37 °C. After three further 5-min washes in PBS, slides were incubated with DAB to terminate color development and followed by three times washes in distilled water. Sections were then counterstained with hematoxylin, dehydrated and permanently mounted.

2.3. Periodic Acid-Schiff (PAS) staining

Paraffin sections were dewaxed, hydrated to deionized water and then immersed in Periodic Acid Solution (PAS kit, Sigma) for 5 min at room temperature. After rinsed in distilled water for several times, slides were immersed in Schiff's Reagent (PAS kit, Sigma) for 15 min at room temperature, and then washed in running tap water for 5 min. Slides were counterstained in Hematoxylin Solution (PAS kit, Sigma) for 90 s and then rinsed in running tap water, dehydrated, cleared and permanently mounted [14].

2.4. RNA extraction and reverse transcription-PCR (RT-PCR)

Total RNA of the placenta was extracted using Trizol Reagents (Invitrogen), and genomic DNA was removed using the RNase-free DNase (Promega) before cDNA was prepared using M-MuLV Reverse Transcriptase (New England BioLabs). After cDNA was purified with QIAquick PCR Purification Kit (Qiagen), PCR were performed.

2.5. cDNA microarray analysis

Cxcl14+/– mice were mated with Cxcl14+/– mice, the embryos of pregnant females were collected on E13.5. After genotyping, the maternal part of Cxcl14–/– and Cxcl14+/+ placentas were dissect out to extract total RNA respectively ($n = 3$) [18]. Total RNA was extracted using PureYield™ RNA Midiprep System (Promega). Microarray hybridization was performed using a Mouse NimbleGen cDNA Microarray Kit (Roche), at CapitalBio Co., Ltd. (Beijing,China). Primary data were scaned using NimbleGen MS200 (Roche), and then extracted using NimbleScan system (Roche).

2.6. Statistical analysis

Values were presented as means ± SEM. All the data in the experiments were analyzed using one-way ANOVA as appropriate. P values at <0.05 were considered statistically significant.

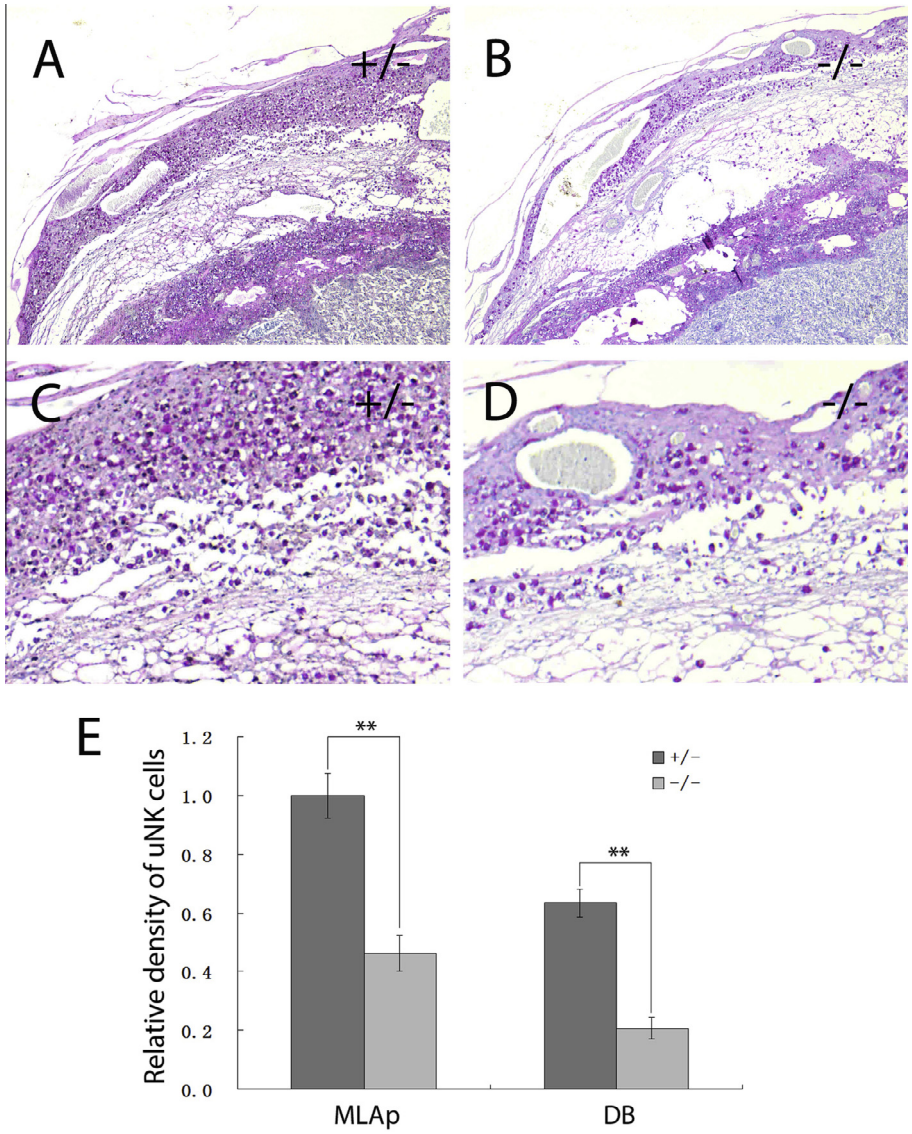

Fig. 3. Cxcl14 deficiency reduced the number of uNK cells in MLAp and decidua basalis of E13.5 pregnant uterus. (A–D) PAS staining of E13.5 Cxcl14$^{+/-}$ and Cxcl14$^{-/-}$ placental sections. Cxcl14$^{+/-}$ and Cxcl14$^{-/-}$ placentas were collected from littermates (X32, X32, 100X and 100X). (E) The density of uNK cells in MLAp and decidua was calculated as a percentage of the total number of uNK cells in the relative area of MLAp and decidua. The area of MLAp and decidua was analyzed with Image-Pro Plus software at E13.5. The number of uNK cells was quantified as the PAS positive nuclei in MLAp and decidua. Six images were randomly selected with uniform staining from each section of placenta for analysis, $n = 11$.

3. Results

3.1. Cxcl14 strongly expressed in mesometrial lymphoid aggregate of pregnancy and decidua basalis

To address the expression of Cxcl14 during pregnancy, we performed RT-PCR to test the transcriptional expression of Cxcl14 in placenta during pregnancy, and found that Cxcl14 show a stronger expression in placenta at mid-gestation stage, and then declined at E17.5 (Fig. 1A), which is consistent with published microarray data from GSE1986 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE1986) and GSE9954 [20]. In addition, we performed RT-PCR with the cDNA from early embryo, and Cxcl14 exhibited a clear expression (Supplementary Fig. S1B). To further establish the expression pattern of Cxcl14, immunohistochemistry was conducted on E9.5 implantation site and E11.5, E13.5 placentas from wild-type mice. Strong staining of Cxcl14 was detected in mesometrial lymphoid aggregate of pregnancy (MLAp) and decidua basalis (DB). On E9.5 implantation site, Cxcl14 was prominently located in decidua basalis with a weak signal (Fig. 1B–D). But later on E11.5 and E13.5 placentas, Cxcl14 displayed a stronger expression in MLAp (Fig. 1E–J), especially on E13.5 pregnant uterus (Fig. 1H–J). The clear expression of Cxcl14 was also detected in labyrinth on E13.5 placenta (data not shown).

3.2. The uNK cells in MLAp and decidua express Cxcl14

To establish which cells in placenta expressing Cxcl14, Periodic Acid-Schiff (PAS) staining was performed on E13.5 serial placental section, which stains the glycoprotein in uterine natural killer cells (uNK cells) granules ([14]). PAS positive signal was localized in uNK cells, which also expressed Cxcl14 protein (Fig. 2). This expression pattern of Cxcl14 in uNK cells suggested that Cxcl14 was necessary for the function of uNK cells and it might affects uNK cells *in vivo* through paracrine and autocrine, which is a widely existed phenomenon in chemokine [10].

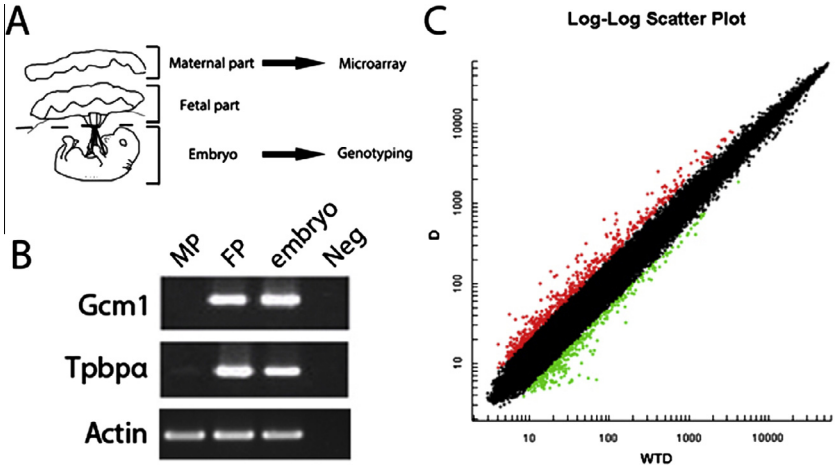

Fig. 4. Schematic representation of experimental procedures for microarray and validation of RT-PCR. (A) After removal of embryo and genotyping, the E13.5 maternal part was dissected out for total RNA extraction and microarray analysis. (B) RT-PCR analysis of labyrinthine marker genes from embryo, maternal and fetal parts of wild type placenta. "MP" represents maternal part of pregnant uterus; "FP" represents fetal part of placenta; "embryo" was selected as positive control; "Neg" represents negative control. Note that the dissection procedure was clean and the maternal part did not contain any fetal part. (C) Pseudocolored microarray scatter plot showed that transcriptional expression of some genes in MLAp and decidua has changed between Cxcl14$^{+/+}$ and Cxcl14$^{-/-}$.

Table 2
Up regulated genes after Cxcl14 knockout from microarray data.

GENE_ID	TRANSCRIPT_ID	GENE_NAME	Ratio
12144.00	AK137022	Blm	2.8
212670.00	AK142380	Catsper2	2.7
170733.00	AK080021	Klra17	2.6
226025.00	AK077331	Trpm3	2.4
21848.00	AK084870	Trim24	2.4
57247.00	AK043258	Zfp276	2.3
16319.00	AK088627	Incenp	2.3
320273.00	AK045519	B230208H11Rik	2.2
72147.00	AK085310	Zbtb46	2.2
22041.00	AK142599	Trf	2.2
18779.00	AK143853	Pla2r1	2.2
319535.00	AK043973	Zfp182	2.1
208922.00	AK044639	Cpeb3	2.1
208647.00	AK086757	Creb3l2	2.1
214058.00	AK122555	Megf11	2.1
240869.00	AK078748	Zbtb37	2.0
70626.00	AK080999	5730522E02Rik	2.0
26893.00	AK138409	Cops6	2.0
21749.00	AK021235	Terf1	1.9
22774.00	AK028305	Zic4	1.9
16975.00	AK030143	Lrp8	1.9
78801.00	AK078221	Ak7	1.9
319405.00	AK082555	D430036J16Rik	1.9
319503.00	AK084679	9930038B18Rik	1.9
219228.00	AK133637	Pcdh17	1.9
83395.00	AK137686	Sp6	1.9

Table 3
Down regulated genes after Cxcl14 knockout from microarray data.

GENE_ID	TRANSCRIPT_ID	GENE_NAME	Fold change
20185.00	AK137632	Ncor1	
64451.00	AK050040	Dip2a	0.42
20185.00	AK137632	Ncor1	0.4
229543.00	AK040147	Ints3	0.5
66614.00	AK078828	Gpatch4	0.5
228366.00	AK081797	Gyltl1b	0.5
320615.00	AK082462	Dopey1	0.5
319518.00	AK086050	4930402E16Rik	0.5
19210.00	AK086136	Ptdss1	0.5
18552.00	AK086210	Pcsk5	0.5
100126229.00	AK087850	Gm12758	0.5
94216.00	AK090269	Col4a6	0.5
381259.00	AK133112	Als2cr4	0.5
234725.00	AK133449	Zfp612	0.5
66384.00	AK135508	Srp19	0.5
100048895.00	AK140384	9330020H09Rik	0.5
208111.00	AK141688	C330019L16Rik	0.5

3.3. Cxcl14 deficiency reduce the number of uNK cells in MLAp and DB

To determine whether Cxcl14 deficiency in embryo modifies the uNK cells, placentas from Cxcl14 null mice and their congenic controls were studied. We crossed the Cxcl14$^{(flox/flox)}$ mice with CAG-Cre$^{(Tg/+)}$ mice in C57BL/6 background to obtain the heterozygous mice, then intercrossed Cxcl14$^{+/-}$ females with Cxcl14$^{+/-}$ males to generate Cxcl14$^{-/-}$ embryos and placentas. RT-PCR of genotyping with embryos was performed, and the results demonstrated that genomic DNA and mRNA of Cxcl14 are absent in Cxcl14$^{-/-}$ embryo (Supplementary Fig. S1). By crossing Cxcl14$^{+/-}$ females with Cxcl14$^{+/-}$ males and Cxcl14$^{+/-}$ females with Cxcl14$^{-/-}$ males, we collected enough E13.5 placentas. After PAS staining of all the placental sections, we analyzed the uNK cells in MLAp and DB areas with Image-Pro Plus software respectively, and found that the density of uNK cells in MLAp and DB areas significantly declines in Cxcl14$^{-/-}$ pregnant uteri compared with Cxcl14$^{+/-}$ pregnant uteri (Fig. 3) (n = 11), and this was not result from the decreased proliferation of uNK cells (Supplementary Fig. S2) (n = 8).

3.4. Microarray analysis of the transcription profile in maternal parts of Cxcl14$^{+/+}$ and Cxcl14$^{-/-}$ placenta

Since Cxcl14 deficiency of the embryo altered the uNK cells in MLAp and DB, we decided to figure out whether transcriptional expression of some genes in these two areas had changed in Cxcl14$^{-/-}$ pregnant uteri. As the receptors of Cxcl14 and its related signaling are still unknown, we then performed a cDNA microarray between Cxcl14$^{+/+}$ and Cxcl14$^{-/-}$ placentas to understand the possible transcriptional mechanism related with Cxcl14 in MLAp and DB. We collected E13.5 Cxcl14$^{+/+}$ and Cxcl14$^{-/-}$ placentas from littermates in which uNK cells in MLAp and DB of Cxcl14$^{-/-}$ pregnant uteri exhibited an obvious defect on that stage. According to the procedure shown in the schematic diagram (Fig. 4A), after genotyping of the embryos, we dissected the placenta into two parts: the maternal part that contained MLAp and DB; the fetal part con-

sisted of spongiotrophoblast and labyrinthine layer (Fig. 4A) [1,21]. Following with the total RNA extraction of the maternal parts, microarray hybridization was performed and the primary data was scanned and extracted (described in Section 2). At the same time, we also extracted the total RNA of maternal and fetal parts respectively from wild-type mice to perform the RT-PCR to check the expression of labyrinthine marker genes. The cDNA of maternal parts did not express Tpbpα which is a typical marker gene of spongiotrophoblast [3,15], and Gcm1 which is a typical marker gene of labyrinth, did not expressed either (Fig. 4B) [3,15]. This result of RT-PCR demonstrated that the dissection procedure of the maternal parts was clean without contamination from the fetal parts. Pseudocolored microarray scatter plot showed that transcriptional expression of some genes change between $Cxcl14^{+/+}$ and $Cxcl14^{-/-}$ placentas in MLAp and decidua (Fig. 4C). Some up regulated genes were shown in Table 2 and down regulated genes were shown in Table 3, and most of these genes were little studied. Since the receptors of Cxcl14 and its related signaling are still unknown, these genes may correlate with Cxcl14, and further study with them may bring a clue to understand Cxcl14 and its signaling.

4. Discussion

The uNK cells are unique subset of NK cells, not identical to the most NK cells in peripheral circulation [1,11,13]. About 40% of the NK cells in the decidua are $CD56^{bright}CD16^{-}$ NK cells [11]. In the peripheral circulation, these $CD56^{bright}CD16^{-}$ NK cells are minor NK population, about 5–10% of total NK cells. The functions of uNK cells during pregnancy are mostly unknown. Recent studies showed that uNK cells, but not peripheral NK cells, regulate trophoblast invasion *in vitro* and *in vivo* [11]. The uNK cells produce interleukin-8 and interferon-inducible protein-10 chemokines to control the invasion of trophoblast. Also, uNK cells secrets angiogenic factors and induce vascular growth in the decidua. The number of uNK cells increase dramatically during placentaion. Although most data suggests that the expansion of uNK cells is accomplished through recruitment of NK cell precursors from the spleen and bone marrow, the necessary molecules involved within this process are not clear.

Cxcl14 is an important chemotactic factor for immune cells [16]. Previous studies suggested that Cxcl14 is involved in chemotactic of immature dendritic cells [16]. Here we found the impaired population of uNK cells in MLAp and DB of $Cxcl14^{-/-}$ pregnant uteri, indicating its new function in chemotactic of NK cells. This is consistent with the recent study showing Cxcl14 could stimulate the migration of activated NK cells [17]. Since the uNK cells do not actively proliferate in E13.5 placenta [3,13], the impaired population of uNK cells in MLAp and DB of $Cxcl14^{-/-}$ pregnant uteri in our study was not similar to the observation in placenta growth factor knockout mice, where the decreased proliferation of uNK cells was observed [19]. We speculated the drop of Cxcl14 at protein level in embryo after Cxcl14 knockout might impair the recruitment of NK cells from maternal circulation. Since uNK cells expressed Cxcl14 endogenously, Cxcl14 might play an important role to stabilize uNK cells in placenta too. However, the interaction between maternal and fetal signaling with Cxcl14 was still elusive.

Former studies showed that the signaling mediated by CXCR4 and CCR5 receptors was necessary for the recruitment of NK cells to the decidua [7], and Cxcl12 was involved in the homing process of uNK cells [7]. Here in our study we first found the decreased population of decidual uNK cells after Cxcl14 knockout. As Cxcl14 and Cxcl12 are highly conserved chemokines of CXC family [8], the overlapping signaling of Cxcl14 and Cxcl12 may underlie the molecular pathway that mediate the homing of uNK cells. Since the receptors and signaling closely related with Cxcl14 are still unknown, we performed microarray analysis to examine the signaling change in decidua after Cxcl14 knockout. We found the expression of many genes were up and down regulated in decidua after Cxcl14 knockout, indicating their interaction with Cxcl14 and potential function in the regulation the homing of uNK cells in decidua. Above all, our study first demonstrated the effect of Cxcl14 in recruitment of uNK cells in pregnant uterus and it helps us to understand the function of Cxcl14 *in vivo*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.106.

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