Prevention of embryo loss in non-obese diabetic mice using adoptive ITGA2⁺ ISG20⁺ natural killer-cell transfer

Yi Lin^{1,2}, Huiqi Wang², Wenjing Wang², Shan Zeng², Yanmin Zhong² and Da-Jin Li³

¹Department of Obstetrics and Gynecology, School of Medicine, Institute of Obstetrics and Gynecology, Renji Hospital, Shanghai Jiaotong University, Shanghai 200001, People's Republic of China, ²College of Life Science and Technology, Institute of Tissue Transplantation and Immunology, Jinan University, Guangzhou 510632, People's Republic of China and ³Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai 200011, People's Republic of China

Correspondence should be addressed to Y Lin; Email: yilinonline@gmail.com
D-J Li; Email: djli@shmu.edu.cn

Abstract

Both regulatory T cells and regulatory natural killer (NK) cells may play essential roles in the maintenance of pregnancy. In this study, we show that a significantly high percentage of spontaneous embryo loss was observed in both allogeneic and syngeneic pregnant non-obese diabetic (NOD) mice. The percentage of embryo loss in allogeneic pregnant mice was further increased by the administration of anti-asialo ganglio-*N*-tetraosylceramide to deplete NK cells, but was decreased by the adoptive transfer of ITGA2⁺ ISG20⁺ (CD49b⁺ CD25⁺) NK cells from normal mice. No such trend was observed in syngeneic pregnant NOD mice. The pattern of CXCR4 (specific receptor for CXCL12) expression on NK cells was analyzed and NK-cell migration was confirmed by *in vitro* and *in vivo* migratory assays. Since CXCL12 production by murine trophoblast cells was confirmed previously, our findings suggest that the recruitment of peripheral CXCR4-expressing ITGA2⁺ ISG20⁺ NK cells into pregnant uteri may be important in the regulation of feto-maternal tolerance.

Reproduction (2009) **137** 943–955

Introduction

The mechanisms by which the allogeneic feto-placental unit is not rejected by the maternal immune system are being intensively investigated, and it is now becoming clear that decidual lymphocytes have unique functions in local cytokine production, endovascular invasion, and placental development. Recent research has also shown that both regulatory T (Treg) cells and regulatory natural killer (NK) cells may play essential roles in pregnancy maintenance (Saito *et al.* 2007). Under physiological conditions, NK cells are the dominant cell population until mid-gestation in the pregnant uterus of both humans and mice. Since ITGA2 (CD49b) antigen (its mAb is specific for ITGA2) is extensively expressed by NK cells but generally not expressed by other kinds of cells, it is commonly used as a pan-NK-cell marker (Arase *et al.* 2001).

It is widely believed that some NK-cell subsets play critical roles in the establishment and maintenance of pregnancy (Croy *et al.* 2002, Lin *et al.* 2006). Based on NK-cell deficiency models, previous studies found that an absence of NK cells during pregnancy is associated with reproductive deficits (Guimond *et al.* 1997, 1998, Lin *et al.* 2006). Additionally, depletion of residual NK

cells in non-obese diabetic (NOD)/severe combined immunodeficiency mice enhances the percentage of embryo loss (Shultz *et al.* 2003, Lin *et al.* 2005).

NK cells can be classified into four subsets by their cytokine profiles: NK1 cells produce IFNG and TNF; NK2 cells produce IL4, IL5, or IL13; NK3 cells produce TGFβ; and NKr1 cells produce IL10. All four of these subsets of NK cells were previously found in uterine cells derived from both humans (Higuma-Myojo *et al.* 2005) and mice (Lin *et al.* 2009b), and are believed to play their unique roles during pregnancy. However, the roles that these NK subsets play in allo-pregnancy tolerance remain to be clarified.

Additionally, the mechanisms by which peripheral NK cells migrate into pregnant uteri have yet to be determined. The chemokine receptor CXCR4 was first identified as an orphan receptor (Loetscher *et al.* 1994) whose ligand is stromal cell-derived factor-1 (SDF1, systematic name CXCL12; Zlotnik & Yoshie 2000). CXCL12 was originally isolated from murine bone marrow stromal cells and characterized as a pre-B-cell stimulatory factor (Nagasawa *et al.* 1994). Although most chemokines are pleiotropic and activate multiple receptors, CXCL12 actions are mediated exclusively via binding to CXCR4 (Jaleel *et al.* 2004). CXCL12 is a potent

chemotactic factor for NCAM1^{bright}CD16[−] NK cells, and modulates cell signaling for placental trophoblast cells in humans (Jaleel *et al.* 2004, Wu *et al.* 2004, 2005).

NOD mice are characterized by a NK-cell deficiency and suffer from a high level of embryo loss. NOD mice litters are 50% smaller than non-immunodeficient controls (Formby *et al.* 1987), which implies that ~50% of NOD embryos are rejected by the maternal immune system during pregnancy. This level is significantly higher than the frequency of rejections observed in non-immunodeficient murine strains due to murine chromosome abnormalities (4–5%; Clark *et al.* 1998). Additionally, a recent study has found that uterine NK-cell numbers were reduced in the decidua basalis of diabetic NOD females, whereas IFNG production was elevated. This suggests that peripheral NK cells in these mice have aberrant homing functions (Burke *et al.* 2007).

In this study, the percentage of spontaneous embryo loss was detected in allogeneic pregnant NOD × C57BL/6 mice (NOD females mated with C57BL/6 males) and syngeneic pregnant NOD × NOD mice. Additionally, inherent NK cells were depleted in these mice. In some cases, exogenous ITGA2⁺ISG20⁺ (CD49b⁺ CD25⁺) NK cells or ITGA2⁺ISG20[−] NK cells were adoptively transferred into these mice to investigate the effects of NK cells on pregnancy tolerance. Expression of CXCL12 by trophoblast cells from NOD mice was confirmed in a recent report (Lin *et al.* 2009a). In the present study, the expression pattern of CXCR4 on NK-cell subsets was detected. Both *in vitro* and *in vivo* migratory assays were performed to examine the homing function of peripheral NK cells into the pregnant uterus. If NK-cell depletion results in the failure of pregnancy and exogenous NK-cell transfer can prevent embryo loss, the notion that NK-cell subsets are a prerequisite for pregnancy success will be supported.

Results

Effects of NK-cell depletion on embryo loss

As shown in Fig. 1, the resorption rate of embryos in NOD × C57BL/6 mice in solvent control groups was $29.7 \pm 7.7\%$ (i.p. injection with RPMI1640 medium) and $34.7 \pm 8.9\%$ (i.v. injection with RPMI1640 medium), which was significantly higher than the rates from the corresponding BALB/c × C57BL/6 solvent control groups (3.5 ± 2.4 and $4.8 \pm 3.1\%$ respectively; $P < 0.01$ for both). NK-cell depletion with anti-ASGM1 increased embryo loss in both the BALB/c ($22.2 \pm 5.5\%$; $P < 0.05$) and NOD mice ($59.1 \pm 6.4\%$; $P < 0.01$).

In syngeneic pregnant mice, the resorption rate in all BALB/c groups was significantly lower than the corresponding NOD groups. However, no substantial influence on embryo resorption was detected after NK-cell depletion. Following the depletion of NK cells with anti-ASGM1, the resorption rate was $7.8 \pm 3.5\%$ for BALB/c and $46.9 \pm 8.3\%$ for NOD mice. These percentages were not significantly different from those in the corresponding control groups i.p. injected with solvent ($4.5 \pm 1.7\%$ for BALB/c and $37.5 \pm 3.7\%$ for NOD mice; Fig. 1).

As highlighted in the regions R1 and R2 of Fig. 1, the P values in R1 (all values are $P < 0.05$ or $P < 0.01$) and R2 (empty, indicating no statistically supported difference) indicated the difference of the resorption rate between the syngeneic and allogeneic BALB/c breedings as well as the syngeneic and allogeneic NOD breedings. Depletion of NK cells increased the resorption rate in allogeneic BALB/c breedings (3.5 ± 2.4 and $22.2 \pm 5.5\%$; $P < 0.05$), but had no effect on the syngeneic BALB/c breedings (4.5 ± 1.7 and $7.8 \pm 3.5\%$; $P > 0.05$).

None of the mice used in our experiments were diabetic over the course of observation.

Figure 1 Effects of NK-cell depletion or transfer on embryo resorption. $n=8$ for each group. P values are indicated. i.p. injection. i.v. injection. ITGA2⁺ISG20⁺ and ITGA2⁺ISG20[−] cells transferred. ITGA2⁺ISG20[−] and ITGA2⁺ISG20⁺ cells transferred. Anti-ASGM1 and anti-ASGM1 was injected to deplete NK cells *in vivo*. R1 and R2: in general, P values in R1 indicate that NK-cell depletion or transfer influenced the percentage of embryo resorption in allogeneic pregnant models. By contrast, the corresponding R2 is empty, which means that there is no statistically supported difference before and after NK-cell depletion or after transfer in syngeneic pregnant models. These results indicate that NK cells may play specific roles in allogeneic pregnancy tolerance.

Effects of ITGA2⁺ISG20⁺ or ITGA2⁺ISG20⁻ cell transfer on embryo loss

Adoptive transfer of ITGA2⁺ISG20⁺ cells into allogeneic pregnant NOD mice significantly decreased the percentage of embryo loss (34.7 ± 8.9 vs $11.0 \pm 3.4\%$; $P < 0.01$), but not in syngeneic NOD breedings (28.9 ± 9.9 and $42.1 \pm 11.0\%$; $P > 0.05$). Conversely, no change in embryo loss was observed when ITGA2⁺ISG20⁻ cells were transferred ($29.1 \pm 6.6\%$; Fig. 1). Furthermore, the percentage of embryo loss in allogeneic pregnant BALB/c mice was not significantly changed after ITGA2⁺ISG20⁺ or ITGA2⁺ISG20⁻ cell transfer (resorption rates 6.3 ± 3.2 and $5.0 \pm 3.3\%$ respectively).

In syngeneic pregnant models, the resorption rate in all BALB/c groups was significantly lower than corresponding NOD groups (Fig. 1). However, no substantial influence on embryo resorption was detected for ITGA2⁺ISG20⁺ NK-cell adoptive transplantations ($7.9 \pm 3.4\%$ in transferred group versus $7.7 \pm 3.3\%$ in control BALB/c mice; $42.1 \pm 11.0\%$ in transferred group versus $28.9 \pm 9.9\%$ in control NOD mice) or ITGA2⁺ISG20⁻ NK-cell adoptive transplantations ($11.0 \pm 4.5\%$ for BALB/c and $47.9 \pm 7.7\%$ for NOD mice; Fig. 1).

Comparison of placental NK-cell subset composition between BALB/c and NOD mice

In the present study, IFNG, IL4, TGFB, and IL10 were used as the representative cytokines for NK1, NK2, NK3, and NKr1 subsets respectively (Lin *et al.* 2009b). As shown in Fig. 2A, the percentage of NK1 cells present in the placental ITGA2⁺ cell population of allogeneic pregnant NOD mice was significantly higher than in BALB/c mice ($P < 0.01$). By contrast, the percentages of NK3 and NKr1 cells were significantly lower in NOD mice than in BALB/c mice ($P < 0.01$ for both). No significant difference was observed in the percentage of the NK2 subset between allogeneic pregnant BALB/c and NOD mice.

In syngeneic pregnant models, the percentage of NK1 cells was significantly higher in NOD mice than in BALB/c mice (41.2 ± 4.5 vs $5.3 \pm 0.7\%$; $P < 0.05$). Additionally, the percentage of NK3 cells ($2.3 \pm 0.7\%$ in NOD versus $10.4 \pm 1.3\%$ in BALB/c; $P < 0.05$) and NKr1 cells ($12.0 \pm 1.5\%$ in NOD versus $23.7 \pm 1.2\%$ in BALB/c; $P < 0.01$; Fig. 2A) was lower.

The absolute number of ITGA2⁺ cells purified from virgin BALB/c and C57BL/6 spleens was significantly higher than from virgin NOD mice ($P < 0.01$ for both). On the other hand, this absolute number was significantly higher in the placentas from both allogeneic ($(5.5 \pm 0.6) \times 10^6$) and syngeneic pregnant ($(5.8 \pm 0.6) \times 10^6$) BALB/c mice than from virgin BALB/c ($(2.7 \pm 0.4) \times 10^6$), C57BL/6 ($(2.5 \pm 0.3) \times 10^6$) and NOD ($(1.1 \pm 0.1) \times 10^6$) mice. Additionally, the absolute number of ITGA2⁺ cells purified from the placentas of each allogeneic pregnant

Figure 2 Production of intracellular cytokines in placental ITGA2⁺ cells detected by flow cytometry. MACS-purified ITGA2⁺ cells were used. Allo., allogeneic pregnant; Syn., syngeneic pregnant; SP, spleen; PL, placenta. (A) Data summary of flow cytometry. $n = 3$ for each group. The “%” mark on the y-axis indicates the percentage of cell staining positive for the cytokines listed on the x-axis. (B) Absolute number of MACS-purified ITGA2⁺ cells from each mouse. $n = 6$ for each group. P values are indicated.

BALB/c mouse was significantly higher than the absolute number of ITGA2⁺ cells from each NOD mouse ($P < 0.01$). Similar results were obtained in syngeneic pregnant models ($P < 0.01$; Fig. 2B).

Identification of placental FOXP3⁺ cells

As shown in Fig. 3A, a fraction of placental PTPRC⁺ cells were positive for FOXP3 in both allogeneic pregnant BALB/c and NOD mice, although the percentage was significantly higher in BALB/c mice ($P < 0.01$). More importantly, FOXP3 was expressed in both placental CD3⁺ cells and ITGA2⁺ cells in BALB/c mice (2.4 ± 0.2 and $11.0 \pm 0.5\%$ respectively) and NOD mice (1.2 ± 0.1 and $5.5 \pm 1.2\%$ respectively). The percentage of FOXP3⁺ cells in both the CD3⁺ population and the ITGA2⁺ population of BALB/c mice was significantly higher than that of NOD mice ($P < 0.01$ and $P < 0.05$ respectively). Additionally, the percentage of FOXP3⁺ cells in the ITGA2⁺ population was significantly higher than that found in the CD3⁺ population of both mouse strains ($P < 0.01$ in BALB/c and $P < 0.05$ in NOD mice). These results suggest that more uterine NK cells express FOXP3 than mature uterine T cells.

In syngeneic pregnant models, similar results were observed (Fig. 3A). A small fraction of FOXP3⁺ cells

Figure 3 Identification of FOXP3⁺ cells. Placental lymphocytes (PL) were detected. (A) Data summary of flow cytometry indicates that FOXP3 was expressed in PL CD3⁺T cells and PL ITGA2⁺ NK cells from both allogeneic and syngeneic pregnant BALB/c and NOD mice. However, the percentage of FOXP3⁺ cells was markedly lower in PTPRC⁺, CD3⁺, and ITGA2⁺ cells derived from allogeneic pregnant NOD than those derived from BALB/c mice. The percentage of FOXP3⁺ cells was also markedly lower in ITGA2⁺ cells derived from syngeneic pregnant NOD than those from BALB/c mice. $n=3$ for each group. (B) Data summary of absolute cell number. $n=6$ for each group. P values are indicated.

were detected in uterine PTPRC⁺ cells ($6.0 \pm 0.6\%$ in BALB/c versus $2.8 \pm 0.9\%$ in NOD mice; $P > 0.05$), CD3⁺ cells ($3.5 \pm 1.0\%$ in BALB/c versus $1.5 \pm 0.4\%$ in NOD mice; $P > 0.05$), and ITGA2⁺ cells ($9.1 \pm 1.2\%$ in BALB/c versus $4.5 \pm 0.4\%$ in NOD mice; $P < 0.05$).

The absolute number of purified uterine PTPRC⁺ cells and ITGA2⁺ cells was significantly higher in both allogeneic and syngeneic pregnant BALB/c mice than corresponding allogeneic and syngeneic pregnant NOD mice ($P < 0.01$ for all). However, the absolute number of CD3⁺ cells in allogeneic pregnant BALB/c mice was significantly lower than that in allogeneic pregnant NOD mice ($P < 0.05$; Fig. 3B), suggesting that more CD3⁺ cells infiltrate the pregnant uterus of allogeneic pregnant NOD mice than the corresponding control BALB/c mice.

In addition, more PTPRC⁺FOXP3⁺ cells, CD3⁺FOXP3⁺ cells, and ITGA2⁺FOXP3⁺ cells were detected in both allogeneic and syngeneic pregnant BALB/c mice than corresponding NOD mice (Fig. 3B).

Confirmation of the existence of CD3⁺ITGA2⁺FOXP3⁺ cells

As shown in Fig. 4, we confirmed the existence of uterine CD3⁺ITGA2⁺FOXP3⁺ cells by using magnetic-affinity cell sorting (MACS)-purified uterine CD3⁺ITGA2⁺ cells and tri-color flow cytometry. Since CD3⁺ cells have been excluded by negative MACS, including any CD3⁺FOXP3⁺ Treg cells, these CD3⁺ITGA2⁺FOXP3⁺ cells are considered a novel cell subset.

The percentage of CD3⁺ITGA2⁺FOXP3⁺ cells in CD3⁺ITGA2⁺ population was 11.5 ± 1.2 and $6.4 \pm 0.8\%$ ($P < 0.01$) in allogeneic pregnant BALB/c and NOD mice respectively, and 6.4 ± 2.2 and $2.8 \pm 1.5\%$ in syngeneic pregnant BALB/c and NOD mice respectively (Fig. 4E1). The percentage of CD3⁺ITGA2⁺ cells in ITGA2⁺ cell population was 99.9 ± 0.1 and $99.8 \pm 0.2\%$ in allogeneic pregnant BALB/c and NOD mice respectively, and 99.9 ± 0.1 and $99.8 \pm 0.2\%$ in syngeneic pregnant BALB/c and NOD mice respectively (Fig. 4E2). This suggests that most of the CD3⁺ cells have been excluded by negative MACS purification. In addition, the percentage of CD3⁺FOXP3⁺ cells in FOXP3⁺ cell population was merely 0.2 ± 0.1 and $0.1 \pm 0.1\%$ in allogeneic pregnant BALB/c and NOD mice respectively, and 0.1 ± 0.0 and $0.2 \pm 0.1\%$ in syngeneic pregnant BALB/c and NOD mice respectively (Fig. 4E3). This further confirms that most CD3⁺FOXP3⁺ cells have been excluded from FOXP3⁺ cells by negative MACS purification.

Status of CXCR4 expression on NK cells

As shown in Fig. 5A, the percentage of CXCR4⁺ cells was significantly higher among placental ITGA2⁺ ISG20⁺ population than in ITGA2⁺ISG20⁺ population isolated from both allogeneic pregnant BALB/c (76.9 ± 1.3 vs $14.6 \pm 1.1\%$) and NOD mice (81.4 ± 2.4 vs $6.0 \pm 1.6\%$; $P < 0.01$ for both). However, the percentage of CXCR4⁺ splenic ITGA2⁺ISG20⁺ cells isolated from virgin NOD mice was significantly lower than both

virgin BALB/c ($P<0.01$) and C57BL/6 mice ($P<0.01$). Additionally, splenic ITGA2⁺ISG20⁺ cells from virgin BALB/c expressed a higher percentage of CXCR4 than splenic ITGA2⁺ISG20⁻ cells from the same mice ($P<0.01$). Similarly, the percentage of CXCR4⁺ splenic ITGA2⁺ISG20⁺ cells from virgin C57BL/6 mice was also significantly higher than found in splenic ITGA2⁺ISG20⁻ cells from the same mice ($P<0.01$).

No such trends were detected in virgin NOD mice. These results suggest that the chemokine CXCL12 expressed by placental trophoblast cells is more likely to attract CXCR4⁺ cells to migrate into the pregnant uterus (Wu *et al.* 2004, 2005, Lin *et al.* 2006). Therefore, the splenic CXCR4⁺ITGA2⁺ISG20⁺ cells in BALB/c or C57BL/6 mice may be one of the candidate sources in the recruitment of uterine NK cells during allogeneic pregnancy.

By contrast, only a low level of CXCR4 expression was detected among the splenic ITGA2⁺ISG20⁺ and ITGA2⁺ISG20⁻ cells from NOD mice (4.8 ± 0.3 and $4.0\pm0.1\%$ respectively). This may contribute to the reduction in uterine NK cells present in the decidua basalis of allogeneic pregnant NOD mice. Most specifically, the insufficiency of CXCR4 expression among splenic ITGA2⁺ISG20⁺ NK cells may be related to the aberrant homing function of peripheral NK cells migrating to the pregnant uterus of allogeneic pregnant NOD mice.

CXCR4 expression in syngeneic pregnant models was also examined (Fig. 5A). The percentage of CXCR4⁺ cells in uterine ITGA2⁺ISG20⁺ groups was significantly higher in both allogeneic and syngeneic pregnant BALB/c (76.9 ± 1.3 vs $14.6\pm1.1\%$ for allogeneic pregnant and 94.6 ± 2.2 vs $3.5\pm0.7\%$ for syngeneic pregnant mice; $P<0.01$ for both) and NOD mice (81.4 ± 2.4 vs $6.0\pm1.6\%$ for allogeneic pregnant and 97.4 ± 2.0 vs $2.5\pm0.4\%$ for syngeneic pregnant mice; $P<0.01$ for both) than in ITGA2⁺ISG20⁻ groups (Fig. 5A).

The absolute number of ITGA2⁺ISG20⁺ cells and ITGA2⁺ISG20⁻ cells was also compared (Fig. 5B1). The absolute number of ITGA2⁺ISG20⁺CXCR4⁺ cells and ITGA2⁺ISG20⁻CXCR4⁺ cells is shown in Fig. 5B2.

More ITGA2⁺ISG20⁺ cells and more ITGA2⁺ISG20⁻ cells were detected in BALB/c and C57BL/6 mice than corresponding NOD mice, both in virgin mouse spleens ($P<0.01$ for both for ITGA2⁺ISG20⁺ cell population; $P<0.05$ for both for ITGA2⁺ISG20⁻ cell population) and placentas (in allogeneic pregnant mice, $(3.5\pm1.1)\times10^6$ in BALB/c versus $(1.0\pm0.3)\times10^6$ in NOD mice for ITGA2⁺ISG20⁺ cells; and $(2.0\pm0.6)\times10^6$ vs $(0.8\pm0.3)\times10^6$ for ITGA2⁺ISG20⁻ cells; $P<0.01$ for both; in syngeneic pregnant mice, $(3.2\pm1.2)\times10^6$ in BALB/c versus $(0.6\pm0.2)\times10^6$ in NOD mice for ITGA2⁺ISG20⁺ cells; and $(2.2\pm0.6)\times10^6$ vs $(1.0\pm0.5)\times10^6$ for ITGA2⁺ISG20⁻ cells; $P<0.01$ for both; Fig. 5B1).

On the other hand, the absolute number of uterine ITGA2⁺ISG20⁺ cells was significantly higher than ITGA2⁺ISG20⁻ cells in both allogeneic pregnant

($P<0.01$) and syngeneic pregnant ($P<0.05$) BALB/c mice. By contrast, more uterine ITGA2⁺ISG20⁻ cells were detected in syngeneic pregnant NOD mice than ITGA2⁺ISG20⁺ cells ($P<0.01$; Fig. 5B1). Notably, most of the CXCR4⁺ cells were ITGA2⁺ISG20⁺ in all of these groups, whereas only a small fraction of ITGA2⁺ISG20⁻ cells were CXCR4⁺ ($P<0.01$ for all groups; Fig. 5B2).

CXCL12-modulated in vitro migration of ITGA2⁺CXCR4⁺ cells

As detected in a transwell system, CXCL12 added to the lower chamber was more likely to preferentially attract splenic CXCR4⁺ cells from both strains of mice, whereas the CXCR4⁻ cells remained in the upper chamber (Fig. 6A). The percentage of ITGA2⁺CXCR4⁺ cells that migrated to the lower chamber was significantly higher than that found in the upper chamber. No significant difference was observed for this percentage between BALB/c mice and NOD mice.

However, the absolute number of BALB/c splenic cells that migrated into the lower chamber was significantly higher than the absolute number of NOD cells, even though the same number of ITGA2⁺ cells was plated in the upper chamber and the same amount of CXCL12 was added to the lower chamber (Fig. 6B). This result suggests that CXCL12 more likely to attract splenocytes from BALB/c mice than those from NOD mice, and this may be attributed to the significantly higher percentage of CXCR4 expressing cells among BALB/c mice splenocytes (Fig. 5).

In vivo migration assay of 5,6-carboxyfluorescein diacetate succinimidyl ester-labeled ITGA2⁺ISG20⁺ and ITGA2⁺ISG20⁻ NK cells

5,6-Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled cells were i.v. injected on gestational day 2.5 into NOD×C57BL/6 mice, and placental mononuclear cells were harvested 10 days later. More CFSE-labeled cells were found in the ITGA2⁺ISG20⁺ cell transfer group than in the ITGA2⁺ISG20⁻ cell transfer group (6.7 ± 0.8 vs $0.4\pm0.1\%$, $P<0.01$; $n=3$ /group). No CFSE signal was detected in the two solvent control groups ($0.1\pm0.0\%$ for both, $P<0.01$ versus the ITGA2⁺ISG20⁺ cell transfer group and $P<0.05$ versus the ITGA2⁺ISG20⁻ cell transfer group; Fig. 7). This may be attributed to the significantly higher percentage of CXCR4-expressing cells found among the splenic ITGA2⁺ISG20⁺ cells than among ITGA2⁺ISG20⁻ cells (Fig. 5B2).

Effects of ITGA2⁺ISG20⁺ cell transfer on placental NK-cell composition

As shown in Fig. 8, the placental NK-cell composition was 14.6, 7.3, 16.1, and 25.8% for INFG⁺NK1, IL4⁺NK2,

Figure 4 (legend continued)

Figure 4 Confirmation of the existence of CD3⁻ITGA2⁺FOXP3⁺ cells. CD3⁻ITGA2⁺ cells were positively isolated with microbead-conjugated anti-ITGA2, negatively isolated with microbead-conjugated anti-CD3, and analyzed by tri-color flow cytometry. Allo., allogeneic pregnant; Syn., syngeneic pregnant. (A1, A3, A5, B1, B3, B5, C1, C3, C5, D1, D3, and D5): isotype control for A2, A4, A6, B2, B4, B6, C2, C4, C6, D2, D4, and D6 respectively. Data represent one of three independently conducted experiments. (E1, E2, and E3): data summary of flow cytometric analysis. A small fraction of FOXP3⁺ cells were detected in MACS-purified CD3⁻ITGA2⁺ cells in all four groups. However, the amount of detected cell proportion was different among groups (*P* values are indicated; E1). Almost all the cells purified were CD3⁻ITGA2⁺ (E2), and almost no CD3⁺ cells were left in the FOXP3⁺ cell population, which confirmed the efficacy of negative CD3⁺ cell exclusion by MACS (E3). In E2 and E3, no significant difference was found for the cell percentages among groups. The % mark on the y-axis of E1, E2, and E3 indicates the percentage of FOXP3⁺ITGA2⁺ cells in the ITGA2⁺ population (E1), the percentage of CD3⁻ITGA2⁺ cells in the ITGA2⁺ population (E2), and the percentage of CD3⁺FOXP3⁺ cells in the FOXP3⁺ population (E3).

TGFB⁺NK3, and IL10⁺NK1 subsets in control NOD×C57BL/6 mice respectively. In comparison, the composition after ITGA2⁺ISG20⁺ cell transfer was 14.6, 6.6, 46.5, and 35.8% for NK1, NK2, NK3, and NK1 respectively. The percentage of NK1 and NK2 subsets did not significantly change, but the constitutional ratio for NK3 (*P*<0.01) and NK1 (*P*<0.05) subsets increased markedly.

Effects of NK-cell transfer on the percentage of FOXP3⁺ placental NK cells

As shown in Fig. 9, the percentage of FOXP3⁺ cells in placental NK cells increased markedly after ITGA2⁺ISG20⁺ cell transfer, compared with both the solvent control and ITGA2⁺ISG20⁻ transfer groups (*P*<0.01 for both). By contrast, no significant difference was observed between the latter two groups.

Discussion

Under physiological conditions, more than 70% of placental lymphocytes are NK cells until mid-gestation. They are characterized by the NCAM1^{bright}CD16⁻ phenotype in humans and the ITGA2⁺ phenotype in mice (Wu *et al.* 2005, Trowsdale & Betz 2006). Placental NK cells, especially those accumulated in the decidual basalis, are believed to be involved in mechanisms that influence reproduction. Most specifically, they are thought to mediate a mucosal immunological balancing function that prevents over-invasion of trophoblast cells into the maternal blood supply while still allowing a degree of placental access. A compromise is reached and both maternal and fetal gene systems, including NK-cell-expressed genes and NK-cell receptor genes, might affect this compromise (Moffett-King 2002).

Researchers increasingly believe that Treg cells are involved in the maintenance of feto-maternal immune

tolerance, as well as other kinds of alloimmune tolerance in both humans and mice (Aluvihare *et al.* 2004, Saito *et al.* 2005, Sakaguchi *et al.* 2006). Naturally occurring Treg cells are characterized by surface expression of CD4 and ISG20 and the intracellular expression of the fork head box p3 gene (*FOXP3*; Sakaguchi *et al.* 2006). In humans, primary unexplained infertility was found to be associated with reduced expression of *FOXP3* in endometrial tissue (Jasper *et al.* 2006). In our present study, *FOXP3* and *ISG20* molecules were found to be expressed by both CD3⁺T-cell subsets and ITGA2⁺NK-cell subsets, suggesting that CD3⁻ITGA2⁺FOXP3⁺ cells and ITGA2⁺ISG20⁺ NK cells may play a role similar to CD3⁺ISG20⁺FOXP3⁺ cells in the maintenance of allogeneic murine pregnancy. It was difficult, if not impossible for previous studies, to find these cell subsets without the assistance of MACS. We confirmed the existence of CD3⁻ITGA2⁺FOXP3⁺ NK cells using tri-color flow cytometry to analyze MACS negatively separated CD3⁻ cells and MACS-purified CD3⁻ITGA2⁺ cells. With the assistance of MACS technique, we can further investigate the biological functions of these cells.

According to previous reports, a significantly higher percentage of embryo loss was observed in NK-cell-deficient NOD mice (Formby *et al.* 1987, Burke *et al.* 2007), which is in agreement in our results. In the present study, the proportion of the placental IFNG⁺NK1 subset in ITGA2⁺ NK cells was significantly higher in NOD×C57BL/6 mice than in wild-type (WT) BALB/c×C57BL/6 mice. By contrast, the proportions of the TGFB⁺NK3 subset and IL10⁺NK1 subset were markedly lower. The proportion of the IL4⁺NK2 subset was similar to the level in WT mice. These results suggest that, although NOD mice suffer from NK-cell deficiencies, these mice maintain remnant NK-cell functions. Additionally, depletion of NK cells with anti-ASGM1 significantly enhanced the percentage of embryo loss in

Figure 5 CXCR4 expression by MACS-purified ITGA2⁺ISG20⁺ and ITGA2⁺ISG20⁻ NK cells. (A) Data summary of flow cytometry. The % mark on the y-axis indicates the percentage of CXCR4⁺ cells in the subsets indicated on the x-axis. (B1) Absolute number of MACS-purified ITGA2⁺ISG20⁺ and ITGA2⁺ISG20⁻ cells counted with a hemacytometer. (B2) Absolute number of ITGA2⁺ISG20⁺CXCR4⁺ and ITGA2⁺ISG20⁻CXCR4⁺ cells detected by flow cytometry. Allo., allogeneic pregnant; Syn., syngeneic pregnant. *n*=6 for each group. *P* values are indicated.

both WT and NOD mice (approximately sixfold more resorption in WT mice and twofold more resorption in NOD mice). These results support the assumption that placental NK cells are essential for a success pregnancy.

Furthermore, the percentage of embryo loss was significantly decreased by the adoptive transfer of splenic ITGA2⁺ISG20⁺ cells that were freshly purified from normal mice. In comparison, no such effect was observed when ITGA2⁺ISG20⁻ cells were transferred. These results suggest that the increased spontaneous embryo loss observed in NOD mice may be partially attributed to an insufficiency of ITGA2⁺ISG20⁺ cells in the pregnant uterus.

One of the most important results obtained in our current study is that depletion of NK cells by the injection of ASGM1 markedly increased the embryo resorption rate in the allogeneic pregnant BALB/c × C57BL/6 model, while no such effect was observed in the syngeneic pregnant BALB/c × BALB/c model. Additionally, adoptive transfer of NK cells, especially ISG20⁺ NK cells, significantly decreased embryo loss in allogeneic pregnant NOD × C57BL/6 model, while no such trend was observed in the syngeneic pregnant NOD × NOD model. These results suggest that NK cells, especially ISG20⁺ NK cells, may play an important role

Figure 6 *In vitro* ITGA2⁺CXCR4⁺ cell migration modulated by CXCL12. MACS-purified splenic ITGA2⁺ cells from virgin BALB/c and NOD mice were plated in the upper chamber of a transwell system with 8 μ m pore size, with or without CXCL12 added into the lower chamber. After cultivation for 6 h, cells in the upper and lower chambers were collected and counted. CXCR4 expression was analyzed using flow cytometry. (A) Data summary of flow cytometry. (B) Data summary of the absolute cell number in the lower chamber counted at the end of cultivation.

in the establishment and maintenance of maternal tolerance to allogeneic fetuses.

A recent report has stated that the homing functions of uterine NK cells were aberrant in pregnant NOD mice (Burke *et al.* 2007). This study also reported that reduced NK-cell numbers in the uterus were only found in prediabetic and diabetic NOD mothers, and that the NK-cell numbers were similar in non-diabetic NOD mice. In our previous report, diabetes was observed in 25% of pregnant NOD mice at 20 weeks of age in both syngeneic NOD $\times$ NOD and allogeneic NOD $\times$ C57BL/6-mating combinations (Lin *et al.* 2009a). Although diabetes was not diagnosed in the female NOD mice in our present study, an abnormal immunological status was detected. This included a decrease in both the relative and absolute numbers of ITGA2⁺ISG20⁺ cells, which suggests that these NOD mice may be in a prediabetic status.

Additionally, this study examined the CXCL12-induced migration of CXCR4⁺ NK cells using a transwell system. A significantly high number of CXCR4⁺ cells were found among the migrated cells, whereas this percentage was significantly low among the non-migrated cells. This demonstrates the ability of CXCL12 to specifically induce CXCR4⁺ cell migration. Notably,

although there was essentially no difference in the percentage of CXCR4⁺ cells among the migrated cells, the absolute number of migrated cells was significantly lower in NOD mice than in WT mice. This implies that fewer ITGA2⁺ NK cells were positive for CXCR4 in NOD mice than in WT mice. Data presented in Fig. 5B2 further confirm this observation.

During *in vivo* NK-cell migration assay, the transferred ISG20⁺ NK cells were more readily detected in the uterus than were ISG20⁻ cells. This could be attributed to the higher percentage of CXCR4 expression on ISG20⁺ NK cells than on ISG20⁻ NK cells. Additionally, we show that more ISG20⁺ NK cells were sensitive to CXCL12-producing trophoblast cells than ISG20⁻ NK cells. The CXCL12–CXCR4 pathway may be involved in the homing function of ITGA2⁺ISG20⁺ cells into the pregnant uterus. In allogeneic pregnant NOD mice, the percentage of CXCR4⁺ cells in splenic ITGA2⁺ISG20⁺ cell population was significantly lower than in WT mice. This may contribute to the decreased NK-cell number in the decidual basalis of NOD mice (Burke *et al.* 2007). Furthermore, the decreased embryo loss was not observed in syngeneic pregnant models after ITGA2⁺ISG20⁺ cell transfer. These observations suggest that these NK cells play a role specifically in allogeneic pregnant NOD mice, rather than the syngeneic pregnant NOD mice.

In previous work, CXCL12 expression was confirmed in murine trophoblast cells (Lin *et al.* 2009a). In the present study, CXCR4 expression on splenic ITGA2⁺ NK cells was determined. Both the percentage of CXCR4⁺ cells and the absolute number of CXCR4⁺ cells were significantly higher in the ISG20⁺ subset than in the ISG20⁻ subset. This implies that CXCL12-expressing trophoblast cells may preferentially attract ISG20⁺ NK cells to migrate into the pregnant uterus. Therefore, aberrant NK-cell homing functions in NOD mice may be partially due to a disorder in the CXCL12–CXCR4 axis.

The effects of transferred ITGA2⁺ISG20⁺ NK cells on the composition of NK-cell subsets at the feto-maternal interface were also evaluated. The baseline level of cytokine expression in the solvent control group was different from that in the untreated group in the detected samples (Figs 2 and 8). The reason for this is not clear, although we speculate that this difference may be due to either the process of injection or the difference among individual samples. After transfer, essentially no change was observed in the percentage of IFNG⁺NK1 or IL4⁺NK2 subsets, but the percentage of TGF β ⁺NK3 and IL10⁺NKr1 subsets were significantly increased. These results suggest that adoptive transfer of ISG20⁺ NK cells from normal mice is unable to reduce the high level of uterine NK1 subset proportion in NOD mice. However, it is able to enlarge the pool of NK3 and NKr1 cells, which subsequently shift the NK1/(NK3 plus NKr1) balance from an NK1-dominant state to an NK3- and NKr1-dominant status (Saito *et al.* 2007). In such an

Figure 7 *In vivo* migration assay of CFSE-labeled ITGA2⁺ISG20⁺ and ITGA2⁺ISG20⁻ NK cells. Cell transfer was performed on gestational day 2.5, and placentas were harvested on day 12.5. Data were from NOD×C57BL/6 mice. Fifty thousand cells were detected using flow cytometry without further staining. (A–D) Representative results from one of three independently conducted experiments. (E) Data summary of flow cytometry. The % mark on the y-axis indicates the percentage of CFSE⁺ cells. More CFSE-labeled cells were found in the pregnant uterus following transfer of ITGA2⁺ISG20⁺ cells than following the transfer of ITGA2⁺ISG20⁻ cells (6.7±0.8 vs 0.4±0.1%, ***P*<0.01).

environment, the allogeneic fetus could be tolerated by the maternal immune system. Hence, this shift in NK subsets may be the mechanism that caused the decrease in embryo loss after the adoptive transfer of ITGA2⁺ISG20⁺ cells.

Interestingly, the percentage of FOXP3-expressing cells in uterine NK-cell population was significantly higher after ITGA2⁺ISG20⁺ cell transfer than after ITGA2⁺ISG20⁻ cell and solvent transfers. This percentage essentially did not change after ITGA2⁺ISG20⁻ cell transfer, compared with the solvent control group. It is likely that these novel FOXP3⁺ NK cells are derived from the transferred exogenous ITGA2⁺ISG20⁺ cells. Since these cells express functional molecules including ISG20 and FOXP3, and are therefore similar to CD4⁺ISG20⁺FOXP3⁺ Treg cells, they may have similar functions in the induction of maternal tolerance to allogeneic fetuses (Sakaguchi *et al.* 2006). The detailed mechanisms remain to be clarified. Additionally, since the relationship between NK cells and reproductive failure remains unclear, corresponding research results should be explained cautiously (Rai *et al.* 2005).

In the future, it will be necessary to further investigate uterine NK-cell functions. For example, it is necessary to determine whether the different subsets of NK cells have overlapping cytokine profiles (i.e. do IFNG⁺ cells also produce IL4, TGFB, or IL10). These results will be more valuable in clarifying uterine NK-cell function. Also, it is not clear whether FOXP3 expression within the ITGA2⁺ISG20⁺ population can be linked to the production of a particular cytokine. Multi-colored flow cytometry should be able to show which of the four NK-cell subsets are FOXP3 positive. It is possible that TGFB and/or IL10 expression could be linked to the ITGA2⁺ISG20⁺FOXP3⁺ NK-cell population, just as TGFB and IL10 are linked to populations of

CD4⁺ISG20⁺FOXP3⁺T-regulatory cells (Masuyama *et al.* 2002, Green *et al.* 2003). However, this assumption requires further investigation.

It is not completely determined whether the precursors of uNK cells self-renew in the uterus or are recruited from other sites. Chantakru *et al.* (2002) suggest that NK cells do not self-renew in uterus, whereas cells from secondary lymphoid tissues of a pregnant donor produced a high level of uNK cell reconstitution in NK-cell-deficient mice. By contrast, King *et al.* (1999) implied that NK cells proliferate in peripheral blood, bone marrow, and decidua. The present study suggests that some NK cells immigrate into the pregnant uterus under the modulation of chemokines, and may play essential role in the maintenance of pregnancy.

Materials and Methods

Murine mating combinations

BALB/c, NOD, and C57BL/6 mice (8–12 weeks old) were purchased from the Experimental Animal Center of Zhongshan University (Guangzhou City, China), and kept under specific

Figure 8 Effects of ITGA2⁺ISG20⁺ cell transfer on the composition of uterine NK-cell subsets. Data summary of flow cytometry. The % mark on the y-axis indicates the percentage of uterine ITGA2⁺ NK cells positive for the cytokines indicated on the x-axis. *n*=4 for each group. NS, not significant.

Figure 9 Effects of NK-cell transfer on the percentage of FOXP3⁺ uterine NK cells. MACS-purified ITGA2⁺ISG20⁺ or ITGA2⁺ISG20⁻ cells were transferred on gestational day 2.5, and the percentage of FOXP3⁺ cells was detected on day 12.5. The % mark on the y-axis indicates the percentage of FOXP3⁺ uterine ITGA2⁺ NK cells. Data summary of flow cytometry. $n=4$ for each group. NS, not significant.

pathogen-free conditions. All experimental procedures involving animals were conducted in accordance with the national guidelines for animal usage in research (China), and permission was obtained from the ethics committee of Shanghai Jiaotong University and Jinan University. Institutional Review Board approval was obtained for the publication of this work, and there is no conflict of interest. Allogeneic BALB/c $\times$ C57BL/6 and NOD $\times$ C57BL/6 mating combinations were established as follows: female BALB/c and NOD mice were mated in natural cycling with male C57BL/6 mice. No superovulation was performed. Syngeneic BALB/c $\times$ BALB/c and NOD $\times$ NOD mating combinations were established in the same way. Detection of a vaginal plug was chosen to indicate day 0.5 of gestation.

All mice were tested for the development of diabetes by monthly tail vein blood sampling as well as measuring blood glucose levels using a Eusure blood glucose meter (Eumed Biotech., Jubel City, Hsinchu, Taiwan). Diabetes was defined by blood glucose values exceeding 250 mg/100 ml on two separate occasions (Levisetti *et al.* 2004, Lin *et al.* 2009a).

Purification of splenic and placental NK cells with MACS

Using MACS, splenic NK cells were purified from non-pregnant (virgin) C57BL/6, BALB/c, and NOD mice, and placental NK cells were purified from allogeneic and syngeneic pregnant BALB/c and NOD mice on gestational day 12.5. In brief, murine spleens and placentas, including decidual basalis, were cut into small pieces of $<1 \times 1 \times 1$ mm with ocular scissors under sterile conditions, and collected with a PBS to obtain single cell suspension. Single-cell suspensions were centrifuged through gradient medium (eBioscience Inc., San Diego, CA, USA) to obtain a lymphoid cell suspension (Lin *et al.* 2005, 2006). ITGA2⁺ cells were separated using microbead-conjugated anti-mouse ITGA2 (DX5, CD49B) and MACS separation columns (Mini-size; both from Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer's instructions. The ITGA2⁺ ISG20⁺ cells and ITGA2⁺ISG20⁻ cells were purified from ITGA2⁺ cells using microbead-conjugated anti-mouse ISG20 (Miltenyi Biotec). The purity and viability of the MACS-purified cells routinely exceeded 97 and 95%, as determined by flow cytometry (Lin *et al.* 2008, 2009b) and propidium iodide (Invitrogen) staining respectively (Fischer *et al.* 2002).

Absolute cell numbers

The absolute number of MACS-purified cells, including ITGA2⁺, ITGA2⁺ISG20⁺, and ITGA2⁺ISG20⁻ cells, was calculated using a hemacytometer. The absolute number of other cell subsets, such as FOXP3⁺ cells and CXCR4⁺ cells in PTPRC⁺, CD3⁺, ITGA2⁺, ITGA2⁺ISG20⁺, and ITGA2⁺ISG20⁻ subsets, was analyzed and calculated using FACS Calibur flow cytometer ($n=6$ for each group) because microbead-conjugated anti-mouse FOXP3 and anti-mouse CXCR4 antibodies were not available.

NK-cell depletion and ITGA2⁺ISG20⁺ or ITGA2⁺ISG20⁻ NK-cell transfer

Randomization of sampling was performed as follows: in the ten groups of 80 allogeneic pregnant mice, each mouse was nominated by the serial number to become pregnant. The number 1–80 was randomly selected, and the mouse corresponding to the first selected number was put into group 1, the mouse corresponding to the second selected number was put into group 2, and so on. Thus, ten mice were put into ten groups. In the second cycle, the mouse corresponding to the 11th selected number was put into group 1, the mouse corresponding to the 12th selected number was put into group 2, and so on. Thus, the second ten mice were put into ten groups. In this way, 80 mice were randomly put into ten groups, 8 mice per group. Random sampling was performed using the same method in syngeneic pregnant mice and any other groups in the present study.

Pregnant mice were randomized and either injected i.p. with 50 μ l rabbit anti-mouse asialo ganglio-N-tetraosylceramide stock solution (ASGM1; Wako Pure Chemical Inc., Osaka, Japan) to deplete NK cells (Clark *et al.* 1998, Lin *et al.* 2005) or injected i.v. with either MACS-purified ITGA2⁺ISG20⁺ cells or ITGA2⁺ISG20⁻ cells (2×10^5 cells/case; $n=8$ for each group). Solvent control groups were injected with RPMI1640 medium (Gibco-BRL) via the same route. All injections were made at gestational day 2.5 (Zenclussen *et al.* 2006), and the females were killed on day 12.5 to remove the uterus and calculate the implantation sites. Abortion (resorption) sites were identified by their small size and a necrotic, hemorrhagic appearance when compared with normal embryos and placentas. The percentage of resorption was calculated as the ratio of resorption sites to total implantation sites (resorption plus normal implantation sites), as described previously (Lin *et al.* 2004, Zenclussen *et al.* 2006).

Detection of intracellular cytokines in placental ITGA2⁺ NK cells

MACS-purified placental NK cells were incubated with permeabilization buffer (eBioscience) for 10 min, washed once with PBS, and stained with phycoerythrin (PE)-conjugated anti-mouse ITGA2 and FITC-conjugated anti-mouse IFNG, IL4, or IL10 (eBioscience Inc). For TGFB staining, cells were stained with PE-conjugated anti-mouse ITGA2 and mouse anti-human/mouse TGFB (US Biological, Swampscott, MA, USA),

followed by staining with FITC-conjugated rat anti-mouse immunoglobulin (Caltag Laboratories, Burlingame, CA, USA). Finally, the stained cells were assayed on a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Isotype control antibodies were used to establish isotype controls and single positive controls to exclude false-positive cells (eBioscience and R&D Systems, Minneapolis, MN, USA). All experiments in this study were performed in triplicate unless otherwise stated (Lin *et al.* 2008, 2009b).

Identification of placental FOXP3⁺ cells

Indicated cells were incubated with permeabilization buffer (eBioscience) for 10 min, washed once with PBS, stained with FITC-conjugated anti-mouse PTPRC, ITGA2, or CD3, together with PE-conjugated anti-mouse FOXP3, and analyzed on a FACS Calibur flow cytometer.

Confirmation of the existence of CD3[−] ITGA2⁺ FOXP3⁺ cells

MACS-purified uterine ITGA2⁺ cells were further incubated with microbead-conjugated anti-CD3 (Miltenyi Biotec) to negatively isolate ITGA2⁺CD3[−] cells by using Mini-MACS (Miltenyi Biotec). To confirm the existence of CD3[−]ITGA2⁺FOXP3⁺ cells, purified ITGA2⁺CD3[−] cells were stained with FITC anti-mouse ITGA2, PE anti-mouse FOXP3, allophycocyanin (APC) anti-mouse CD3 (eBioscience), and then detected using tri-color flow cytometry. FITC-, PE-, and APC-conjugated isotype antibodies (eBioscience) were used to establish isotype controls.

Detection of CXCR4 expression on NK cells from spleen and placenta

MACS-purified cells were stained with FITC-conjugated rat anti-mouse ITGA2 (eBioscience) and rat anti-CXCR4 monoclonal antibodies (R&D Systems) for 10 min. The cells were subsequently stained with PE-conjugated goat anti-rat immunoglobulin (Caltag Laboratories) and analyzed using flow cytometry.

In vitro migration assay of ITGA2⁺ cells

A total of 600 µl RPMI1640 culture medium (Gibco-BRL) supplemented with 10% fetal bovine serum (Sigma-Aldrich) in the presence or absence of 100 ng/ml recombinant mouse CXCL12 (R&D Systems) was added into the lower chamber of a 24-well transwell plate with an 8 µm pore size (Corning Costar, Cambridge, MA, USA). Plates were precoated with type 1 collagen (20 ng/ml; Sigma-Aldrich) overnight at 4 °C. The filters were subsequently blocked with 3% BSA in PBS to prevent non-specific migration. A total of 1 × 10⁶ MACS-purified splenic ITGA2⁺ cells obtained from virgin BALB/c (WT) or NOD mice were added to the upper chamber and were allowed to migrate for 6 h at 37 °C. Both the migrated and non-migrated cells were collected and counted using a hemacytometer. The percentage of CXCR4⁺ cells among the

harvested ITGA2⁺ cells was detected by flow cytometry, stained with PE-conjugated anti-mouse ITGA2 and rat anti-CXCR4 monoclonal antibodies (R&D Systems) followed by the staining with FITC-conjugated goat anti-rat immunoglobulin (Caltag Laboratories), and analyzed using flow cytometry.

In vivo migration assay of CFSE-labeled ITGA2⁺ ISG20⁺ and ITGA2⁺ ISG20[−] NK cells

Splenic ITGA2⁺ISG20⁺ and ITGA2⁺ISG20[−] cells from virgin C57BL/6 mice were purified by MACS and labeled with CFSE (Promega). For CFSE staining, a CFSE stock (10 mM in DMSO) stored at −20 °C was thawed, filtered with a 0.22 µm filter unit (Millipore, Carrigrohill, Co., Cork, Ireland) to obtain a germ-free solution, and diluted in PBS until the desired working concentration was attained. Cells were suspended in PBS to 10⁶ cells/ml and incubated for 10 min at 37 °C with CFSE at a final concentration of 1 µM. Cells were then washed and resuspended in culture medium for 15 min to stabilize CFSE staining. After the final wash, 2 × 10⁵ CFSE-labeled cells were i.v. injected into allogeneic pregnant BALB/c and NOD mice on gestational day 2.5. The pregnant mice were killed on day 12.5 for the collection of placental mononuclear cells. To determine whether the CFSE-labeled cells migrated into the pregnant uterus, the CFSE signal in these cells was analyzed by flow cytometry without further staining. Fifty thousand cells were analyzed in each case (Lin *et al.* 2009a).

Effects of ITGA2⁺ ISG20⁺ cell transfer on uterine NK-cell composition

Splenic ITGA2⁺ISG20⁺ cells were purified by MACS from virgin C57BL/6 mice and i.v. transferred into NOD × C57BL/6 mice on gestational day 2.5. The status of intracellular cytokine production, including IFNG, IL4, TGFβ, and IL10, in placental ITGA2⁺ NK cells was detected on day 12.5 using flow cytometry.

Effects of NK-cell transfer on the percentage of FOXP3⁺ cells in placental NK cells

MACS-purified splenic ITGA2⁺ISG20⁺ and ITGA2⁺ISG20[−] cells from virgin C57BL/6 mice were transferred into allogeneic pregnant NOD mice on gestational day 2.5, and the percentage of FOXP3⁺ cells in placental ITGA2⁺ population was detected on day 12.5 using flow cytometry.

Statistical analysis

Flow cytometry data were analyzed using Quad statistics (Lin *et al.* 2006). The resorption rate and cell percentages were compared using independent sample *t*-tests. Results were expressed as means ± S.E.M. (Lin *et al.* 2006).

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

This study was supported by grants from the National Basic Research Program of China (2006CB944007) (to D-J Li), the Key Project of National Natural Science Foundation of China (30730087) (to D-J Li), and the National Natural Science Foundation of China (30672231 and 30872761) (to Y Lin).

References

- Aluvihare VR, Kallikourdis M & Betz AG 2004 Regulatory T cells mediate maternal tolerance to the fetus. *Nature Immunology* **5** 266–271.
- Arase H, Saito T, Phillips JH & Lanier LL 2001 The mouse NK cell-associated antigen recognized by DX5 monoclonal antibody is CD49b ($\alpha 2$ integrin, very late antigen-2). *Journal of Immunology* **167** 1141–1144.
- Burke SD, Dong H, Hazan AD & Croy BA 2007 Aberrant endometrial features of pregnancy in diabetic NOD mice. *Diabetes* **56** 2919–2926.
- Chantakru S, Miller C, Roach LE, Kuziel WA, Maeda N, Wang WC, Evans SS & Croy BA 2002 Contributions from self-renewal and trafficking to the uterine NK cell population of early pregnancy. *Journal of Immunology* **168** 22–28.
- Clark DA, Chaouat G, Arck PC, Mittrucker HW & Levy GA 1998 Cytokine-dependent abortion in CBA $\times$ DBA/2 mice is mediated by the procoagulant fgl2 prothrombinase. *Journal of Immunology* **160** 545–549.
- Croy BA, Chantakru S, Esadeg S, Ashkar AA & Wei Q 2002 Decidual natural killer cells: key regulators of placental development. *Journal of Reproductive Immunology* **57** 151–168.
- Fischer K, Anderesen R & Mackensen A 2002 An improved flow cytometric assay for the determination of cytotoxic T lymphocyte activity. *Journal of Immunological Methods* **259** 159–169.
- Formby B, Schmid-Formby F, Jovanovic L & Peterson CM 1987 The offspring of the female diabetic ‘nonobese diabetic’ (NOD) mouse are large for gestational age and have elevated pancreatic insulin content: a new animal model of human diabetic pregnancy. *Proceedings of the Society for Experimental Biology and Medicine* **184** 291–294.
- Green EA, Gorelik L, McGregor CM, Tran EH & Flavell RA 2003 CD4 $^{+}$ CD25 $^{+}$ T regulatory cells control anti-islet CD8 $^{+}$ T cells through TGF- β -TGF- β receptor interactions in type 1 diabetes. *PNAS* **100** 10878–10883.
- Guimond MJ, Luross JA, Wang B, Terhorst C, Danial S & Croy BA 1997 Absence of natural killer cells during murine pregnancy is associated with reproductive compromise in TgE26 mice. *Biology of Reproduction* **56** 169–179.
- Guimond MJ, Wang B & Croy BA 1998 Engraftment of bone marrow from severe combined immunodeficient (SCID) mice reverses the reproductive deficits in natural killer cell-deficient tg ϵ 26 mice. *Journal of Experimental Medicine* **187** 217–223.
- Higuma-Myojo S, Sasaki Y, Miyazaki S, Sakai M, Siozaki A, Miwa N & Saito S 2005 Cytokine profile of natural killer cells in early human pregnancy. *American Journal of Reproductive Immunology* **54** 21–29.
- Jaleel MA, Tsai AC, Sarkar S, Freedman PV & Rubin LP 2004 Stromal cell-derived factor-1 (SDF-1) signalling regulates human placental trophoblast cell survival. *Molecular Human Reproduction* **10** 901–909.
- Jasper MJ, Tremellen KP & Robertson SA 2006 Primary unexplained infertility is associated with reduced expression of the T-regulatory cell transcription factor Foxp3 in endometrial tissue. *Molecular Human Reproduction* **12** 301–308.
- King A, Gardner L & Loke YW 1999 Co-stimulation of human decidual natural killer cells by interleukin-2 and stromal cells. *Human Reproduction* **14** 656–663.
- Levisetti MG, Suri A, Frederick K & Unanue ER 2004 Absence of lymph nodes in NOD mice treated with lymphotoxin- β receptor immunoglobulin protects from diabetes. *Diabetes* **53** 3115–3119.
- Lin Y, Zeng Y, Zhao J, Zeng S, Huang J, Feng Z, Di J & Zhan M 2004 Murine CD45 $^{+}$ CD86 $^{+}$ cells isolated from para-aortic lymph nodes in an abortion-prone model. *Journal of Reproductive Immunology* **64** 133–143.
- Lin Y, Chen Y, Zeng Y, Wang T & Zeng S 2005 Lymphocyte phenotyping and NK cell activity analysis in pregnant NOD/SCID mice. *Journal of Reproductive Immunology* **68** 39–51.
- Lin Y, Liang Z, Chen Y & Zeng Y 2006 TLR3-involved modulation of pregnancy tolerance in double-stranded RNA-stimulated NOD/SCID mice. *Journal of Immunology* **176** 4147–4154.
- Lin Y, Xu L, Jin H, Zhong Y, Di J & Lin Q 2009a CXCL12 enhances exogenous CD4 $^{+}$ CD25 $^{+}$ T cell migration and prevents embryo loss in non-obese diabetic mice. *Fertility and Sterility* [in press]. DOI: 10.1016/j.fertnstert.2008.01.109.
- Lin Y, Zhong Y, Saito S, Chen Y, Shen W, Di J & Zeng S 2009b Characterization of natural killer cells in nonobese diabetic/severely compromised immunodeficient mice during pregnancy. *Fertility and Sterility* [in press]. DOI: 10.1016/j.fertnstert.2007.08.087.
- Lin Y, Zhong Y, Shen W, Chen Y, Shi J, Di J, Zeng S & Saito S 2008 TSLP-induced placental DC activation and IL-10 $^{+}$ NK cell expansion: comparative study based on BALB/c $\times$ C57BL/6 and NOD/SCID $\times$ C57BL/6 pregnant models. *Clinical Immunology* **126** 104–117.
- Loetscher M, Geiser T, O'Reilly T, Zwahlen R, Baggiolini M & Moser B 1994 Cloning of a human seven-transmembrane domain receptor, LESTR, that is highly expressed in leukocytes. *Journal of Biological Chemistry* **269** 232–237.
- Masuyama J, Kaga S, Kano S & Minota S 2002 A novel costimulation pathway via the 4C8 antigen for the induction of CD4 $^{+}$ regulatory T cells. *Journal of Immunology* **169** 3710–3716.
- Moffett-King A 2002 Natural killer cells and pregnancy. *Nature Reviews. Immunology* **2** 656–663.
- Nagasawa T, Kikutani H & Kishimoto T 1994 Molecular cloning and structure for a pre-B-cell growth-stimulating factor. *PNAS* **91** 2305–2309.
- Rai R, Sacks G & Trew G 2005 Natural killer cells and reproductive failure – theory, practice and prejudice. *Human Reproduction* **20** 1123–1126.
- Saito S, Sasaki Y & Sakai M 2005 CD4 $^{+}$ CD25 high regulatory T cells in human pregnancy. *Journal of Reproductive Immunology* **65** 111–120.
- Saito S, Shiozaki A, Sasaki Y, Nakashima A, Shima T & Ito M 2007 Regulatory T cells and regulatory natural killer (NK) cells play important roles in fetomaternal tolerance. *Seminars in Immunopathology* **29** 115–122.
- Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, Shimizu J, Takahashi T & Nomura T 2006 Foxp3 $^{+}$ CD25 $^{+}$ CD4 $^{+}$ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunological Reviews* **212** 8–27.
- Shultz LD, Banuelos SJ, Leif J, Appel MC, Cunningham M, Ballen K, Burzenski L & Greiner DL 2003 Regulation of human short-term repopulating cell (STRC) engraftment in NOD/SCID mice by host CD122 $^{+}$ cells. *Experimental Hematology* **31** 551–558.
- Trowsdale J & Betz AG 2006 Mother's little helpers: mechanisms of maternal-fetal tolerance. *Nature Immunology* **7** 241–246.
- Wu X, Li DJ, Yuan MM, Zhu Y & Wang MY 2004 The expression of CXCR4/CXCL12 in first-trimester human trophoblast cells. *Biology of Reproduction* **70** 1877–1885.
- Wu X, Jin LP, Yuan MM, Zhu Y, Wang MY & Li DJ 2005 Human first-trimester trophoblast cells recruit CD56 bright CD16 $^{-}$ NK cells into decidua by way of expressing and secreting of CXCL12/stromal cell-derived factor 1. *Journal of Immunology* **175** 61–68.
- Zenclussen AC, Gerlof K, Zenclussen ML, Ritschel S, Zambon Bertoja A, Fest S, Hontsu S, Ueha S, Matsushima K, Leber J & Volk HD 2006 Regulatory T cells induce a privileged tolerant microenvironment at the fetal-maternal interface. *European Journal of Immunology* **36** 82–94.
- Zlotnik A & Yoshie O 2000 Chemokines: a new classification system and their role in immunity. *Immunity* **12** 121–127.

Received 27 September 2008

First decision 4 December 2008

Revised manuscript received 14 March 2009

Accepted 25 March 2009