CXCL12 enhances exogenous CD4$^+$CD25$^+$ T cell migration and prevents embryo loss in non-obese diabetic mice

Yi Lin, M.D.,[a] *Liang Xu, M.D.,*[b] *Haiyan Jin, M.D.,*[c] *Yanmin Zhong, M.Sc.,*[a] *Jingfang Di, M.Sc.,*[a] *and Qi-de Lin, M.D.*[b]

[a] Institute of Tissue Transplantation and Immunology, College of Life Science and Technology, Jinan University, Guangzhou; [b] Department of Obstetrics and Gynecology, Affiliated Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai; and [c] Department of Obstetrics and Gynecology, School of Medicine, Jinan University, Guangzhou, People's Republic of China

Objective: To investigate the possible role of CXCL12 in the migration of regulatory T (Treg) cells.
Design: Animal model–based study.
Setting: Academic.
Animal(s): Pregnant non-obese diabetic (NOD) mice were compared with non-immunodeficient mice.
Intervention(s): In vivo and in vitro CXCL12 induction.
Main Outcome Measure(s): Flow cytometric analysis and Treg cell migratory assay.
Result(s): A significantly high percentage of spontaneous embryo resorption was observed in both syngeneic and allogeneic pregnant NOD mice. The percentage of embryo loss in allogeneic pregnant NOD mice was significantly decreased by treatment with Treg cells and CXCL12 injection; however, no such effect was observed in syngeneic pregnant NOD mice. In addition, the migration of Treg cells induced by CXCL12 was confirmed by both in vitro and in vivo migratory assays. CXCR4, the specific receptor for CXCL12, was expressed more intensively on Treg cells than on non-Treg CD3$^+$ T cells, whereas CXCL12 was dominantly expressed in cytokeratin 7$^+$ trophoblast cells at an early stage of gestation, and its expression reduced gradually during pregnancy.
Conclusion(s): The higher level of embryo loss in allogeneic pregnant NOD mice may be due to the lack of Treg cells. CXCL12 can cause CXCR4$^+$ Treg cells to migrate into the pregnant uterus and establish a beneficial microenvironment for the fetus. (Fertil Steril® 2009;91:2687–96. ©2009 by American Society for Reproductive Medicine.)

Key Words: Chemokine, immune modulation, pregnancy tolerance, reproductive immunology, rodent model

Non-obese diabetic (NOD) mice are commonly used as a mouse model of insulin-dependent type 1 diabetes mellitus. A considerable percentage of NOD mice are insulin-deficient owing to the loss of pancreatic β-cells because of an autoimmune assault, and they show fertility impairment with poor implantation and low embryo viability (1–3). A previous study demonstrated that insulin-dependent diabetes occurs spontaneously in 9% and 80% of female NOD mice by 12 and 29 weeks of age, respectively. The litter is 50% smaller in NOD mice than in non-immunodeficient controls (4). This result implies that in NOD mice approximately 50% of 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, which is 4% to 5% (5).

The critical role of regulatory T (Treg) cells in the maintenance of maternofetal immune tolerance in both human and mice is gaining wider acceptance (6, 7). Naturally occurring Treg cells are characterized by the surface expression of CD4 and CD25 and the intracellular expression of the fork head box p3 gene (*Foxp3*) (8). Previous reports suggested that Treg cells can prevent diabetes in NOD mice (9, 10). However, it is not completely clear whether they play a role in the maternofetal tolerance in pregnant NOD mice.

The chemokine receptor CXCR4 was first identified as an orphan receptor (11); its ligand was later found to be the stromal cell-derived factor-1 (SDF-1; systematic name, CXCL12) (12). Stromal cell-derived factor-1 was originally isolated from murine bone marrow stromal cells and characterized as a pre-B cell stimulatory factor (13). Although most chemokines are pleiotropic and activate multiple receptors, SDF-1 functions are mediated exclusively by its binding to CXCR4 (14, 15). Stromal cell-derived factor-1 is a potent chemotactic factor for T cells (16) and modulates cell signaling in placental trophoblast cells (15).

Received November 9, 2007; revised and accepted January 15, 2008; published online April 1, 2008.
Y.L. has nothing to disclose. L.X. has nothing to disclose. H.J. has nothing to disclose. Y.Z. has nothing to disclose. J.D. has nothing to disclose. Q.-d.L. has nothing to disclose.
Supported by grants from the National Natural Science Foundation of China (no. 30530740 to Q.L., no. 30672231 to Y.L., and no. 30730087, partly to Y.L.).
Y. Lin and L. Xu share first authorship of this article.
Reprint requests: Qi-de Lin, M.D., Department of Obstetrics and Gynecology, Affiliated Renji Hospital, School of Medicine, Shanghai Jiaotong University, 145 Shandong Zhong Road, Shanghai, 200001, People's Republic of China (E-mail: shlinqide@hotmail.com).

In this study the percentage of spontaneous embryo loss in both syngeneic and allogeneic pregnant NOD mice was determined, and exogenous Treg cells were adoptively transferred into pregnant NOD mice in an attempt to investigate their effect on pregnancy tolerance. Furthermore, CXCL12 was injected to evaluate the possible role of this chemokine in the modulation of Treg cell migration into the pregnant uterus.

MATERIALS AND METHODS

Mice

BALB/c, C57BL/6, and NOD mice were obtained from the Experimental Animal Center of Zhongshan University (Guangzhou City, China) and bred under specific pathogen-free conditions. All the experimental procedures involving animals were in accordance with the national guidelines for animal usage in research (China), and permission was obtained from the ethics committee of Jinan University. Institutional review board approval was obtained for the publication of this work, and there is no conflict of interest. Syngeneic C57BL/6 × C57BL/6 and NOD × NOD and allogeneic BALB/c × C57BL/6 and NOD × C57BL/6 mating combinations were established. The time at which the vaginal plug was observed was designated as day 0.5 of pregnancy. Abortions commonly occur during the early stages of pregnancy; the status of local immunity at these stages is more important to the pregnancy outcome than that at later stages; and we expected to isolate a greater number of immunocompetent cells at an earlier gestation point. Therefore, the resorption rate of the embryos was calculated on gestational day 9.5 in the present study (17, 18). In brief, hysterolaparotomy was performed to collect embryo-depleted placentas and the associated decidual tissues, including the decidua basalis. The resorbed embryos were macroscopically identified by their small size (<20% of the average size), hemorrhage (a dark brown blood clot at the implantation site), and necrosis (5, 18–20). The embryo resorption rate was calculated with the following formula: resorption rate (%) = [number of resorbed embryos/total (resorbed + viable) number of embryos] × 100 (5, 18–21).

The development of diabetes was followed in all the mice by monthly tail vein blood sampling and blood glucose measurement with 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 (22).

Spontaneous Embryo Loss in Pregnant NOD Mice at Different Ages

The percentage of spontaneous embryo resorption was calculated on gestational day 9.5 in pregnant NOD × NOD and NOD × C57BL/6 mice at ages of 12, 16, and 20 weeks; pregnant C57BL/6 × C57BL/6 and BALB/c × C57BL/6 mice of the same ages were used as non-immunodeficient controls (5, 18, 22–25).

Flow Cytometric Analysis of Treg Cells and CXCR4$^+$ T Cells

We purified CD3$^+$, CD3$^+$CD25$^+$, CD3$^+$CD25$^-$ cells, and CD4$^+$ cells from pooled placentas and spleens of the pregnant mice by using magnetic affinity cell sorting (MACS), with microbead-conjugated anti-mouse CD3, CD4, CD25, or combinations of these (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions (18, 22–26). In this study the purity and viability of the MACS-purified cells routinely exceeded 97% and 95%, as determined by flow cytometry (26) and propidium iodide (Invitrogen, Eugene, OR) staining, respectively (27). The percentage of Treg cells in the purified cell populations and that of CXCR4$^+$ cells in purified T cell subsets was evaluated with flow cytometry with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-mouse antibodies (eBioscience, San Diego, CA). For CXCR4 staining, the purified cells were stained with PE-conjugated rat anti-mouse CD3 and CXCR4 monoclonal antibodies (R&D Systems, Minneapolis, MN) for 10 minutes. After washing once with phosphate-buffered saline (PBS), secondary staining was performed with FITC-conjugated goat anti-rat immunoglobulin (Caltag Laboratories, Burlingame, CA), and the cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ). Isotype control antibodies were used to exclude false-positive cells (eBioscience). All experiments in this study were performed in triplicate, unless stated otherwise.

Intracellular CXCL12 Expression in CK7$^+$ Cells

Cytokeratin 7 (CK7) is known as the best marker for placental trophoblast cells (28). The expression pattern of intracellular CXCL12 in the placental CK7$^+$ cells was detected by previously described methods (24). Embryo-depleted placentas were harvested from pregnant mice on gestational days 9.5, 13.5, and 17.5. They were cut into small pieces with ocular scissors. Tissue particles were digested with 25 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid solution containing 0.125% trypsin and 25 U/mL DNAse I (Sigma-Aldrich, St. Louis, MO) at 37°C for three cycles of 10 minutes each in a shaking water bath. An adequate amount of fetal bovine serum (FBS; Sigma-Aldrich) was then added to neutralize the trypsin and DNAse. The cells from pooled harvests were pelleted at 1500 rpm for 10 minutes, suspended in PBS (containing 2 mmol/L ethylenediaminetetraacetic acid [EDTA]), and fractioned by centrifugation in Ficoll-Hypaque density medium (eBioscience) at 2000 rpm for 20 min at 22°C. Trophoblast cells were isolated from the middle layer of the gradient (29). After washing with PBS, the pellet was resuspended in red blood cell lysis solution (150 mmol/L ammonium chloride, 10 mmol/L $NaHCO_3$ and 0.1 mmol/L EDTA) for 10 minutes at 37°C. The cells were then suspended in permeabilization buffer (eBioscience) for 10 minutes at 4°C and washed once with PBS. For detecting the CXCL12$^+$ percentage in the CK7$^+$ cell population, the cells were incubated with both mouse anti-CK7 monoclonal antibody (catalog no. MAB-0166; Maxim, Fuzhou City,