Murine CD200⁺CK7⁺ trophoblasts in a poly (I:C)-induced embryo resorption model

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Abstract

Cytokeratin 7 (CK7) is currently regarded as the best marker for trophoblast cells, while CD200 (OX-2), known as 'tolerance signal', plays an important role in normal pregnancy. In this study, the status of CD200 expression was investigated in BALB/c × C57BL/6 and BALB/c × BALB/c mating combinations designed as allogeneic and syngeneic murine models of induced embryo resorption, in which the resorption rate was boosted by an i.p. injection of poly (I:C), a synthetic double-stranded RNA. The percentage of CD200⁺ cells in the CK7⁺ cell population (CD200⁺CK7⁺ percentage) and the absolute number of these cells were determined with flow cytometry, using trophoblast cells collected at day 8.5 and day 13.5 of gestation. The potential effect of poly (I:C) on CD200 expression was also evaluated by detecting the CD200⁺CK7⁺ percentage in trophoblast cells incubated in the presence or absence of poly (I:C), *in vitro*. The distribution pattern of CD200⁺ cells at the fetomaternal interface was evaluated by immunocytochemical examination. When 10⁴ cells were analyzed at day 8.5 of gestation in each case, no significant difference was observed between the poly (I:C)-treated group and the control PBS group either in the CD200⁺CK7⁺ percentage or in the absolute number of these cells. Similar results were observed both in BALB/c × C57BL/6 mice and in BALB/c × BALB/c mice. However, the CD200⁺CK7⁺ percentage was significantly decreased in the poly (I:C)-treated group when evaluated at day 13.5 of gestation. Accordingly, a dramatically elevated rate of embryo resorption was observed at this time point of pregnancy after the administration of poly (I:C). In addition, the CD200⁺CK7⁺ percentage was significantly lower in trophoblast cells incubated with poly (I:C) at a certain concentration, *in vitro*, while histocytochemical examination showed the CD200⁺ cells mainly scattered in placental tissue adjacent to the interface of the placenta and uterus. This indicates that sufficient expression of the CD200 molecule on CK7⁺ cells at the fetomaternal interface may be necessary for the maintenance of embryos during pregnancy in this rodent model, while poly (I:C) administration may increase embryo resorption, at least partially via direct inhibition of CD200 expression on CK7⁺ cells.

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Introduction

CD200 (OX-2) is a type-1 membrane glycoprotein containing two extracellular Ig-like domains, which is expressed on trophoblast cells and on a subpopulation of uterine decidua cells, in both mice and human beings (Gorczyński *et al.* 2004). The unusual tissue distribution of CD200 suggests the special function of this molecule. The CD200 receptor (CD200R) is known as an immunomodulatory membrane protein. The CD200R gene maps closely to the CD200 gene on human chromosome 3q12–13 (Vieites *et al.* 2003), which displays an expression pattern restricted to myeloid cells, and the engagement of CD200R with CD200 results in inhibition or down-regulation of myeloid cell activity (Hoek *et al.* 2000, Barclay *et al.* 2002). The unusual tissue distribution of CD200 indicates where myeloid cells can be restrictively controlled through cell–cell contact. In mice lacking CD200 (CD200^{−/−}), macro-

phage lineage cells exhibited an activated phenotype and were more numerous (Homola *et al.* 2000). In addition, restricted CD200R expression at the fetomaternal interface suggests CD200 and CD200R interactions may serve important functions determining the outcome of pregnancy (Gorczyński *et al.* 2004).

Polyinosinic-polycytidylic acid (poly (I:C)) is known as a synthetic double-stranded RNA capable of activating macrophages and natural killer (NK) cells (De Fougerolles & Baines 1987, Cavanaugh *et al.* 1996). In abortion-prone CBA/J × DBA/2 mating combinations, the natural resorption rate (RR) of about 20–40% could be boosted to 60% if the females were injected with poly (I:C) (Shimada *et al.* 2003).

Cytokeratin 7 (CK7) has been found to be expressed by the majority of trophoblast cells, but is absent in matrix cells. Therefore, it can be used as an intracellular marker for trophoblast cells in flow cytometric analysis with a

properly designed gate threshold and properly selected monoclonal antibodies (mAbs) (Maldonado-Estrada *et al.* 2004).

In this study, BALB/c $\times$ C57BL/6 (H-2^d $\times$ H-2^b) and BALB/c $\times$ BALB/c (H-2^d $\times$ H-2^d) mice were used as allogeneic and syngeneic mating combinations respectively (Morecki *et al.* 1998, Alner *et al.* 2003). In these mice, an i.p. injection of poly (I:C) was performed to establish induced models of embryo resorption. Using these models, both the percentage of CD200⁺ cells in the CK7⁺ cell population (CD200⁺CK7⁺ percentage) and the absolute number of these cells were determined with flow cytometry, using trophoblast cells collected at day 8.5 and day 13.5 of gestation. In another design, the CD200⁺CK7⁺ percentage was detected in short-term cultured trophoblast cells in the presence or absence of poly (I:C) to evaluate its potential effect on CD200 expression. In addition, the contribution pattern of the CD200 molecule was also investigated with histological and histochemical examination in the embryonic units, defined as individual embryos together with placenta and embracing uterine tissue (Shimada *et al.* 2003).

Materials and Methods

Induced-abortion model

Healthy female BALB/c, male C57BL/6 and male BALB/c mice at age 10–12 weeks were obtained from the Experimental Animal Center of Zhongshan University (Guangzhou City, China) and kept under pathogen-free conditions. Each female mouse was co-caged with one male. The point at which a vaginal plug was detected was designated day 0.5 of gestation. BALB/c $\times$ C57BL/6 and BALB/c $\times$ BALB/c mice were used as allogeneic and syngeneic mating combinations respectively. Both groups of these mice were further divided into two groups: a poly (I:C)-treated group and a control PBS-treated group.

Poly (I:C)-treated group

To induce embryo loss, pregnant mice were given an injection of poly (I:C) (product #P0913, γ -irradiated; Sigma) i.p. at a dosage of 200 μ g/20 g body weight (Shimada *et al.* 2003). Injection was given at day 7.5 of gestation, and pregnant mice were killed at day 8.5 or 13.5. RRs of embryos were calculated as: RR (%) = Number of resorbed embryos/Number of total (resorbed + viable) embryos $\times$ 100.

Control PBS group

As a control, each mouse in this group was injected i.p. with PBS at day 7.5 of gestation. Placentas from both groups were collected at day 8.5 or 13.5 of gestation and employed when fresh in further analyses. A portion of placenta from day 8.5 of gestation collected under sterile conditions was used to isolate trophoblast cells for

cultivation in the presence or absence of poly (I:C) and a portion of placenta from day 13.5 of gestation was taken together with adjacent uterine tissue (intact uterine wall) for histological and histochemical examination, while the other placental tissues were pooled to isolate trophoblast cells for flow cytometric analysis.

Isolation of trophoblast cells

Placentas, depleted of embryos and decidua, were harvested at both day 8.5 and day 13.5 of gestation and cut carefully into small pieces with ocular scissors. Tissue particles were digested three times with 25 mM HEPES solution containing 0.125% trypsin and 25 U/ml DNase I (Sigma) at 37°C for 10 min of each cycle in a shaking water bath. An adequate amount of fetal bovine serum (Sigma) was added to neutralize trypsin DNase. The cells from pooled harvestings were pelleted at 1500 r.p.m. for 10 min, resuspended in PBS (containing 2 mM EDTA), and fractionated on Ficoll–Hypaque density medium (density 1.077 $\pm$ 0.002 g/ml) by centrifugation at 2000 r.p.m. for 20 min at 22°C. Trophoblast cells were isolated from the middle layer of the gradient (Kliman *et al.* 1986). After washed with PBS, the pellet was resuspended in red blood cell lysis solution for 10 min at 37°C (150 mM ammonium chloride, 10 mM NaHCO₃ and 0.1 mM EDTA).

Cell staining and flow cytometry

The isolated trophoblast cells were resuspended in permeabilization buffer containing 0.1% saponin and 0.09% sodium azide (Catalog #00-8333-56; eBioscience, San Diego, CA, USA) for 1 h at 4°C, then repelleted and the supernatant flicked out. Twenty microliters of rat anti-mouse CD200 (Catalog #552512; BD Bioscience Pharmingen, San Diego, CA, USA) and mouse anti-CK7 mAbs (Catalog #MAB-0166; Maxim, Fuzhou City, China) were added at 10⁶ cells/10 μ l to the trophoblast cell suspension. After incubation overnight at 4°C, the cells were washed with PBS and then incubated with both fluorescein isothiocyanate (FITC)-labeled anti-rat IgG antibody produced in goat (Catalog #A110-109F; Bethyl, Montgomery, TX, USA) and biotinylated anti-mouse IgG (Catalog #SA1026; Boster, Wuhan City, China) for 1 h at 4°C in the dark. The cells were washed with PBS and then, in the case of biotinylated anti-mouse IgG, incubated with phycoerythrin (PE)-labeled streptavidin (Catalog #12-4312; eBioscience) for 1 h, still at 4°C in the dark. After being washed twice with PBS, the pellet was resuspended in 1 ml 4% paraformaldehyde in phosphate buffer (pH 7.3), and analyzed by flow cytometry (FACS Calibur; BD). Ten thousand cells were examined in each case (Clark *et al.* 2003, Zenclussen *et al.* 2003).

As negative controls, adequately designed gate thresholds were set to capture trophoblast cells using appropriate FITC- and PE-labeled isotype control antibodies. The double-negative controls were derived from

trophoblast cells stained with both FITC- and PE-labeled isotype control antibodies (Catalog #EB1-4321-82 and EB12-4719-73; eBioscience) (Clark *et al.* 2003, Zenclussen *et al.* 2003).

The percentage of CD200⁺ cells in the CK7⁺ cell population was calculated as: percentage CD200⁺ cells in CK7⁺ cell population = CD200⁺CK7⁺ cell number/(CK7⁺ cell number) × 100

In the 10⁴ cells analyzed, the absolute numbers of CD200⁺CK7⁺, CD200[−]CK7⁺ and total CK7⁺ cells were calculated respectively as follows: CD200⁺CK7⁺ cell number = CD200⁺CK7⁺ cell percentage × 10 000; CD200[−]CK7⁺ cell number = CD200[−]CK7⁺ cell percentage × 10 000; CK7⁺ cell number = CK7⁺ cell percentage × 10 000.

Cultivation of trophoblast cells with or without poly (I:C)

Under sterile conditions, trophoblast cells were isolated from pooled placentas at day 8.5 of gestation with methods described above. Isolated trophoblast cells were seeded in DMEM supplemented with 10% fetal calf serum and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) at a density of 4×10^5 cells/cm² in six-well culture dishes. Cells were maintained under standard tissue culture conditions at 37°C in a humidified incubator with 5% CO₂ in air. The medium was changed after overnight incubation. At this time point, poly (I:C) was added in a series of concentrations including 0, 1, 5 and 25 µg/ml. From then on, the cells were incubated in the presence or absence of poly (I:C) for 6 days. At the end of cultivation, trophoblast cells were harvested with 0.125% trypsin digestion, followed by staining with anti-CD200 and anti-CK7 mAbs using methods described above. The expression pattern of CD200 on the CK7⁺ cell population was analyzed by comparison of CD200⁺CK7⁺ cell percentages determined with flow cytometry, using methods described above.

Immunocytochemical analysis

Placentas harvested at day 13.5 of gestation, together with the adjacent uterine tissue (intact uterine wall containing decidua and muscle), were embedded in paraffin. Immunocytochemical examination was performed to evaluate the distribution pattern of CD200⁺ cells at the feto–maternal interface. Briefly, paraffin sections (thickness 5–6 µm) were dewaxed in xylene and rehydrated through a descending ethanol series. Non-specific endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 30 min at room temperature. All staining required a microwave boiling in citrate buffer at pH 6.0 for 15 min. Sections were then washed with PBS (pH 7.4) and treated with 5% BSA/PBS for 30 min to block non-specific binding of antibodies. At a dilution of 1:100, rat anti-mouse CD200 mAb was applied to sections for 1 h at room temperature. This was followed by biotinylated anti-rat Ig (Catalog #SA1026; Boster) for

30 min at room temperature, and an avidin–biotin–peroxidase detection system (Catalog #SA1026; Boster) for a further 30 min at room temperature. The sections were then developed with diaminobenzidine for 3–5 min to generate a brown-colored product. Finally, the sections were lightly counterstained with hematoxylin for nuclear localization (Knoeller *et al.* 2003).

Both in BALB/c × C57BL/6 and in BALB/c × BALB/c mating combinations, CD200⁺ cells were counted for ten high-power fields (× 400) ($n = 8$ in each group). Comparison was performed between poly (I:C)-treated groups and control PBS groups as well as between allogeneic and syngeneic mating combinations.

Statistical analysis

Data of flow cytometry were analyzed using Quad statistics (Arck *et al.* 1999). The RR of embryos was analyzed using a χ^2 test. The percentage and the absolute number of cells were analyzed using Student's *t*-test, and the results were shown as means ± S.E.M. On the sections stained immunocytochemically, the number of positive cells, counted under high-power fields, was also analyzed using Student's *t*-test and shown as means ± S.E.M.

Results

Embryo resorption

The RR of embryos was examined at day 8.5 ($n = 6$ in each group) and day 13.5 of gestation ($n = 8$ in each group) (Table 1).

RR in BALB/c × C57BL/6 mice

In these mice, the RR at day 8.5 was 7.0% (3 out of 43) in the poly (I:C)-treated group, not significantly different from that in the control group (5.8%; 3 out of 52) ($P > 0.05$). However, RR at day 13.5 was 37.0% (20 out of 54) in the poly (I:C) group, which was significantly higher than that in the control (9.1%; 5 out of 55) ($P < 0.01$).

RR in BALB/c × BALB/c mice

In these mice, RR at day 8.5 was 3.6% (2 out of 56) in the poly (I:C) group, which was not significantly different from that in the PBS group (5.6%; 3 out of 54) ($P > 0.05$). In contrast, RR at day 13.5 was 29.0% (18 out of 62) in the poly (I:C) group. Such a level was significantly higher than that in the PBS group (5.8%; 4 out of 69) ($P < 0.01$).

Comparison of RR between BALB/c × C57BL/6 and BALB/c × BALB/c mice

In the absence of poly (I:C)-stimulation, RR at day 8.5 was 5.8% (3 out of 52) in BALB/c × C57BL/6 mice, not significantly different from that in BALB/c × BALB/c (5.6%; 3 out of 54; $P > 0.05$). Similarly, at day 13.5, RR

Table 1 The CD200⁺CK7⁺/CK7⁺ cell percentage and the absolute number of CD200⁺CK7⁺, CD200⁻CK7⁺ and CK7⁺ cells in the poly (I:C)-treated group and control PBS group detected at day 10.5 and 13.5 of gestation.

Mating combination	Treatment	d	n	RR	CD200 ⁺ CK7 ⁺ /CK7 ⁺ (%)	CD200 ⁺ CK7 ⁺	CD200 ⁻ CK7 ⁺	CK7 ⁺
BALB/c × C57BL/6	Poly (I:C)	8.5	6	7.0% (3/43)	11.7 ± 2.1	1102 ± 175	8447 ± 379	9549 ± 246
BALB/c × C57BL/6	PBS	8.5	6	5.8% (3/52)	10.0 ± 1.9	835 ± 213	7381 ± 925	8216 ± 1034
<i>P</i> value ^a	—	—	—	>0.05 ^b	0.545	0.357	0.311	0.238
BALB/c × BALB/c	Poly (I:C)	8.5	6	3.6% (2/56)	4.6 ± 1.5	371 ± 139	8014 ± 868	8384 ± 864
BALB/c × BALB/c	PBS	8.5	6	5.6% (3/54)	5.5 ± 1.1	462 ± 99	8068 ± 663	8530 ± 667
<i>P</i> value ^a	—	—	—	>0.05 ^b	0.665	0.605	0.961	0.896
BALB/c × BALB/c	Poly (I:C)	13.5	8	37.0% (20/54)	6.3 ± 2.2	140 ± 39	3633 ± 820	3773 ± 794
BALB/c × BALB/c	PBS	13.5	8	9.1% (5/55)	36.1 ± 3.3	1941 ± 286	3261 ± 227	5202 ± 426
<i>P</i> value ^a	—	—	—	<0.01 ^b	0.000	0.000	0.668	0.135
BALB/c × BALB/c	Poly (I:C)	13.5	8	29.0% (18/62)	8.5 ± 1.7	701 ± 176	7348 ± 758	8048 ± 829
BALB/c × BALB/c	PBS	13.5	8	5.8% (4/69)	26.1 ± 2.8	1886 ± 393	4930 ± 702	6816 ± 1039
<i>P</i> value ^a	—	—	—	<0.01 ^b	0.000	0.016	0.035	0.370

d: gestational day. Poly (I:C)-treated group vs control PBS group: *P* value, ^aStudent's *t*-test, ^bχ² test. Data from day 8.5 vs day 13.5.

in BALB/c × C57BL/6 mice (9.1%) was not significantly different from that in BALB/c × BALB/c (5.8%, *P* > 0.05, see Table 1).

The findings were the same in poly (I:C)-treated mice: RR was 7.0 vs 3.6% (*P* > 0.05) at day 8.5, and 37.0 vs 29.0% (*P* > 0.05) at day 13.5, as compared between BALB/c × C57BL/6 and BALB/c × BALB/c mice respectively.

Double-negative control

In flow cytometry, gate thresholds were set to capture trophoblast cells (Fig. 1). As discussed previously (Clark *et al.* 2003), the disaggregated placenta contained at least two distinct CD200⁺ immunomodulatory populations, and the more potent activity was associated with a moderately large-(medium) sized rather than small-sized cells or very large cells. As shown in Fig. 2A1, B1, C1 and D1 and Fig. 3A1 and B1, most spots were double-negative when stained with appropriate FITC- and PE-labeled isotype control antibodies, serving here as negative controls for the trophoblast cells isolated from allogeneic (BALB/c × C57BL/6) and syngeneic (BALB/c × BALB/c) mating combinations at day 8.5 and day 13.5 of gestation.

Figure 1 Gate threshold set by isotype control staining. Trophoblast cells captured in R2 were used in further flow cytometric analysis. FSC-H: forward scatter count height. SSC-H: side scatter count height.

CD200⁺CK7⁺ cells

CD200⁺CK7⁺ cells isolated from BALB/c × C57BL/6 mice

No significant difference was observed at day 8.5 of gestation in either the percentage or absolute number of CD200⁺CK7⁺ cells in the CK7⁺ population between the poly (I:C) group and the PBS group (Table 1, Fig. 2A2 and A3).

The CD200⁺CK7⁺/CK7⁺ cell percentage was 6.3 ± 2.2% and 36.1 ± 3.3% in poly (I:C)-treated and PBS-treated BALB/c × C57BL/6 mice at day 13.5 of gestation respectively (*P* < 0.001). When 10⁴ cells were analyzed, the absolute number of CD200⁺CK7⁺ cells was 140 ± 39 in the poly (I:C) group and 1941 ± 286 in the PBS group (*P* < 0.001) (Table 1, Fig. 2C2 and C3).

CD200⁺CK7⁺ cells isolated from BALB/c × BALB/c mice

No significant difference was observed at day 8.5 in the percentage or absolute number of CD200⁺CK7⁺ cells in the CK7⁺ population between the poly (I:C) and the PBS group (Table 1, Fig. 2B2 and B3).

The CD200⁺CK7⁺/CK7⁺ cell percentage was significantly lower in the poly (I:C) group than the PBS group at day 13.5 (*P* < 0.001). The absolute number of CD200⁺CK7⁺ cells was also significantly lower in the poly (I:C) group than the control PBS group (*P* = 0.016) (Table 1, Fig. 2D2 and D3).

CD200⁻CK7⁺ cells

CD200⁻CK7⁺ cells isolated from BALB/c × C57BL/6 mice

In these mice, the CD200⁻CK7⁺ cell number in the poly (I:C) group was not significantly different from that in the PBS group when 10⁴ cells were examined for each mouse at either day 8.5 or 13.5 of gestation.

Figure 2 Flow cytometric analysis of CD200 expression in the CK7⁺ cell population from placentas harvested at day 8.5 and 13.5 of gestation. Row A (A1, A2, A3): derived from BALB/c x C57BL/6 mice at day 8.5 of gestation; row B: from BALB/c x BALB/c at day 8.5; row C: from BALB/c x C57BL/6 at day 13.5; row D: from BALB/c x BALB/c at day 13.5. A1–D1 (A1, B1, C1 and D1): double-negative control. A2–D2: poly (I:C)-treated group. A3–D3: control PBS group.

CD200[−]CK7⁺ cells isolated from BALB/c x BALB/c mice

At day 8.5 of gestation, the CD200[−]CK7⁺ cell number in the poly (I:C) group was not significantly different from that in the PBS group. However, the number was higher in the poly (I:C) group when examined at day 13.5 ($P = 0.035$).

CK7⁺ cells

CK7⁺ cells isolated from BALB/c x C57BL/6 mice

When 10⁴ cells were examined per mouse at day 8.5 of gestation, CK7⁺ cell number derived from the placentas of poly (I:C)-treated mice was not significantly different from

Figure 3 Flow cytometric analysis of CD200 expression in CK7⁺ trophoblast cells incubated in the presence or absence of poly (I:C). Trophoblast cells were isolated from mice at day 8.5 of gestation. Column A: derived from BALB/c × C57BL/6 mice; column B: from BALB/c × BALB/c. A1, B1: double-negative control. A2, B2: trophoblast cells incubated with poly (I:C) at a concentration of 1 µg/ml. A3, B3: poly (I:C) at 5 µg/ml. A4, B4: poly (I:C) at 25 µg/ml. A5, B5: without poly (I:C).

that from the PBS group. A similar result was observed at day 13.5 (Table 1 and Fig. 2).

CK7⁺ cells isolated from BALB/c × BALB/c mice

The CK7⁺ cell number from poly (I:C)-treated mice was not significantly different from that of PBS-treated mice, at either day 8.5 or 13.5 (Table 1 and Fig. 2).

In vitro influence of poly (I:C) on CD200 expression in CK7⁺ cell population

After incubation with poly (I:C) for 6 days, the trophoblast cells were harvested and analyzed with flow cytometry (Figs 3 and 4).

In BALB/c × C57BL/6 mice at day 8.5 of gestation, the CD200⁺CK7⁺ cell percentage was $8.7 \pm 0.5\%$ after cultivation for 6 days at the absence of poly (I:C), significantly higher than those incubated with poly (I:C) at a concentration of 1 µg/ml ($P < 0.001$), 5 µg/ml ($P = 0.011$) or 25 µg/ml ($P < 0.001$). However, no significant difference was observed among CD200⁺CK7⁺ cell percentages derived from cells incubated with poly (I:C) at 1, 5 and 25 µg/ml (Figs 3 and 4).

Finding were a little different in BALB/c × BALB/c mice at day 8.5 of gestation. After incubation with poly (I:C) at the level of 1 µg/ml for 6 days, the CD200⁺ cell percentage in the CK7⁺ population was not different from those incubated without poly (I:C). However, the percentage could be dramatically decreased when the concentration of poly (I:C) was increased to 5 or 25 µg/ml ($P = 0.001$) (Figs 3 and 4).

Immunocytochemical analysis of CD200⁺ cells

The distribution pattern of CD200⁺ cells in the embryonic units was evaluated at day 13.5 of gestation by immunocytochemical examination. Both in BALB/c × C57BL/6 and BALB/c × BALB/c mice, CD200⁺ cells were visible in the placentas derived from the poly (I:C)-treated group as well as the control PBS group. However, median CD200⁺ cell number per high-power field (× 400) was significantly smaller in the poly (I:C) group than in the PBS group (4.0 ± 0.5 vs 16.4 ± 2.1 in BALB/c × C57BL/6, and 3.1 ± 0.5 vs 14.1 ± 1.8 in BALB/c × BALB/c, $P < 0.001$) (Table 2). In addition, it appeared that these CD200⁺ cells were mainly scattered in the placental tissue adjacent to the interface of the placenta and uterus (Fig. 5).

In these sections, efforts were directed to determine the identity of these CD200⁺ cells. It appeared that they mainly belonged to cytotrophoblast cells, while a small subset of decidua cells was also found to express this antigen (Fig. 5).

Discussion

CK7 is expressed specifically by trophoblast cells and trophoblast-derived tumor cells. By virtue of the exclusive

Figure 4 *In vitro* effect of poly (I:C) on CD200 expression on CK7⁺ cells. Unit of poly (I:C) concentration: µg/ml. Cell percentage: the percentage of CD200⁺CK7⁺/CK7⁺ cell after incubation in the presence or absence of poly (I:C).

CK7 expression on trophoblast cells, it has therefore been proposed that CK7 could be used as the first choice marker with which to monitor the purity of purified cytotrophoblast cells (Maldonado-Estrada *et al.* 2004). Clark *et al.* (2003) assessed the role CD200 molecules may play in human pregnancy. But at the time of their study, a suitable reagent was not available for intracellular staining of CK7. Instead of anti-CK7 mAbs, they used a number of mAbs, including anti-CK5, -CK6, -CK8, -CK17, -CK18 and -CK19. Both CK- and CD200-positive cells were observed in their study. However, their examination was merely performed on the placentas derived from normal human pregnancy, and therefore, the relationship between CD200 expression and murine embryo resorption remained to be answered.

Is CD200 expression in trophoblast cells necessary for the maintenance of embryos during pregnancy? Has the CD200 expression pattern been altered in the murine model of poly (I:C)-induced resorption? In this study, no significant difference was observed at the earlier stage of pregnancy (i.e. day 8.5) in either the CD200⁺CK7⁺ percentage or the absolute number of CD200⁺CK7⁺ cells between the poly (I:C)-treated group and the control PBS group. Accordingly, the RR in poly (I:C)-treated mice was not significantly different from that in the PBS group at this time point of pregnancy. Similar results were obtained both in BALB/c x C57BL/6 and in BALB/c x BALB/c mice. In contrast, when detected at day 13.5, a dramatically

Table 2 The number of CD200⁺ cells from placentas at day 13.5 of gestation counted under ten high-power fields (× 400) in immunocytochemical analysis.

Mating combination	Poly (I:C) group	PBS group	P value ^a
BALB/c x C57BL/6	4.0 ± 0.5	16.4 ± 2.1	<0.001
BALB/c x BALB/c	3.1 ± 0.5	14.1 ± 1.8	<0.001
P value ^b	NS	NS	—

n = 8 for each group. *P* value ^apoly (I:C)-treated group vs control PBS group, ^bBALB/c x C57BL/6 vs BALB/c x BALB/c mice. NS: not significant.

Figure 5 Distribution pattern of CD200⁺ cells in the embryonic unit harvested at day 13.5 of gestation. Data from BALB/c x C57BL/6 mice. CD200⁺ cells were visible in placenta tissue adjacent to the interface of placenta and uterus, but positive cells were fewer in the poly (I:C)-treated group. UT: uterus; PL: placenta; IT: interface of placenta and uterus. (A–D): poly (I:C) group; (E–H): PBS group. (A, B, E, F) Hematoxylin and eosin staining. (C, D, G, H) Immunocytochemical staining. (B, D, F, H) at × 400 are locally amplified fields of (A, C, E, G) (× 100) respectively.

decreased level of CD200⁺CK7⁺ cell percentage could be observed in the poly (I:C) group, together with a boosted rate of embryo resorption. These results support the idea that adequate CD200 expression in CK7⁺ trophoblast cells may prevent embryo resorption, while the administration of poly (I:C) seems to be a suppressive factor on CD200 expression in CK7⁺ cells.

Could poly (I:C)-administration directly or indirectly inhibit the expression of CD200 on CK7⁺ cells? In order to answer to this, trophoblast cells harvested at day 8.5 of gestation were cultivated for 6 days in the presence or absence of poly (I:C). At the end of cultivation, the CD200⁺CK7⁺ percentage was detected with flow cytometry to evaluate the potential effect of poly (I:C) on CD200 expression. It seemed that poly (I:C) could directly inhibit the expression of CD200 on CK7⁺ cells if added at a certain level in medium (not lower than 1 µg/ml for cells from BALB/c x C57BL/6 mice and not lower than 5 µg/ml for cells from BALB/c x BALB/c mice). It implies that poly (I:C) can directly abrogate the effect of CD200 at the

feto–maternal interface, and result in the increase of embryo resorption.

Interestingly, the trophoblast cells isolated from BALB/c $\times$ C57BL/6 mice seemed to be a little more sensitive to poly (I:C)-stimulation than those from BALB/c $\times$ BALB/c mice *in vitro*. In addition, a dose-dependent response could be observed in the effect of poly (I:C) on cells from BALB/c $\times$ BALB/c mice, but such a trend was not apparent in the effect of poly (I:C) on cells from BALB/c $\times$ C57BL/6 mice. The reasons remain unclear. It may be due to the diversity of sensitivity of these cells to stimulation by poly (I:C). In human beings and mice, maternal recognition of paternally derived MHC may be beneficial to pregnancy outcome. Because sensitization to paternal MHC occurs commonly in pregnancies without any deleterious effects, it was proposed that normal pregnancy requires maternal recognition of, and response to, paternally derived fetal antigens, while abnormal pregnancy may result from failure of the maternal immune system to recognize or respond to fetal antigens. Inadequate recognition and response may be associated with feto–maternal histocompatibility, i.e. fetuses whose paternally derived MHC do not differ from maternal MHC. Increased MHC sharing among couples with poor reproductive outcome is consistent with this hypothesis (Ober & van der Ven 1997). Therefore, we expected that the sensitivity to poly (I:C)-stimulation in BALB/c $\times$ BALB/c mice may be different from that in BALB/c $\times$ C57BL/6. However, no statistically supported differences were observed either in the natural RR or in the induced RR between these mating combinations in our current study. MHC sharing may be a potential factor influencing the sensitivity of trophoblast cells to the stimulation of poly (I:C), but its significance could not be described completely in our design with a rather small case number. In addition, the effects of poly (I:C), when a rather high dose is given, may be strong enough to mask the role that MHC sharing may play in the mechanism of pregnancy tolerance. Properly designed research on an enlarged scale and with a wide range of poly (I:C) concentrations may be helpful in clarifying whether poly (I:C) can down-regulate CD200 expression in CK7⁺ cells derived from BALB/c $\times$ C57BL/6 mice in a dose-dependent manner, *in vitro*.

In histological and immunocytochemical examination, we also observed that the CD200⁺ cell number could be dramatically decreased after poly (I:C) administration in the embryonic units, and these positive cells were mainly located in regions near the interface of the placenta and uterus, but absent or fewer in uterine tissue or placental tissue far from the feto–maternal interface. These results strengthened the notion that CD200 molecule may be involved in pregnancy tolerance as a 'tolerance signal' to prevent embryos from being resorbed (Clark *et al.* 2003).

Recent reports indicate that CD200 and CD200R interaction may be a critical event in inhibiting or down-regulating the function of myeloid cells, such as macrophages, infiltrating at the feto–maternal interface (Barclay *et al.* 2002, Gorczynski *et al.* 2004). On the other hand, poly

(I:C) is believed to be an activator of NK cells and macrophages (Shimada *et al.* 2003). Our current study implies that poly (I:C) may boost embryo rejection by abrogating the effect of CD200 and CD200R interaction. Notably, it was reported recently that in BALB/c macrophages, poly (I:C), as a stimulus for toll-like receptor 3 (TLR3), could strikingly induce the expression of an NKG2D ligand, RAE-1, in an innate immune response to infection (Hamerman *et al.* 2004). It was also reported that the stimulation of poly (I:C) may lead to antigen cross-presentation between dendritic cells and CD8⁺T cells via a TLR3-involved mechanism in anti-microbial immunity (Datta *et al.* 2003). However, the detailed mechanisms of induced embryo resorption in which poly (I:C) may be involved remain to be clarified in future research.

Now that the administration of poly (I:C) may have a suppressive effect on CD200 expression in CK7⁺ cell population, is it a specific suppressive effect, or a general one on both CD200⁺CK7⁺ cells and CD200[−]CK7⁺ cells? To answer this, the absolute numbers of CD200[−]CK7⁺ and total CK7⁺ cells were calculated in 10⁴ cells for each case with flow cytometry. It showed in general that the number of CD200[−]CK7⁺ cells and CK7⁺ cells were not significantly altered after the administration of poly (I:C). These results indicate that poly (I:C) injection is likely to be a suppressive factor with a specific effect on CD200 expression.

As discussed in a previous report (Clark *et al.* 2003), the cell isolation method they used to isolate human trophoblast cells (similar to ours) did not isolate intact multinucleated syncytiotrophoblast cells, but a significant percentage of the cells isolated could have been nucleated syncytial fragments. Further studies will be needed to evaluate CD200 expression on intact multinucleated syncytiotrophoblast cells.

In summary, it seems that the disruption of CD200–CD200R interaction by down-regulating CD200 expression with poly (I:C) could increase the susceptibility to embryo resorption in mice normally resistant to spontaneous abortion. It may be valuable to use these murine models of poly (I:C)-induced resorption and properly selected cell markers to clarify the detailed mechanisms of pregnancy tolerance in further studies.

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