

Changes in expression of the CD200 tolerance-signaling molecule and its receptor (CD200R) by villus trophoblasts during first trimester missed abortion and in chronic histiocytic intervillitis

David A. Clark^{1,2}  | Jennifer M. Dmetrichuk² | Elizabeth McCready^{2,3} | Sukhbinder Dhesy-Thind⁴ | Jorge L. Arredondo²

¹Department of Medicine, McMaster University, Hamilton, Ontario, Canada

²Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

³Genetics Services, Hamilton Regional Laboratory Medicine Program, Hamilton Health Sciences, Hamilton, Ontario, Canada

⁴Department of Oncology, McMaster University, Hamilton, Ontario, Canada

Correspondence

David A. Clark, McMaster University, Hamilton, Ontario, Canada.
Email: clarkd@mcmaster.ca

Problem: Expression of CD200 at the fetomaternal interface is associated with successful murine and human pregnancy. CD200 binding to CD200 receptors on lymphomyeloid cells suppresses inflammation and induces Tregs. CD200 receptors are also expressed on mouse and human placental trophoblast cells. What is the expression of CD200 and CD200R in human missed abortions which have preserved Treg levels and in chronic histiocytic intervillitis (CHI) where maternal inflammatory cells cause IUGR?

Methods: Immunohistochemistry for CD200, CD200R, and Ki67 using human placental sections from missed abortions, term placenta, and CHI. PCR testing was done for trisomy in missed abortion.

Results: CD200 and CD200R were expressed by human villus trophoblasts from 2 weeks post-implantation to term. Cytotrophoblast proliferation (Ki-67⁺ count) decreased at term. In first trimester missed abortion cases, CD200⁺CD200R⁺ villus trophoblasts accompanied missed abortion of non-trisomic male fetuses. CD200⁺ and Ki67⁺ trophoblast proliferation was preserved in CHI with maternal inflammatory cell infiltration but CD200R was greatly decreased.

Conclusion: Residual CD200 activity may prevent completion of abortions via induction of Treg cells. In CHI, infiltrating maternal effector T cells may block Treg induction. An autocrine role for CD200-CD200R interaction versus inhibition of soluble CD200 by soluble CD200R is discussed.

KEYWORDS

CD200, CD200 receptor, chronic histiocytic intervillitis, missed abortion, tolerance, villus trophoblast

1 | INTRODUCTION

First trimester miscarriage (abortion) is a common event occurring sporadically in 5%–15% of pregnancies, and a subset of women have recurrent losses.¹ In the latter group, a variety of putative “causes,” some more convincing than others, have been hypothesized based

on “association” but in the majority of cases, embryo loss is either due to chromosome abnormalities in the embryo or to “unexplained” factors, which are currently thought to be due to abnormalities in the mother’s innate and/or adaptive immune system.^{1,2} Indeed, women who have had a live born male followed by subsequent recurrent losses (secondary recurrent miscarriages) have reported to manifest

adaptive immunity to the male H-Y antigen.³ A general state of Th1 T-cell activation and innate immune system activation (characterized by Th1 cytokine production) has also been noted in women with “unexplained” primary recurrent miscarriages, and potential activating stimuli such as bacterial flora with pathogen-associated molecular patterns (PAMPs) combined with psychological stress are proposed to be important.^{1,2} Fortunately, there are inbred laboratory mouse models of recurrent loss, such as the CBA×DBA/2 model and B10×B10A model, which allows for the role of innate and adaptive immune factors to be more easily dissected due to very low rates of karyotype abnormal embryos, a short 21-day gestation, techniques for detailed analysis of the immune system, and ability to ethically obtain uterine and other tissues for study prior to the onset of the abortion process.^{4,5} Even with recent development of the trophoblast retrieval and isolation from the cervix (TRIC) procedure,⁶ one is loath to sample uterine contents, except for chorionic villus biopsy for genetics where TRIC is not available, until it is evident that a pregnancy is and has failed based on loss of a fetal heart±symptoms of pain, uterine bleeding, and expulsion of decidua and fetal parts. Uterine tissues are then usually compared to tissues from elective terminations of normal progressing pregnancies, but in the case of an inevitable and ongoing miscarriage, any difference compared to normal pregnancy tissue may be the result of the inflammatory process at the fetomaternal interface and not the cause of the loss, and although such artifacts can be minimized to some extent by immediately emptying the uterus when non-viability is diagnosed and symptoms are only just beginning, one can never be certain.

A possible solution to the study of the evolution of a human pregnancy loss is provided by the occurrence of missed abortions where there are no symptoms or evidence of uterine bleeding. In this situation, Nakashima et al. have shown lack of infiltration into the decidua of IL-17⁺ cells.⁷ IL-17 is a pro-inflammatory Th1 cytokine which arises from Th17 cells; the latter cells develop when naïve activated CD4⁺ maternal T cells or FoxP3⁺ Tregs cells arising from these CD4⁺ T cells differentiate into IL-17-producing effector T cells, and the pro-inflammatory IL-6 cytokine facilitates this process.^{1,8} It is therefore not surprising that Treg cell levels in decidua are low in *inevitable spontaneous abortion* of normal karyotype embryos compared to normal levels in elective termination decidua and in decidua associated with abnormal karyotype embryos.^{9,10} Abortion of normal karyotype embryos is associated with increased Th1 T cells and an increase in activated blood NK cells, and the Th1 cytokines TNF- α and IFN- γ contributed by blood NK cells and M1 macrophages have been proposed to play an important role at the fetomaternal interface in the demise of these normal embryos, albeit the likely role of Treg cells is to suppress the inflammatory process rather than to mitigate a hypothetical fetomaternal “immune conflict” by creating a mythical state of maternal-fetal tolerance.^{1,8} By contrast, in the decidua of *missed abortion* cases, which have not become inevitable, with a normal karyotype embryo Ebina et al. reported an *increase* in Foxp3⁺CD4⁺CD25^{high} Tregs and an *increase* in CD8⁺IFN- γ ⁺ “effector” T cells.¹¹ So is missed abortion a distinct entity unrelated to the pathogenesis of spontaneous abortion, or are

there factors in missed abortions that *prevent progression* to clinical abortion?

In spontaneous clinical abortions, expression of CD200 molecules was reduced in villus syncytiotrophoblasts, and mRNA was decreased in whole villi,¹² so if missed abortion is a distinct process one might expect to see no reduction in CD200. However, a reduction in CD200R1 in villus syncytiotrophoblasts and villus cytotrophoblasts was more dramatic so CD200/CD200R1 ratios increased.¹² Based on CD200^{-/-} mouse data, CD200 exerts potent anti-inflammatory and anti-autoimmune effects which includes suppression of IL-17-producing cells.^{13,14} The CD200 knockout is on a B6 background, such mice are raised in SPF isolator cage facilities, and actual litter size data has not been published. However, in conventional facilities, B6 mice with intact CD200 have low rates of abortion, can be aborted by administration of a sufficient dose of LPS, and this increase in abortion rate can be countered by further increasing CD200 expression.¹⁵ By contrast, the CBA/J mice mated to DBA/2 males (where there is inadequate seminal plasma peptide induction of Treg cells at mating to suppress inflammatory responses) spontaneous abortion rates (which are augmented if CD200 is blocked from day 8.5 of pregnancy). Furthermore, the abortions are prevented if soluble CD200Fc is administered.^{1,8,16} Thus, in the mouse, sufficient expression of CD200 can counter pathways leading to embryo elimination. However, presence of CD200R1 on lymphomyeloid cells is required for CD200 to have any effects.^{1,14,15,17} It is unclear what CD200R1 expression on CD200-expressing cells might do. Wang et al. have suggested CD200 acts on CD200R1 on syncytiotrophoblasts to ensure pregnancy success,¹² so an autocrine effect is possible which could be compromised if CD200R1 expression is too low. But CD200 is also released in soluble form (sCD200),¹⁸ and similarly CD200R1 may be released, binding of sCD200 to cell surface or a soluble CD200R1 could reduce the effective CD200 concentration and action on the usual lymphomyeloid target cells as well as on syncytiotrophoblasts that make β -hCG. The net difference, CD200-CD200R1, could provide a potentially useful estimate of net CD200 activity.

In this paper, we provide a more comprehensive survey of expression of CD200 and CD200R1 on placental villus tissue from 5, 6, 8, and 9 weeks gestation first trimester villus trophoblasts from missed abortion patients and compare the results to expression by villus trophoblasts at term. We also examined the relationship between CD200 and CD200R1 expression by first trimester villus trophoblasts and embryonic genome in missed abortion cases. Finally we examined expression of CD200 and CD200R1 in a case of chronic histiocytic intervillitis (CHI) at 36 weeks gestation and compared expression to normal healthy term placenta.

2 | MATERIALS AND METHODS

2.1 | Patient materials

Anonymous archived human placental tissues were selected from the Department of Pathology and Molecular Medicine’s tissue bank by

one of the co-authors (JLA). Tissue was from elective terminations of first trimester missed abortion, from third trimester placenta after delivery at term, and from a placenta with CHI at 36 weeks gestation, and had been fixed in 10% formalin and embedded in paraffin. The study was approved by the Hamilton Integrated Research Ethics Board. The only clinical information available was what was provided on the pathology requisition form that accompanied placental tissues and which could be used without compromising patient anonymity, plus additional information for the 5 and 8 week missed abortion cases where the inpatient chart could be accessed by JLA who was the pathologist reporting those cases.

2.2 | Immunohistochemistry staining

The method described previously was used.¹⁷ Immunostaining was performed in the Department of Pathology's Immunohistology Facility in the Michael DeGroot Centre for Learning and Discovery at McMaster University using 4 μ sections cut from paraffin blocks affixed to positively-charged slides. Antigen recovery was done by heating in EDTA buffer pH8 (for anti-CD200 and anti-CD200R1) or citrate buffer pH 6 (for Ki67) in a Biacore Digital Decloaking Chamber using factory settings. Rabbit antihuman CD200 (RB846) serum and pre-immune control rabbit serum is described in detail elsewhere and was used at a 1/1000 dilution.¹⁹ This anti-CD200 serum has been shown to give stronger yet identical staining compared to an antigen-affinity-purified rabbit anti-CD200 at 1/250 dilution.¹⁹ Antigen-affinity-purified antihuman CD200R1 (hereafter designated as anti-CD200R as there is only one confirmed CD200R protein in humans unlike the mouse) (Antibodies Online ABIN1715098 Lot# 9A13M60, KLH-huCD200R1 synthetic peptide) was used at 1/250 and as a control, antigen-affinity purified rabbit anti-KLH (ABIN401183 Lot #30477, whole KLH) was used at 1/250. Rabbit mAb anti-Ki-67 was used at 1/250. Rabbit IgG was detected using ENVISION and AEC followed by hematoxylin. The slides were scanned using Imagescope and photographed at 400 \times . Staining intensity for CD200 and CD200R was measured using NIH Image J 1.4 software. Using the Image J drawing tool to include syncytiotrophoblasts and cytotrophoblasts, the differential staining intensity red low and blue high measured in pixels of at least 13 villi was obtained and the isotype-control background was subtracted. It was not possible to separately analyze syncytiotrophoblasts and cytotrophoblasts.

2.3 | PCR typing

Detection of trophoblast chromosome abnormalities using QF-PCR technology, which detects >94% of relevant abnormalities, was done as described elsewhere.^{20,21}

2.4 | Statistics

The significance of correlations was determined using Fisher's Exact test, and Student's *t* test was used to test gestation-related

differences in Ki-67⁺ cell frequency and differences in intensity of CD200 and CD200R staining. A 1-tail *P* < .05 was considered statistically significant.

3 | RESULTS

Figure 1 shows immunohistochemical stains of CD200 on placental villi at the 5th, 6th, 8th, and 9th weeks of human gestation based on embryo Carnegie stage, and at term. The 5 and 9 week villi have previously been shown and new areas of the placenta on each case included here with the new anti-KLH control antibody control for anti-CD200R.²² It can be seen that syncytiotrophoblasts brush border membranes in direct contact with maternal blood stained intensely, and at term, when villus cytotrophoblasts have disappeared, staining the basal region of syncytiotrophoblasts was evident. There was also staining of first trimester cytotrophoblasts underlying syncytiotrophoblasts. CD200R also stained in both trophoblast populations, although in one sample cytotrophoblasts were more prominently stained. A case of CHI at 36 weeks compared to normal term placenta is also shown. With respect to syncytiotrophoblasts expression of CD200, it is evident that CD200 expression was increased and CD200R expression was decreased. However, CD200R expression by the infiltrating histiocytes was striking greater than CD200 expression.

Table 1 summarizes quantitative analysis of trophoblast proliferation based on Ki-67⁺ staining was assessed on at least five low power photomicrographs from which mean villus number and Ki-67⁺ cells number was calculated. In the four missed abortion cases, trophoblast cells were dividing consistent with their continued viability. At term, Ki67⁺ staining was reduced, and this was reproduced using a second term placenta. In a case of CHI at 36-week gestation, the number of Ki67⁺ cells was similar to term placentae but the number of identifiable villi was significantly reduced, with many identifiable villi showing loss of trophoblast layer due to histiocyte invasion on one side of the villus. The ratio of Ki67⁺ cells/villus was greater than in term placenta consistent with continued survival and growth of viable trophoblasts.

Table 2 summarizes quantitative analysis of syncytiotrophoblasts plus cytotrophoblast CD200 and CD200R staining based on at least 13 villi photographed at 200 \times rather than 400 \times magnification to facilitate having a sufficient number of whole villi in each field. These photomicrographs were larger than what is displayed in the Figure 1 montage so it was easy to precisely encompass the trophoblast layer using the Image J drawing tool so as to enable quantitation of staining intensity. In the Figure 1, 6-week case it can be seen that occasionally there can be a villus where anti-CD200R stained primarily villus cytotrophoblasts but this was not the norm. In all missed abortion cases, in term placenta, and in CHI, CD200 and CD200R were detected. In the 9 week missed abortion sample, both CD200 and CD200R dropped, but in all cases CD200 > CD200R. CD200 > CD200R persisted to term and was also seen in CHI where CD200R was very low. Cytotrophoblast proliferation (Ki67⁺ cell count) dropped after the

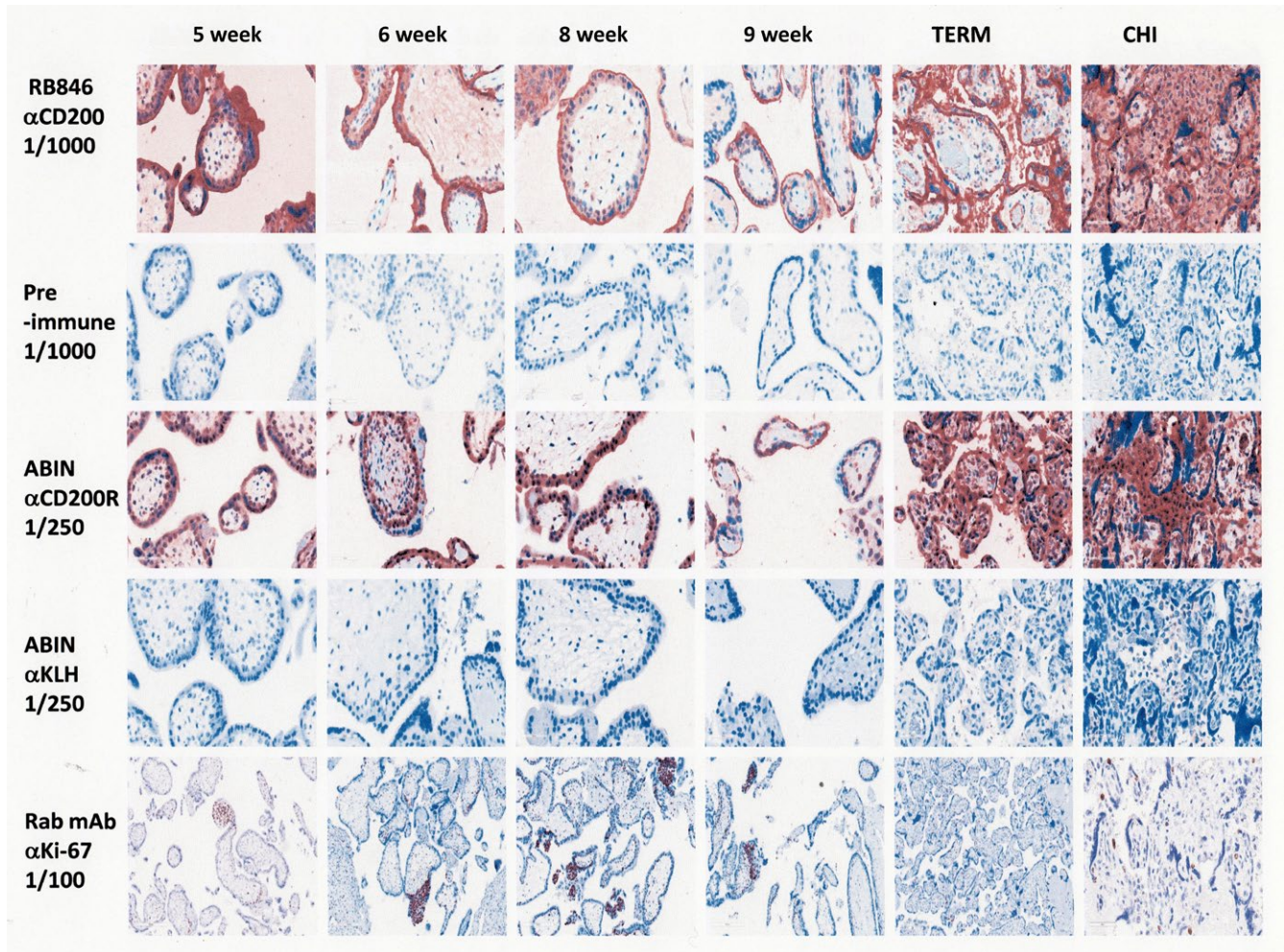


FIGURE 1 Composite results of immunohistochemical staining for CD200 and CD200R with relevant control antibody plus Ki67 staining is illustrated at 400× magnification

first trimester but was preserved even in CHI where villus damage was taking place.

4 | DISCUSSION

As set out in the introduction, at least 50% of miscarriages are attributed to karyotype abnormalities present in the embryo and its trophoblast, and a trisomy, which can be recurrent, is a frequent “cause” of spontaneous abortions. In the 4 missed abortion cases we studied, we expected the 6 and 9 weeks pregnancies to be karyotype abnormal as difference between CD200 and CD200R was significant and not different from the result in the successful term placenta control. Conversely, we hypothesized that the 5 and 8 week missed abortions, where CD200 was not statistically greater than CD200R staining, could represent putative “embryo failure due to immune rejection.” Neither chromosome karyotype nor microarray testing were available for the studied missed abortion cases. However, by rapid aneuploidy testing using PCR, none of the missed abortion placental villi for which PCR typing was possible showed evidence of trisomy. Chromosomal

microarray analysis is more sensitive than PCR for detecting chromosome abnormalities, but may also identify abnormalities that are not lethal.^{23,24} Of nine women having preimplantation embryo analysis on non-transferred embryos at the pre-morula stage for genetic abnormalities, 70% had abnormalities, and so the implantation rate per embryo should not have exceeded 30% but all nine women had a live birth. The usual results in this center for screened women were 23.3% from 60 embryos.^{23,24} But in fact live birth rates per embryo transferred (which is more stringent than implantation rate) can reach 50% suggesting many embryos that have abnormal blastomeres may succeed under optimal circumstances.²⁵

The importance of CD200 and CD200Rs expression in successful mouse pregnancy was outlined in the Introduction. In our human study as shown in Table 2, CD200 expression was slightly but significantly reduced in missed abortion and CHI villi while CD200R was only reduced in the 9 week missed abortion case and in our CHI case. It is tempting to speculate that CD200 expression can be reduced to a level insufficient to prevent embryonic failure but that continued expression of that reduced level of CD200 in our missed abortion cases might have sufficed to inhibit infiltration of Th17 cell or their

TABLE 1 Ki-67⁺ proliferating villus trophoblasts at different times in gestation

| Carnegie stage (gestation age) | Genetics | # of 1.6 mm ² villus fields | # villi per field | # Ki-67 ⁺ cells per field | # Ki-67 ⁺ cells per villus |
|--------------------------------|--------------------|--|-------------------|--------------------------------------|---------------------------------------|
| 11 (5 wk) | 46 XY ^a | 5 | 22.3±3.2 | 53.8±16.1 | 2.4 |
| 12 (6 wk) | 46 XY ^b | 4 | 21.3±3.1 | 66.8±4.3 | 3.1 |
| ES (8 wk) ^c | NA ^c | 4 | 16.0±2.3 | 126±3.5 | 7.9 |
| 19 (9 wk) | 46 XY ^d | 4 | 24.5±5.8 | 65.0±7.8 | 2.0 |
| Term 1 | ND | 4 | 117±7.5 | 21.3±1.9 | 0.18 |
| Term 2 | ND | 4 | 54.8±7.8 | 31.5±5.8 | 0.57 |
| CHI | ND | 4 | 26.8±4.8 | 29.3±6.0 | 1.1 |

^aNormal male fetus missed abortion. Patient had three prior spontaneous losses. Fibrin clotting in decidual vessels suggests possible thrombophilic cause. ND not done.

^bEmbryo CR length 5.0 mm.

^cEmbryo seen on ultrasound at 5 weeks gestation had disappeared by 8 weeks in spite of rising βhCG. Empty sac (ES) was removed by dilatation and curettage at 8 weeks gestation based on last normal menstrual period. Three attempts to obtain embryonic DNA free of contaminating maternal DNA were unsuccessful, so no result could be obtained.

^dEmbryo CR length 2.0 cm had been implanted in a one horn of a unicornuate uterus.

TABLE 2 Expression intensity of CD200 and CD200R on placental villus trophoblasts

| Carnegie stage (gestational age) | CD200 | CD200R | Ratio ^a | CD200-CD200R |
|----------------------------------|---------------------------------|-------------------|--------------------|--------------|
| 11 (5 wk) | 57.110±2.701 (14) ^{b*} | 54.937±2.139 (14) | 1.04 | 2.172±3.439 |
| 12 (6 wk) | 50.578±3.210 (13)** | 39.972±4.620 (13) | 1.27 | 10.606±5.626 |
| ES (8 wk) | 51.696±2.293 (14)** | 48.753±3.070 (14) | 1.06 | 2.943±3.832 |
| 19 (9 wk) | 35.251±2.690 (14)*** | 17.560±3.013 (14) | 1.99 | 17.691±2.009 |
| Term placenta | 64.937±2.428 (13) | 41.866±2.490 (13) | 1.55 | 23.071±3.478 |
| 36 wk CHI | | | | |
| Villi | 49.917±2.037 (13)** | 16.283±1.676 (13) | 3.07 | 33.634±2.638 |
| Intervilli | 53.884±1.335 (13) | 56.245±1.450 (13) | 0.96 | -2.361±1.971 |

^aRatio of CD200/CD200R.

^bMean±1 SEM. Parentheses show number of villi scored.

*P<.025, **P<.0025, ***P<.00025 reduced compared to term placenta control (Student's t test).

differentiation from Tregs,¹ and thereby explain why the abortions in our series were missed and did not proceed to completion. It is known that many missed abortions will resolve if observed and it is quite possible that the two cases with low CD200-CD200R values were in the process of resolution mediated by allowing infiltration of Th17⁺ maternal cells. We used normal term placenta as a successful pregnancy control as we did not have any healthy normal karyotype pregnancy termination villi (which are unfortunately not removed by dilatation and curettage so as to retain decidua but rather are traumatically removed and disrupted by vacuum extraction), nor did we have trophoblasts obtained by TRIC prior to the onset of an active pregnancy loss to compare to our missed abortion material.⁶ Additionally, we did not have any villi from early progressing spontaneous abortions tested for anomalies using our QF-PCR technology. In CHI, which is associated with fetal growth restriction, CD200 staining intensity although lower than in term placenta was much less reduced than was staining of CD200R (i.e., the CD200/CD200R ratio was increased) and in areas where a part of villus walls were destroyed, and particularly where villi had been replaced with fibrinoid, more neutrophils than expected

from mere presence of maternal blood could be seen but we have no markers to indicate if these cells were activated (Arredondo and Clark, unpublished observations). In the infiltrate, CD200 was also detected but CD200R expression was much increased compatible with known role for of CD200R on histiocytes, and the CD200-CD200R value was "negative." Nevertheless, although CD200 is known to suppress inflammation and rejection by inhibiting NK cells, promoting M1→M2 macrophage transition, and by stimulating regulatory T-cell generation, inflammation in CHI had not been suppressed by the CD200 which was present.^{1,12,16,22} In a mouse model of second-third trimester pregnancy loss triggered by fetal Class II MHC presentation of the 2WIS peptide, activated CD4⁺ Th1 T cells identified by production of IFN-γ has been reported to block Treg generation,²⁶ and that could account for the failure of CD200 in CHI pregnancies to stimulate enough Treg activity to prevent inflammatory cell infiltration. In missed abortions with no loss of Treg cells, the IFN-γ⁺ T cells are CD8⁺, so obviously IFN-γ is not a cytokine that alters Tregs.⁷ Up to 5% of the infiltrate in CHI are T cells, their phenotype and function known, and B cells and NK cells are usually absent.²⁷ However, CHI is known to begin

in the first trimester and most cases represent first trimester abortions.²⁷ An alternative interpretation to that provided by the 2WIS mouse model described above is that sufficient expression of CD200 in first trimester placenta villi affected by CHI prevents infiltration by a significant number of abortogenic IL-17-dependent neutrophils in the 1/3 of affected embryos that survive into the second and third trimester. Chronic villitis, in which T cells invade villi, is related to CHI and in some cases, both pathologies may be seen together. It is tempting to speculate that fetal antigen-driven inflammation can overcome protective effects of CD200 and CD200-induced Tregs.

The above data suggest that free CD200 as calculated from CD200-CD200R may not be the only determinant of pregnancy failure or CHI that leads to fetal growth restriction. Although there are other immunoregulatory molecules along with CD200 that may be important, such as PDL-1 and galectin-1 (as demonstrated using mice),²⁸⁻³³ the alternative proposed role for simultaneous expression of CD200 and CD200R on the same cell is autocrine effects. Such effects are common in immunology. For example, IL-1 β can bind to IL-1 β R on the same monocytes that produced it, and increase secretion of IL-1 β .³⁴ Further, activated T cells produce IL-2 which binds to upregulated high-affinity IL-2R promoting proliferation and differentiation.³⁵ HER2⁺ breast cancers produce IL-6 that binds to IL-6R promoting tumor growth.³⁶ Breast cancer cell production of VEGF-A binds to VEGF-R2 on the same cells to promote growth.³⁷ Autocrine effects of TGF- α and a variety of other mediators are important in liver cell regeneration.^{38,39} The malignant Reed-Sternberg cell in Hodgkin's lymphoma expresses the immune check point inhibitor PDL1 (and also PDL2) that can bind to PD1 on the same cell with an as yet undescribed effect.⁴⁰ Wang et al. reported that CD200 acting of CD200R⁺ syncytiotrophoblasts (that produce β -hCG in humans) enhances pregnancy success but few details were provided.¹² Syncytiotrophoblast CD200 could have an autocrine self-stimulating effect as well as paracrine effects on CD200R⁺ cytotrophoblasts. However, the reduction in Ki67⁺ trophoblasts and Ki67⁺ cells/villus at term was associated with a slightly higher CD200-CD200R value than in missed abortion villi, but in CHI, the highest CD200-CD200R difference obtained in our study was associated with an increase in Ki67⁺ cells/villus, so it seems unlikely that CD200 regulates cytotrophoblast cell proliferation. Determining the functional effects of CD200 binding to CD200R on different villus trophoblast populations will require in vitro studies of purified cell populations followed by in vivo validation. Whilst our present study involves a small number of samples, the data are sufficiently intriguing to encourage further investigations. One cannot extract proteins and perform Western blots using formalin fixed material as formalin cross-links tissue proteins, and similarly, one cannot test the effects of CD200:CD200R signaling and selective activation of downstream subcellular pathways without having purified viable trophoblast tissue for in vitro studies. Quantifying mRNA in individual cells in formalin-fixed paraffin-embedded tissues is more informative than whole villus mRNA data but is not for us a readily available technology. Further work will be necessary to evaluate CD200-CD200R interactions at the placental-maternal interface.

NOTE ADDED IN PROOF

After this article was accepted, we found the same levels of CD200 immunostaining in villi from an elective termination of a PCR disomic normal pregnancy at 7 weeks gestation as measured in 2 term placentae but significantly lower staining in an additional 8 weeks gestation missed abortion sample stained for CD200 at the same time.

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