Cell-Surface CD200 May Predict Efficacy of Paternal Mononuclear Leukocyte Immunotherapy in Treatment of Human Recurrent Pregnancy Loss

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Keywords
CD200, immunotherapy, infertility, recurrent spontaneous abortion, tolerance, transfusion-related immunomodulation

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Problem
The allogeneic leukocytes in transfused blood can modulate the recipient’s immune system so as to induce TGF-β-producing suppressor cells, and the cell-surface CD200 tolerance-signaling molecule on mononuclear dendritic cells is required for this effect. A subset of couples with unexplained recurrent pregnancy loss appears to benefit from transfusion of allogeneic paternal blood leukocytes (LIT), and considerable effort has been devoted to characterizing those who may benefit. Some data has been accumulated for LIT as sole therapy in patients with classical spontaneous abortions with respect to dose-response, duration of protection, need for boosting, excluding patients with autoimmunity, and inefficacy of paternal mononuclear cells stored at 4°C overnight before use which causes loss of cell-surface CD200. Recent data emphasize an important role of expression of the CD200 tolerance-signaling molecule on cells used to prevent abortions both in mice and humans.

Method of study
An observational study of outcome as a function of the number of CD200+ paternal mononuclear cells was performed. Fourteen patients constituted the pilot group. Patients with autoimmunity who had failed inspite of treatment with IVIG + Heparin + Aspirin ± Prednisone were allowed to have paternal mononuclear cells added to their therapy. CD200 on purified paternal blood mononuclear cells was measured by flow cytometry.

Results
The number of CD200+ cells administered was significantly greater in women achieving pregnancy \((39.2 \times 10^6 \text{ versus } 20.8 \times 10^6), P < 0.025\) and in those who achieved a live birth \((50.2 \times 10^6 \text{ versus } 20.8 \times 10^6), P < 0.005\) compared to those who did not achieve pregnancy, and % of paternal cells that were CD200+ was greater \((11–12.5\% \text{ versus } 5.6\%), P < 0.01\). Amongst those achieving pregnancy which failed, the CD200+ cell dose was not significantly different from the non-pregnant group \((30.5 \times 10^6 \text{ versus } 20.8 \times 10^6)\).

Conclusion
The number of CD200+ paternal mononuclear leukocytes may be an important determinant of subsequent reproductive outcome in a subset
Introduction

Paternal mononuclear leukocyte immunotherapy (LIT) appears to improve the live birth rate in a subset of women with unexplained recurrent miscarriages, specifically women with primary recurrent miscarriages, no evidence of autoantibodies such as ANA or ACL, and loss of normal karyotype embryos. The mechanism of pregnancy failure is thought to be similar to the pathogenesis of recurrent resorptions in the CBA × DBA/2 mouse model where there is an excess of Th1 > Th2, cytokine activation at the feto–maternal interface, activation of fgl2 prothrombinase, thrombin, complement, and infiltration by polymorphonuclear leukocytes. An activation of maternal immune effector T and NKT cells, and lack of Treg-mediated tolerance is antecedent to Th1 cytokine excess. These events are countered by the CD200 tolerance signaling molecule (an antithesis for CD80/86 and CD40 costimulatory molecules). Whilst some place considerable emphasis on rationale for a treatment, empirical clinical trial data represents the key evidence with respect to whether or not a therapy works. In this respect, it is critical to know the characteristics of patients who respond and those who have no benefit, or a negative effect of treatment.

CD200 is expressed on trophoblasts and by cells in maternal decidua, and acts by binding to CD200 receptors. Blocking CD200 in vivo in the CBA × DBA/2 model augments the rate of abortions, whereas CD200Fc administration blocks abortions; similarly, in LPS-driven abortions in B6 background mice, up-regulating CD200 prevents losses, consistent with its anti-inflammatory effects. CD200 is also expressed on lymphomyeloid cells, and expression on allogeneic dendritic cells is proposed to account for the phenomenon of transfusion-related immunomodulation (TRIM). TRIM can suppress the inflammatory response and via CD200, activate suppressor cells in the recipient that make transforming growth factor-β (TGF-β). Administration of BALB/c mononuclear cells bearing paternal DBA/2-type paternal antigens to CBA/J female mice prevents most of the abortions in the CBA × DBA/2 model, but this protection is abrogated by treating the immunizing cells with anti-CD200 or by storing the cells overnight at 4°C which depletes cell surface CD200. Similarly, in recurrent miscarriage patients, paternal mononuclear leukocytes stored at 4°C lost surface CD200 and therapy proved ineffective, whereas cells stored at 37°C, which increases surface CD200 expression, have been reported to be highly effective in a recent randomized controlled clinical trial.

Most of the literature on factors determining efficacy of LIT have focused on patient selection. In most RCTs, the time of onset of the pregnancy loss has not been strictly delimited. Based on data from Mowbray, LIT appears effective if given before the 6th week of gestation, and this data has suggested that losses prior to the 6th week, including chemical pregnancies, merit an alternative treatment. However, similar immune abnormalities are reported in women with classical recurrent miscarriages and losses prior to 6 weeks including ‘implantation failure’ (which can be detected as chemical pregnancies or late ‘heavy’ menses), and Check et al. reported an improved pregnancy rate and live birth rate in IVF failure patients who were given LIT. There has been some data on properties of the treatment maneuver, including dose–response and duration of protection, but no information with respect to the dose of CD200 cells and outcome has been generated. It was hypothesized that higher dosed of CD200 paternal mononuclear leukocytes would be associated with a higher rate of reproductive success. Conversely, reproductive failure would be associated with a lower dose of CD200 cells, and a loss where a high dose of CD200 paternal cells had been administered would be due to either a karyotype abnormal embryo or untreated autoimmunity. An observational study was performed to test these predictions about CD200 cell dose.

of patients. A lower % CD200 cell number may also reflect hitherto unappreciated paternal factors bearing on reproductive success. It is feasible to recruit women to enter observational studies and to obtain useful data as a foundation for further studies. More complete patient characterization in a larger study is needed.
Materials and methods

Patients

Women with unexplained recurrent pregnancy loss were referred by their specialist Obstetrician Gynecologist to the author’s Reproductive Medicine Clinic in the Department of Medicine, McMaster University Medical Center, 1200 Main St. West, Hamilton, Ontario. Patients with three or more spontaneous abortions and no live births with their current partner, and negative tests for ANA and ACL antibodies, with both husband and wife negative for VDRL, Hepatitis B antigen, anti-Hepatitis C, HIV and HTLV were offered a trial of paternal mononuclear leukocyte therapy (without charge) in a study approved by the Hamilton Health Sciences/McMaster University Research Ethics Board, 1290 Main St. West, Hamilton, Ontario. Anti-CMV was measured in all couples. Informed consent was required, and the consent document provided full details of background data, controversies, rationale for the study, possible risks both real and theoretical, and alternatives. Patients with autoimmune abnormalities and patients with secondary recurrent miscarriages who had re-aborted in spite of intravenous immunoglobulin infusions (IVIG) + heparin (H) and aspirin (A) ± prednisone (P) were also eligible to have paternal mononuclear cell LIT added to their current therapy. All patients were tested for LAC, anti-thyroid antibodies, ANA, ENA, anti-smooth muscle/anti-parietal cell/anti-mitochondrial antibodies, and anti-thyroid and anti-thyroglobulin antibodies, and anti-reagin (VDRL) antibodies. Patients with autoimmune abnormalities were initially offered IVIG + HA in a separate trial. Some patients had a panel of APL antibodies measured at Millenova Laboratories (Chicago, IL, USA) if they could afford the cost. This more extensive test panel was not available in Canada. All patients had ABO and Rh typing of both partners and a check for anti-husband erythrocyte antibodies in the woman.

Immunotherapy

The Mowbray method was used. The McMaster clinic program was conducted under the Immunotherapy Oversight Committee chaired by the Head of Transfusion Medicine at Hamilton Health Sciences and Director of the Canadian Blood Services Program in Hamilton. Standard Operating Procedures (SOPs) were in place for all procedures. In accordance with the Mowbray protocol, one unit (400–450 mL) of blood was collected from the husband in ACD anticoagulant, centrifuged to isolate the buffy coat, and the buffy coat was purified by centrifugation over a cushion of sterile Ficoll-Paque. The purified cells were washed, counted, and an aliquot was taken for staining for cell surface CD200 by flow cytometry as described elsewhere. The purified cells were held at 4°C on ice in 4–5 mL phosphate-buffered saline, and at time of use, a second aliquot was taken for a repeat CD200 determination by flow cytometry. Prior to attempting conception, two-thirds of the cells were given i.v. and 1/3 were divided among two intradermal and two subcutaneous sites using both forearms. The cell dose was limited to 400 million cells based on previous dose–response data. All Rh patients received anti-Rh globulin (120 µg) i.v. immediately prior to paternal cells. For patients with autoimmunity who were continuing to receive IVIG + HA ± P, IVIG represented intravenous immunoglobulins (Gamagard or Gammunex, 0.4 g/kg i.v. every 4 weeks or 40 g i.v. every 3 weeks for IVFET patients), A represented aspirin 81 mg p.o. daily, H represented 5000 units of standard heparin s.c. b.i.d., and P represented 5 mg of prednisone taken bid until a positive pregnancy test and then 10 mg b.i.d. until 12 weeks of gestation when the prednisone was rapidly tapered to zero to avoid complications. All treatments were commenced prior to conception and with the exception of P, were continued to term. Patients were followed by thrice weekly βhCG values, a one weekly estradiol + progesterone level, and by serial ultrasound examinations to 12 weeks gestation. If the serial βhCG trajectory fell below the expected curve and ultrasound showed absent fetal heart, the uterus was emptied and the products of conception were sent for karyotyping by culture in accordance with RCOG guidelines and the recommendation from Christiansen et al. for investigational studies, and for FISH staining for common trisomies was performed where possible.

Statistics

Pregnancy was defined as one or more βhCG values >5. The endpoint was live birth. Patients were classified based on outcome. The absolute number of CD200+ cells was used to determine the treatment dose. The mean and S.E.M. was calculated. The null
hypothesis based on previous published results was patients who became pregnant and had a live birth had not received a larger dose of CD200+ paternal cells.4,5,12 The significance of differences between groups of patients was determined using Student’s t-test. The data was plotted using Prism Graphpad.

Results

The characteristics of the 14 patients in this study are provided in Table I. Spontaneous losses with the current partner are shown. Remote elective terminations are not listed. There were no ectopic pregnancies. Results of immune testing are also given, along with the total dose of CD200+ cells administered. There was no difference in % CD200+ cells immediately after purification and at the time the cells were administered (after a short <2-hr period of storage on ice.) (data not shown).

Fig. 1 summarizes the CD200+ cell dose in different categories of patients. Patients achieving pregnancy had received a significantly larger number of CD200+ cells than those not achieving a pregnancy (39.22 ± 6.79 x 10^6 versus 20.82 ± 3.90 x 10^6, P < 0.025). In pregnant patients who had a live birth, the CD200+ cell dose was also significantly greater than in the no pregnancy (infertile) group.

Table I Characteristics of the Individual Patients in the Study

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>History</th>
<th>Immune testing</th>
<th>PreLIT Rx</th>
<th>Result</th>
<th>PBL dose</th>
<th>CD200+ cell dose</th>
<th>Result</th>
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<tr>
<td>1</td>
<td>32</td>
<td>AAAA Skin rash in Cuban sun</td>
<td>Neg.</td>
<td>IVIGHA</td>
<td>A46XY</td>
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<td>66.9 x 10^6</td>
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<td>2</td>
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<td>Neg. (oPA*)</td>
<td>IVIGHA</td>
<td>A46XX</td>
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<tr>
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<td>ANA*RF+ AT+</td>
<td>IVIGHA</td>
<td>A46XX</td>
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<td>23.1 x 10^6</td>
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<td>A46XX</td>
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<td>16.3 x 10^6</td>
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<tr>
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<td>IVIGHA</td>
<td>A46XX</td>
<td>400 x 10^6</td>
<td>14.4 x 10^6</td>
<td>A &gt;6 weeks</td>
</tr>
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<td>6</td>
<td>39</td>
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<td>IVIGHA</td>
<td>A46XX</td>
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<td>54.6 x 10^6</td>
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<td>400 x 10^6</td>
<td>24.9 x 10^6</td>
<td>Infert</td>
</tr>
<tr>
<td>9</td>
<td>42</td>
<td>AAA infert</td>
<td>ÑNK*</td>
<td>IVIGHA</td>
<td>A46XX</td>
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<td>40.9 x 10^6</td>
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<tr>
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</tr>
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<tr>
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<td>AA+ infert/IVF</td>
<td>ANA<em>ATA</em></td>
<td>IVIGHA</td>
<td>A46XX</td>
<td>400 x 10^6</td>
<td>6.6 x 10^6</td>
<td>Infert</td>
</tr>
</tbody>
</table>

aPatient had a live birth after LIT and a second pregnancy without a boost that miscarried (A).
bPatient had a live birth with LIT >3 years previously. Note that pre-LIT therapy was continued in all patients.
cTwins – one stillborn and one died immediately after birth. A subsequent pregnancy with live birth occurred after a metropasty.
dPrevious live birth on IVIG + A >3 years previously.
eElevated NK activity at Millenova Labs. Where patient had arranged her own testing.
fAt least one miscarriage + history of late heavy menses (occult losses).
gTwo early losses (chemical pregnancies after IVFET) + six IVFET failures (negative βhCG).
In the group that became pregnant and aborted, the CD200⁺ cell number was intermediate and was not significantly greater than in the infertile group in contrast to pregnant patients who achieved a live birth (30.46 ± 10.60 × 10⁶ versus 20.82 ± 3.90 × 10⁶).

The pregnant patients with success had received a larger number of CD200⁺ paternal cells than those who became pregnant and failed, but this difference did not achieve statistical significance in part due to insufficient numbers for adequate statistical power; the pregnant and fail group also showed considerable heterogeneity in CD200⁺ cell dose (as reflected in the larger S.E.M. value compared to the pregnant and success group), and in timing of the pregnancy failure. Three patients had classical abortions beginning after 6 weeks gestation, and two had received >50 million CD200⁺ cells. One lost a karyotype normal male embryo, and a subsequent pregnancy succeeded with IVIG. A second lost a karyotype normal female embryo. Both had received >50 million cells. Karyotyping of one other patient was not successful for technical reasons: she had received <20 million CD200⁺ cells. Two other patients receiving similar low doses had losses before 6 weeks.

Fig. 2 shows that the difference in CD200⁺ cell dose among the patients set out in Table I was largely determined by the husband. In some cases, the % CD200⁺ cells was low, and in other cases, total cell recovery from 1 unit of blood was <400 million. Patient 12 had a history of one definite miscarriage and a number of late heavy menses suggestive of occult losses. If she is excluded from the calculations in Fig. 1, the patients achieving pregnancy still received significantly more CD200⁺ paternal cells (P < 0.05) and the % CD200⁺ cells in their husbands blood (Fig. 2) was still significantly greater than for their husbands of the infertile outcomes (P < 0.025).

### Discussion

The data in this study show that patients achieving a successful pregnancy had received a larger number of CD200⁺ cells than patients not achieving pregnancy. It was an a priori prediction of this study that women achieving successful pregnancy would have received more CD200⁺ cells than women aborting their pregnancies (or conversely, that a higher dose of CD200⁺ cells would be associated with a better reproductive outcome). This was, in fact, the trend but the difference did not reach P < 0.05 due to small sample size (inadequate power), and the larger degree of variability in those who re-aborted as set out in the Results.

Pregnancy failure in treated patients can have several causes, and this explains the increased variability in the CD200⁺ doses in this group. Indeed, as noted, three patients had classical losses >6 weeks gestation (one putatively autoimmune as the loss was normal XY karyotype and IVIG led to a subsequent success) and two had losses <6 weeks. What was unexpected was that those achieving pregnancy had received a larger number of CD200⁺ cells than those who did not achieve pregnancy (or conversely, that a larger dose of CD200⁺ cells was associated with achieving pregnancy). The effect of LIT in increasing the chance of achieving pregnancy was similar to that of Check et al. who reported improved pregnancy rates in IVF-failure patients receiving LIT with their next cycle (38.3%, 70.3% and 90% with LIT versus 28.7%, 45.9% and 60% for untreated controls in three different reports) but a significant increase had not been seen in RSA patients in the RMITG study where pregnancy rates were only slightly greater (78%) in the LIT-treated patients compared to the controls (75%). However, autoimmune testing in this study was quite limited, and infertility (requiring IVF) was not the major problem in these patients. In the Ober et al. study, 79.1% of immunized women achieved pregnancy compared to 74.1% in the control group; this study used only 250 million cells and these cells were

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**Fig. 2** Percentage of paternal mononuclear leukocytes that were CD200⁺ in patient groups with different outcomes. P, achieved pregnancy; PS, pregnancy resulted in a livebirth; PF, pregnancy failed; NL, no pregnancy/implantation occurred and hence, ‘infertile’. 

#### Significantly greater than NP, P < 0.013; +++Significantly greater than NP, P < 0.0025; ++Not significantly greater than NP, P > 0.05.
stored overnight (which reduces cell surface CD200 levels and efficacy in preventing miscarriages, although perhaps not for the improvement in pregnancy rate). In comparison to the RMITG and Ober et al. reports, the patients in this study were a select group (Table I), and most were receiving additional treatments for autoimmune problems when given LIT. Interestingly, Winger et al. recently reported improved live birth rates in an observational study of women with primary RSA where LIT was added to IVIG + HA. Similar effects were noted where anti-TNF-α was substituted for LIT. In TRIM studies in mice, allogeneic blood cells treated with anti-CD200 led to an enhanced TNF-α response in the recipient [see ref. (11) and D. A. Clark, unpublished data].

A live birth rate of 4/14 may not seem particularly impressive, given that for the average number of prior losses, has this been a population of primary recurrent miscarriage patients without a positive ANA or ACL, one would have expected this number of live births without LIT based on an intention-to-treat analysis. However, the patients in this study differ by having evidence of autoimmunity in 8/14, and such patients may have a worse prognosis untreated. Indeed, a worse prognosis has been documented for women with an ANA or ACL autoantibody by Nielsen and Christiansen. In other words, the patients reported in this study represented a highly selected group with a worse prognosis. In patients with autoimmunity, it has been argued that LIT alone may be inappropriate, but LIT + auto-immune therapy has not been formally addressed in a full paper. Further, only 9/14 (64%) patients in this study achieved a pregnancy, compared to 91% in the study mentioned above. If one corrects for the infertility in this study population, a 40% live birth rate would have been expected, which compares favorably with a 53% success rate in primary recurrent miscarriage patients and no ANA or ACL.

As the sample size is small in this study, there is insufficient power to determine if 40% and 53% are different; such a comparison should be properly made using groups of patients with the same phenotypes, and that would require a much larger study. Additionally, Clifford et al. reported a high rate of successful pregnancy in a group of women treated with supportive care, but women with autoimmunity (and a putatively worse prognosis) had been excluded. An additional explanation for a low success rate suggested by this study is inefficacy of CD200+ cell doses <40 x 10⁶ but in an observational study, one can only demonstrate associations. It is not possible to say that a larger CD200+ cell dose would have improved the chance of a pregnancy without randomizing to high or low CD200+ cell dose, a randomized phase II trial. Where the % CD200+ cells was low in the donor blood, a mirror increase in the non-CD200+ population may have been a factor in the infertility. Indeed, in TRIM studies in mice, treatment of allogeneic blood cells with anti-CD200 to neutralize the subpopulation of dendritic cells with CD200 not only abolished the TRIM effect (detected a stimulation of tumor nodule growth via TGF-β effects), it led to inhibition of nodule formation and an increase in TNF-α+ cells in the recipients (DA Clark, unpublished data). As not all of the patients who received a low dose of CD200+ cells had received a total of 400 x 10⁶ cells, it seems unlikely that deleterious effects of CD200- blood mononuclear cells was responsible to the reduced pregnancy success rate in patients receiving lower dosed of the CD200+ cells. Alternately, low % CD200+ cell numbers may be telling us something about the husbands of pregnancy failure patients. These husbands may be less likely to father pregnancies that succeed for non-immunological reasons. Recurrent unexplained miscarriages have been proposed to be partner specific, and a rationale for LIT has been a more efficient induction of an anti-abortive response (in women whose innate immune system is poised to reject embryos) and the husbands antigens have been viewed as insufficiently immunogenic when delivered by the normal route. However, a deficient tolerance signal rather than lack of immunogenicity could explain partner specificity, and little attention has been given to reproductive performance of the male partner in previous relationships. In this study, none of the women had had miscarriages with another partner.

These hypotheses are testable in a randomized phase II design, where CD200+ cell dose is the variable. For example, if all women received a total of 50 x 10⁶ CD200+ paternal cells, would there be a different outcome in those who had more non-CD200+ cells rather than fewer? If there were not enough CD200+ cells recovered from 400 mL of the partner’s blood, would a repeat treatment to achieve the 50 x 10⁶ total be more effective? Would the outcome differ for all types of patients if 50 x 10⁶ CD200+ cells were set as the target? (Recurrent pregnancy failure is a syndrome that can have many causes, and to suggest that a particular therapy such
LIT should benefit everyone is not unlike demanding that a single antibiotic should cure all types of pneumonia.) Up-regulating CD200 expression by culturing paternal cells at 37°C overnight has been reported in a RCT to achieve a high livebirth rate, but other effects of cell culture cannot be excluded, and culturing at 37°C risks bacterial contamination.5,14 A similar problem could arise if one attempted to purify large numbers of CD200+ blood cells by cell-sorting procedures. In a mouse model of partner-specific recurrent miscarriage, large doses of exogenously administered soluble CD200 (OX-2Fc) was able to prevent losses, but similar preparations are not available for humans at present.7 In the TRIM model, syngeneic lymphoid cells which express CD200 but no alloantigens are inactive in comparison to allogeneic blood cells;11 whilst the explanation for this observation is speculative, it could pertain to a role of alloantigen in bringing antigen + physiological levels of CD200 together in the encounter with cells of the recipient’s immune system. Another conclusion from this pilot study is that patients in future studies will need more extensive laboratory testing to adequately characterize them. It is important to have better diagnosis of subsets that benefit from LIT alone or added to other treatments as in this study, and subsets that do not. Some of the patients in the study had an APL antibody or elevated NK cell activity documented by testing at a US laboratory. In this study, the full panel of APL, and NK activity testing, was not possible due to cost to the patient. As the patients are clearly heterogeneous, there is merit in broad inclusion criteria with more complete immunological characterization as possible so that the effect of CD200+ cell dose in definable subsets can be elucidated.

Previous observational studies have suggested LIT that suppressed circulating NK activity was likely to be associated with successful pregnancy.44 In a recent IVIG study we did, the importance of measuring the in vivo effect of treatment on blood NKT cells as a predictor of success or failure was emphasized.45 Allogeneic leukocyte administration may also induce immunoregulatory cells that suppress NK/NKT and Th1 cytokine production.11,44 In the present observational study of CD200+ cells in LIT, we did not have a surrogate biological marker to assess effect on physiology of the recipient.11,14,44 Such endpoints need to be included in future studies. There may be patients who fail to respond to CD200+ cells, or, who have a subnormal response. To define what represents a clinically relevant response in itself requires an observational approach. However, only an observational study with a panel of immune assays can define responses that correlate with success or failure. Such assays will be an essential part of any phase III trial.

There are at least five conclusions one can draw from the present small ‘pilot’ observational study: (a) it is feasible to do such studies, and women will agree to participate (which is not the situation where there is randomization to a placebo); (b) it is worth investigating the relationship between dose of CD200+ cells and outcome further; (c) more detailed measurements (laboratory and/or clinical) should be done including more extensive autoantibody testing, NK and NKT analysis, Th1/Th2/Th3 cytokine responses of maternal PBL and salivary cortisol levels among other measures of ‘stress level’, both before and after therapy,46 particularly given the importance of stress as a putative trigger of pregnancy failure and infertility and the claim by Clifford and others that stress relieve may prevent miscarriages;5,31,42 (d) the treatment design should be altered or expanded to examine effects of altering total cell dose and dose of CD200+ cells; (e) a much larger sample size should be used to increase statistical power and to allow comparison of groups of phenotypically similar groups of patients (e.g. it would have required twice as many patients to achieve P < 0.05 for success versus failure for CD200+ cell dose in Fig. 1, and an even larger sample to show a difference in % of donor cells that were CD200+). However, the data in this study prove that the approach is feasible and can yield potentially valuable information. Indeed, some striking differences were noted which points to a way forward. The most important step is to independently repeat the observations made in this study using a larger number of patients. A pilot study is useful in that it provides an effect estimate that can be used to decide sample size required for a new study. Those centers who continue to use LIT may have a sufficient patient volume to repeat this study using the needed larger sample size and may have a more extensively characterized patient population. Phase III studies will not become ethical without the necessary observational phase II data, and even then, women will only be willing to enter phase III trials if there is equipoise between assigned treatment arms. Phase III trial designs that lack a solid basis obtained from
phase II studies and which lack adequate laboratory and clinical endpoints and follow-up as discussed above are unlikely to meet with the favor of regulatory bodies.

This study was designed to test the a priori hypothesis that higher doses of CD200$^+$ cells were more likely to be associated with a successful pregnancy, and the result was positive. It should not be taken as proof that LIT works, as clearly under certain circumstances it did not. There are both patient and treatment variables that require additional study, and a goal of this study is to stimulate such work. LIT is a biological therapy, related to transfusion-induced immunomodulation,\textsuperscript{44} which belongs in the realm of ‘tissue therapy’.\textsuperscript{5} The use of complex mixtures has been replaced by use of the purified active agent, particularly in the field of endocrinology. It is of interest that the magnitude of improvement in take-home-baby rate with LIT may be achieved using more specific alternatives, such as anti-TNF-$\alpha$ drugs.\textsuperscript{3,39,40} It would be an important step forward if the same patient population that benefits from LIT were the one that benefits from anti-TNF-$\alpha$ therapy. If that approach is too expensive, one might consider evaluating therapy with a better defined ‘tissue’ with anti-abortive effects commercially available as Intralipid.\textsuperscript{47} However, there will only be progress if such potential treatments are part of well designed studies the results of which are published.

References


37 Bruno DL, Burgess T, Ren H, Nouri S, Pertile MD, Frances DI, Norris F, Kenney BK, Schouten JA, Choo KH, Slater HR: High-throughput analysis of chromosome abnormalities in spontaneous...


