Intravenous Immunoglobulin Treatment Increased Live Birth Rate in a Spanish Cohort of Women with Recurrent Reproductive Failure and Expanded CD56$^+$ Cells

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Problem
Natural killer (NK, CD3$^-$CD56$^+$/CD16$^+$) and NKT-like cells (CD3$^+$CD56$^+$/CD16$^+$) activity is considered among the key factors for reproductive success. In the absence of immunological screening, beneficial effects of intravenous immunoglobulin (IVIG) in preventing recurrent reproductive failure (RRF) have not been reported. Here, we analyse the IVIG influence on pregnancy success in women with RRF and circulating NK or/and NKT-like cells expansion.

Method of study
One hundred fifty-seven women with previous recurrent miscarriage and/or recurrent implantation failure after *in vitro* fertilization were consecutively studied. Sixty-four patients with CD56$^+$ cell expansion, no apparent underlying disease and who maintained their desire to conceive were selected. Forty of them received IVIG during pregnancy.

Results
Overall, the clinical pregnancy rate for the women under IVIG therapy was 92.5% and the live birth rate was 82.5%. Significantly lower pregnancy and live birth rates (25% and 12.5%, respectively) were observed for the patients with recurrent pregnancy loss and NK/NKT-like cells expansion without IVIG. After three cycles of IVIG, NK cell percentages decreased significantly and these values persisted throughout gestation.

Conclusion
Intravenous immunoglobulin therapy for women with RRF and NK or NKT-like cell expansion was a safe and beneficial therapeutic strategy that associated with high clinical pregnancy and live birth rates.

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Introduction

Recurrent miscarriage (RM), defined by the occurrence of three or more consecutive spontaneous pregnancy losses before twenty weeks of gestation, affects about 1% of the child-bearing population.[1,2] Recognized causes of RM are parental chromosomal abnormalities, anatomical uterine alterations, maternal coagulopathies or endocrine disorders.[3] However, in almost half of RM cases, the underlying cause remains unknown.[4] The RRF concept includes RM occurring both after natural pregnancy and following artificial fertilization techniques.[5] Indeed, RM and implantation failure after *in vitro* fertilization recurrent implantation failure (RIF), two important causes of emotional distress for the couples trying to conceive, might share common pathogenic mechanisms. Yet, their assessment is challenged by the difficulties in the identification and characterization of the underlying causes and the lack of reliable biomarkers.

Natural killer (NK) cells have been recently identified as relevant immunological factors involved in reproductive success. Of note, uterine natural killer (uNK) cells (mostly $CD56^{bright}CD16^{-}$) represent the dominant immune cells in the decidua (around 70% of maternal lymphocytes), and they seem to contribute to the normal implantation process; uNK cells have been involved in both the immune tolerance to a semiallogeneic foetus and remodelling of spiral uterine arteries.[6,7] By contrast, the increased proportions and cytotoxic activity of blood type NK cells (mostly $CD56^{dim}CD16^{+}$) and their presence in the endometrium of pregnant women have been related to pregnancy loss.[8,9] The circulating, blood type NK cell proportions seem to mirror the presence of cytotoxic $CD56^{dim}CD16^{+}$ NK cells within the endometrium, and thus, they emerge as a useful tool for defining a subgroup of RM patients that could benefit from immunomodulatory therapy.[10–12] Furthermore, increasing evidence links the local and peripheral production of Th2- and regulatory T-cell cytokines with normal pregnancy, while a shift towards a Th1/Th17-cytokine profile has been associated with miscarriage events.[3] Dysregulation of the local cytokine milieu might be related to the presence of activated, cytotoxic NK cells in the endometrium that could eventually trigger miscarriage. The recently established uNK control upon T cells in the decidua, inducing Th1 and Th17 apoptosis and a Th2 cytokine shift, supports this hypothesis.[13] An imbalance between blood type, cytotoxic ($CD56^{dim}CD16^{+}$) and uterine $CD56^{bright}CD16^{-}$ NK cells could negatively influence on this T-cell regulation in the decidua.

Both classical (invariant) and non-classical NKT cells have been also identified in human decidua.[14] In a broader sense, cell subtypes that resemble both NK and T cells can fall under this category.[15] NKT cells have been involved in the modulation of both innate and adaptive immunity. Although their role at the maternal–foetal interface has been poorly explored, currently available data suggest that they coordinate functional interactions among decidual lymphocytes.[14] Their dual capacity to mediate both pro-inflammatory and tolerogenic immune responses underlines their putative contribution to pregnancy outcome. Blood NKT cells but not decidual NKT cells are markedly biased towards a Th2 profile in normal pregnancy.[16] Thus, in a similar way as NK cells, pro-inflammatory NKT cells presence in the endometrium could trigger a shift towards Th1 cytokine production and eventually contribute to pregnancy loss.

Intravenous immunoglobulin (IVIG) counts among the therapeutic options for women suffering RIF and RM of unknown aetiology associated with NK/NKT-like cell expansions.[5,8,17] Although the mechanism of action of IVIG in this context has not been completely elucidated, it was proposed that it can shift the cytokine balance from Th1/Th17 towards a Th2-regulatory profile. The association between TNF-alpha and IL-17 upregulation and infertility or implantation failure, and the restoration of TNF-alpha/IL-10 ratio after IVIG treatment support this hypothesis.[18–20] Furthermore, evidence suggests that IVIG can cause a decrease in the number and the cytotoxic activity of blood NK cells in *in vitro* models, and this down-modulation was associated with pregnancy success.[8,21,22]

Here, we have evaluated the clinical effects of IVIG therapy on live birth outcome in a series of women suffering RM or RIF, with expanded NK ($CD3^{-}CD56^{+}/CD16^{+}$) or NKT-like ($CD3^{+}CD56^{+}/CD16^{+}$) cell subsets.

Materials and methods

Subjects

One hundred and fifty-seven women with history of RM, infertility or RIF were consecutively referred to our Clinical Immunology Unit from May, 2005 to May, 2011. Thirty-two healthy pregnant (mean age,

32.3 ± 3.1) and 34 non-pregnant women (mean age, 28.7 ± 5.5) with regular menstrual cycles and previously confirmed fertility were also studied, as control groups for analysis of NK/NKT-like cell proportions. A full fertility screening was performed for all women and their partners. This included complete clinical history, physical examination, hormonal analysis, cytology, partner spermiogram, chromosomal abnormality screening and pelvic ultrasound scan to assess ovarian morphology and the uterine cavity, and hysterosalpingography, followed by hysteroscopy or laparoscopy, if indicated. Genetic evaluation included karyotype of parents and tests for inherited thrombophilic disorders (factor V Leiden, prothrombin G20210A mutation, serum homocysteine, and deficiencies of the anticoagulants protein C, protein S, and antithrombin III). Hormonal analysis was performed for thyroid-stimulating hormone (TSH), thyroxin (T4), prolactine and progesterone, and immunological screening included measurement of antinuclear antibodies (by indirect immunofluorescence; Innova Diagnostics, San Diego, CA, USA), anticardiolipin and anti-beta-2-glycoprotein (IgG or IgM) antibodies (Orgentec Diagnostika Gmbh, Mainz, Germany) and lupus anticoagulant, antithyroid antibodies (antiperoxidase and antithyroglobulin antibodies, Phadia ABl; Uppsala, Sweden), antitransglutaminase-2 (IgA, Phadia AB), and NK/NKT-like cells proportions in blood.

Only women with either NK or NKT-like cells expansion and no apparent infectious or lymphoproliferative diseases were selected for the current study. Expansion of blood NK cells ($CD3^-CD56^+CD16^+$, $CD3^-CD56^+CD16^-$ or $CD3^-CD56^-CD16^+$ lymphocytes) was defined as proportions above 12% and for NKT-like cells ($CD3^+CD56^+/CD16^+$) as proportions above 10% of total lymphocytes.[10,12] These cut-off values were locally validated by analysing a control group of 32 age-matched, healthy pregnant and 34 non-pregnant fertile women.

The study protocol was approved by the Ethics Committee of our Hospital. An informed consent was obtained from each patient for study participation and off-label use prior to the initiation of IVIG treatment.

Blood Cells Immunophenotyping

Lymphocyte subsets were analysed using multiparametric flow cytometry, single platform analysis (TruCount®; FACScalibur BD Biosciences, San Jose, CA, USA). Briefly, blood samples were extracted in appropriate EDTA/heparinised vacuum tubes and processed within 2 hr of collection. Peripheral blood lymphocytes were stained with the following monoclonal antibodies according to the manufacturer recommendations: CD3-fluorescein isothiocyanate (FITC); CD4-Allophycocyanin (APC); CD8-phycoerythrin (PE); CD16/CD56-PE; CD19-APC; and CD45-peridinin chlorophyll protein (PerCP), lysed, then washed and acquired in FACScalibur (BD).

IVIG Therapy Protocol

All women with RM selected for IVIG therapy received 400 mg per kg of body weight (Privigen®; CSL Behring AG, Bern, Switzerland or Flebogamma®; Grifols S.L., Spain) every 3–4 weeks from the date of known pregnancy to 13 weeks of gestation. In patients undergoing *in vitro* fertilization (*IVF*), a first IVIG infusion of 400 mg/kg of body weight was given within 24 hr before the embryo transfer, then at day 15 (after confirmed biochemical pregnancy) and afterwards every 3 weeks during the first trimester of gestation. After gestational week 13, patients with both RM and RIF were given IVIG with a monthly dose of 200 mg/kg of body weight until 35 weeks of gestation.

Statistical Evaluation

Descriptive data are presented as mean ± standard deviation (S.D.). The normality of the distribution of data values was assessed with the Kolmogorof–Smirnov test. A two-sided, paired *t*-student test was used to compare the NK cell levels at different endpoints or between groups. For the comparison of NKT-like cell percentages, we used the nonparametric Wilcoxon test. A two-sided, Chi squared test or the Fisher exact test, when necessary, was used to compare the pregnancy and live birth rates in study patients and control groups. A significance level of 5% was chosen. Statistical analysis was performed using SPSS 15.0. (SPSS Inc., Chicago, IL, USA).

Results

Patients Characteristics

Sixty-four women (40.8%) with expanded NK/NKT-like cell subpopulations, who had either prior recurrent miscarriages and got pregnant (RM group, $n = 24$, 37.5%, range 3–7, mean 3.5 ± 0.9) or recurrent

implantation failure and underwent new *IVF* cycles (RIF group, $n = 40$, 62.5%, range 3–11 *IVF* cycles, mean 5.6 ± 2.5), were selected from the total cohort. The average age at pregnancy confirmation for the selected patients was 37.2 ± 4.2 years (range, 27–44).

Forty patients (62.5%) were scheduled for IVIG therapy (20 of them with RIF), while the remaining 24 (37.5%) did not receive IVIG for diverse reasons (mostly due to economic reasons or because IVIG was not proposed by their attending physician), of which, 20 had RIF (Table I). Extensive gynaecologic, biochemical, haematological and genetic studies ruled out any known cause of infertility. Similarly, none of the selected patients presented any of the screened thrombophilia associated mutations. Overall, the immunological studies did not reveal pathological serum antibody levels. Three patients (two included in the IVIG group) had positive antinuclear antibodies (at titres up to 1/320), and this finding was not associated with any clinical manifestation or other immunological alterations of defined systemic autoimmune disease. One patient (not scheduled for IVIG) had positive antithyroid antibodies and two patients (IVIG group) showed low titres of IgM anti-cardiolipin or anti-beta-2 glycoprotein I antibodies, without fulfilling antiphospholipid syndrome diagnostic criteria; none of them had positive IgG anti-phospholipid antibodies.

Intravenous Immunoglobulin Therapy Effects on Pregnancy Outcomes

Treatment was started in women with natural pregnancies at average gestational week of 3,5 ± 1.8; in patients undergoing *IVF,* first IVIG infusion was given within 24 hr before embryo transfer, as previously described. A positive biochemical and ultrasound pregnancy was confirmed in 37 out of 40 women (92.5%) selected for IVIG. Two patients without positive pregnancy despite IVIG therapy in the *IVF* setting had, so far, one failed embryo transfer and the third one had two failed IVIG-associated *IVF* cycles.

Thirty-four out of the 40 patients scheduled for IVIG (85%) reached the gestational term without significant complications. Two women (5%) miscarried at 7th week of gestation despite IVIG treatment, and one patient with prior unilateral Fallopian tube removal had an ectopic pregnancy. The live birth rate for the IVIG-treated patients was 82.5% (Table II). Of the 34 pregnant women who reached the gestational term, two patients gave birth to twins and one of them had triplets after 2-embryo transfer (by spontaneous division).

Two patients in the RIF control group (10%) had successful implantation after *IVF* and only one of them reached the gestational term and gave birth to a healthy neonate. Two of the four patients with RM without IVIG therapy (50%) miscarried during the first trimester of gestation. The overall pregnancy rate for the patients without IVIG was 25% and the live birth rate was 12.5%, values significantly lower than the corresponding ones calculated for the patients under IVIG therapy (two-sided $P < 0.0001$, OR of 37 and 34, respectively) (Table II).

The low number of patients with confirmed clinical pregnancy in the subgroup without IVIG prompted us to compare the outcomes in our study group of IVIG-treated patients with the control

Table I Sample characteristics

		Mean Age (mean ± S.D.)	Mean recurrent miscarriages (mean ± S.D.)	Mean number of failed IVF (mean ± S.D.)
$CD56^+$ cell expansion and IVIG	RM (20)	35.7 ± 4.4	3.5 ± 1.6	–
	RIF (20)	37.8 ± 4.1	3.9 ± 1.1	5.6 ± 3.2
	Total (40)	36.5 ± 4.2	3.6 ± 1.4	–
$CD56^+$ cell expansion without IVIG	RM (4)	37 ± 5.1	3	–
	RIF (20)	38.3 ± 4.4	4.5 ± 1.5	4.9 ± 1.6
	Total (24)	38.0 ± 4.4	4.6 ± 1.5	–
Control group[a] Ata et al.[23]	RM (175)	32.2	4.3	–
Control group[b] Clark et al.[8]	RIF (39)	36.7	Not known	2.8

[a]Control group of patients with idiopathic RM treated with placebo.
[b]Control group of patients with immunologic alteration (NK cell expansion or positive autoantibodies) which underwent *IVF* without IVIG associated therapy.
RM, recurrent miscarriage; RIF, recurrent implantation failure; IVF, *in vitro* fertilization.

Table II Overall pregnancy outcome

Overall pregnancy outcome	Clinical pregnancy rate (%)	Miscarriage rate[a] (%)	Live birth rate[b] (%)
RM patients with IVIG (20)	–	5	95
RIF patients with IVIG (20)	85	11.8	75
NK cell expansion[c] (33)	93.9	9.1	90.9
NKT-like cell expansion[c] (8)	87.5	0	100
Total sample (40)	92.5	8.1	82.5
RM Control group (4)	–	50	50
RIF Control group (20)	10	50	5
Total control sample (24)	25	50	12.5
Control group, RM, Ata et al.[23] (175)	76	34.6	49.7
Control group, RIF, Clark et al.[8] (39)	30.8	83.3	5.1

aCalculated for women who got pregnant in each series (37 in our series, 12 in Clark et al. and 133 in Ata et al.).
bCalculated for all patients included in each group.
cWomen with NK and NKT-like cell expansion are included in either RM or IVF subgroups.
RM, recurrent miscarriage; RIF, recurrent implantation failure; IVIG, intravenous immunoglobulin.

groups in the meta-analysis of Ata et al.[23] and Clark et al.[8], who included 133 placebo treated pregnant women previously diagnosed of RM[23], and 39 women with prior failed *IVF* and immunological alterations (NK cell expansion in 75% of them and/or positive autoantibodies),[8] respectively. These authors found live birth rates of 65%[23] and 5.1%,[8] respectively, and these values were lower than the live birth rates in our study group (two-sided $P < 0.0001$, OR for the combined placebo treated groups, 8).

We used Privigen® (10% IgG concentration; CSL Behring AG, Bern, Switzerland) as the first-line preparation because of low sodium content, low infusion volume and time; alternatively, Flebogamma® (5% IgG preparation; Grifols S.L., Barcelona, Spain) was used for the IVIG treatment of our patients. Privigen was commercialized in Spain since 2009, and we used Flebogamma in patients enroled prior to this year or at the clinician criteria, considering the chemical differences between these two IVIG preparations. Tolerance was overall excellent, with low incidence of mild adverse events (headache, asthenia) reported by 15% of patients ($n = 6$; four patients with Flebogamma and two with Privigen). No major differences in terms of efficacy and side effects were found between these two IVIG formulations.

Proportions of CD56+ Cells in Patients and Controls

Thirty-three out of the 37 women who got pregnant in the IVIG-treated group had expanded blood NK ($CD3^-CD56^+/CD16^+$) cell percentages prior to pregnancy (range: 12–26%, mean 18.5 ± 4.4) and eight patients had expanded NKT-like ($CD3^+CD56^+/CD16^+$) cells percentages (range: 10–29%, mean 15.7 ± 4.8). Two patients presented expansion of both phenotypically defined NK and NKT-like cells. The average baseline NK percentage among the patients without IVIG was 15.2% (range 12–19%), and only one patient in this group had NKT-like cells above 10%. No other alteration was found in the numbers and/or percentages of other circulating lymphocyte populations studied in blood samples from all patients, either with or without IVIG therapy.

All women in the healthy pregnant control group had NK cell percentages lower than 12% of total lymphocytes before and throughout the whole gestational period (range: 5–10, mean: 9.7 ± 4.6). Similarly, no woman in the healthy non-pregnant control group showed NK cell percentages above 12% at day 1–3 of the menstrual cycle (range: 5–11, mean: 8.8 ± 4.1). As expected, when compared with the patient groups (with and without IVIG), these values were significantly lower (two-sided $P < 0.0001$, for both patient and control groups) (Fig. 1). Furthermore, all pregnant and non-pregnant women in the control groups showed NKT-like cell proportions below 10%.

Intravenous Immunoglobulin Therapy Effects on CD56+ Cell Proportions

After three cycles of IVIG, NK cell percentages diminished significantly with respect to pre-preg-

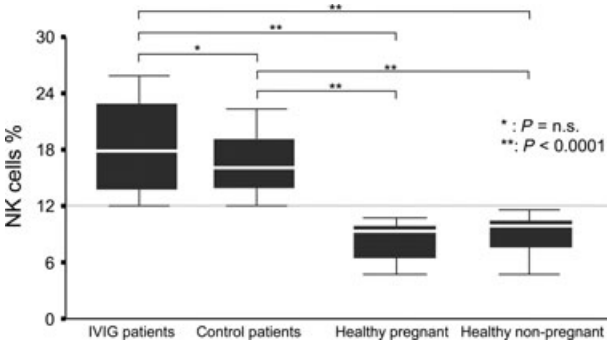

Fig. 1 Baseline (pregestational) Natural killer (NK) cell percentages in patients with RRFs and controls. The circulating NK cell population percentages in four groups are shown: Patients with recurrent gestational failure, NK cell expansion and Intravenous immunoglobulin (IVIG) therapy; Patients with recurrent miscarriage/recurrent implantation failure and NK cells expansion without IVIG; healthy pregnant women; and fertile non-pregnant women at day 1–3 of the menstrual cycle. Each box plot represents the median and the 25 and 75th percentiles. The error bars represent the smallest and largest values in each group.

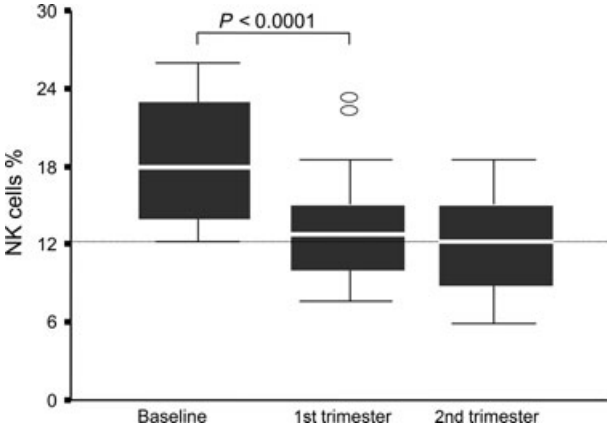

Fig. 2 Overall blood Natural killer (NK)% evolution. Evolution of the circulating NK cell population percentages in our series at three different timelines: Baseline, NK cell percentages before intravenous immunoglobulin therapy; 1st Trimester, NK cell percentages after the first trimester of pregnancy; 2nd Trimester, NK cell percentages at the end of the second trimester of pregnancy. Each box plot represents the median and the 25 and 75th percentiles. The error bars represent the smallest and largest values in each group.

nancy levels ($P < 0.0001$), and this decrease persisted throughout gestation (Fig. 2). Of note, 32 out of 33 (97%) IVIG-treated women with initial NK cell expansion presented a decrease in the NK percentages, and 24 (72.7%) patients reached NK percentages below 12% (Fig. 3). The only woman whose NK percentages were still increased after three IVIG infusions (patient #18) reached percentages of NK cells below 12% after the fifth IVIG cycle and she is now mother of a healthy newborn. All three patients who miscarried had expanded NK cells before pregnancy; no relationship could be established between these cell subsets proportions during the gestational period and the pregnancy evolution.

No significant relationship was found between IVIG treatment and NKT-like cell percentage changes (two-sided $P = 0.6$, data not shown). However, seven out of the eight patients (87.5%) with increased numbers of NKT-like cells had successful implantation after IVIG treatment and their pregnancy course evolved without major incidents.

Pregnancy Outcomes and CD56$^+$ cell Proportions in RM and RIF Subgroups

A stratified analysis considering the RM versus RIF patients revealed similar decreases in the NK cell percentages after IVIG therapy (Fig. 4). The group of RM women showed a higher live birth rate than the RIF patients (95% versus 89%, respectively). Similarly, a higher live birth rate was registered for the RM patients without IVIG when compared with the RIF subgroup (50% versus 5%, two-sided $P = 0.06$) and results in range with the ones obtained for the literature-selected control groups (Table II).

Discussion

We present here a real-life, observational study on a series of women with previous RM or RIF and expanded NK/NKT-like cell proportions, who were treated or not with IVIG throughout gestation. The overall pregnancy and live birth rates in our series suggest that IVIG is beneficial for pregnancy outcome in a selected group of patients, with RRF and increased circulating proportions of CD56$^+$ cells. In contrast to several randomized controlled studies that have failed to demonstrate the efficacy of IVIG on RRF of unknown aetiology,[4,24–26] we have considered IVIG treatment based on an immunological abnormality, that is, expansion of NK/NKT-like cell subsets. The results of our study could be pointing to the necessity to define a group of patients that will benefit from IVIG therapy.

This new series supports the previously identified correlation between IVIG therapy and fall-off of CD56$^+$ cells.[11,27,28] Furthermore, IVIG treatment is associated with high pregnancy and live birth rates,

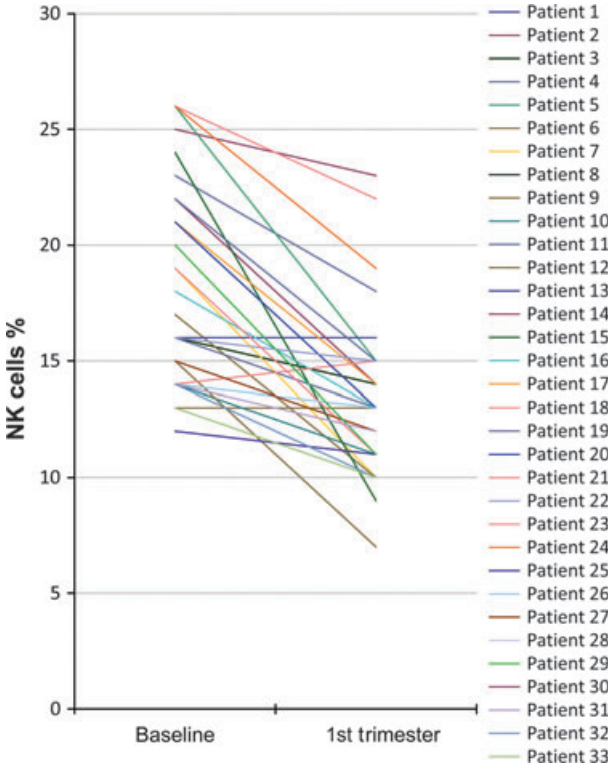

Fig. 3 Circulating Natural killer (NK) cell percentages before and after intravenous immunoglobulin (IVIG). NK cell percentages are shown for each of the 33 patients with NK cell expansion prior to IVIG therapy and positive pregnancy. NK cell percentages are represented at baseline and after the 1st trimester. NK cells cut-off of > 12% at baseline was considered as selection criteria.

supporting the efficacy of this therapeutic option for RM or RIF in women with NK/NKT-like cells expansion. A randomized controlled trial using similar selection criteria would be critical for further strengthening the currently available evidence on IVIG efficiency in patients with RRF and immunological deviations.

A statistically significant decrease in NK percentages after IVIG treatment during pregnancy was seen in more that 90% of the patients in our series, results compatible with other reports.[11,27] This decrease could be a direct effect of IVIG, the effect of pregnancy alone or both. However, Perricone et al.[28] did not see differences in NK cell proportions between RM pregnant and non-pregnant patients without IVIG (and these values were both increased), suggesting that pregnancy does not, by itself, decrease the percentages of NK cells.

Only 72.7% of our IVIG-treated patients reached NK numbers below 12% after the first trimester of

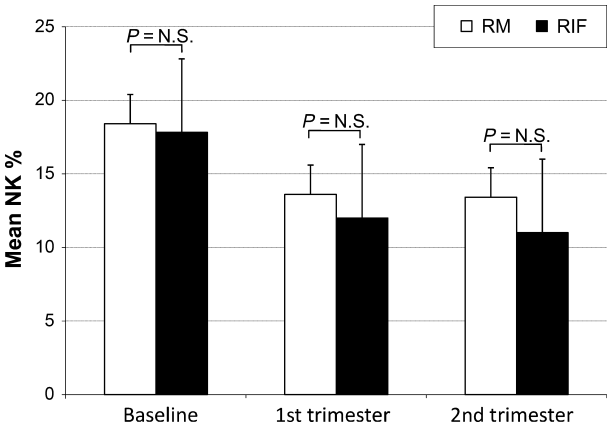

Fig. 4 Evolution of circulating Natural killer (NK) cell percentages in the recurrent miscarriage (RM) and RIF subgroups. Mean NK cell percentages for women with RM and implantation failures after *in vitro* fertilization are shown at different timelines during the gestational period. No significant differences between these two subgroups were found. RM, recurrent miscarriage; RIF, recurrent implantation failure; N.S., not significant.

gestation, while the overall positive pregnancy rate was 92.5%, suggesting that this decrease is not a necessary premise for a successful pregnancy. The pregnancy rates in the group of patients with elevated NKT-like cell proportions were 75%, although no significant decrease in this cell subset was observed. Previous studies have shown that IVIG can induce a decrease in the cytotoxic activity of blood NK cells.[22] *In vivo* and *in vitro* investigations have demonstrated that IVIG induces a functional impairment of $CD56^{dim}CD16^{+}$ NK cell cytotoxic activity and NK cell exhaustion.[21] IVIG relevant effects for pregnancy success might relate to NK/NKT-like cell functional capacity rather than solely limiting to numerical decreases.

Other numerous regulatory and anti-inflammatory effects exerted by IVIG might also contribute to the overall positive outcome.[29] In particular, IVIG has been shown to increase the proportions and activity of T regulatory (Treg) cells,[20] a subpopulation of T cells that diminish in patients with recurrent miscarriages,[30] whereas it decreases Th17 cells numbers,[19] which have been shown to be increased in this pathology.[31] As $CD56^{bright}CD16^{-}$ uNK can induce Th1 and Th17 apoptosis and a Th2-regulatory profile shift,[13] NK cell phenotype changes exerted by IVIG might relate to the changes reported for these T-cell populations. The exact sequence of interactions between IVIG and all these lymphocyte subsets remains to be established in other studies.

Yet, the elevated blood $CD56^+$ cell proportions before pregnancy seem to be a valuable marker that could correlate with elevated cytotoxic activity of these cells. Previous studies described low NK cell percentages in both healthy non-pregnant and pregnant women,[28] considering the whole gestation period, further supporting the use of $CD56^+$ cells expansion as biomarker for pregnancy failure. Our control group for NK cells confirms these results.

Van den Heuvel et al.[12] analysed the influence of IVIG on pregnancy outcome in women with RM and $CD3^+CD56^+$ NKT-like cell expansion and found an important decrease in the percentages of this cell subset and a high live birth rate after IVIG in these patients. We have not observed a decrease in NKT-like cell numbers after IVIG, although the pregnancy rate in our group of IVIG-treated patients with NKT-like cell expansion was similar to that reported by these authors. NKT cells are potent activators of other subsets of lymphocytes and their activation might be one of the initial steps in a complex immune cascade that eventually trigger miscarriage. The shift towards a Th2 profile of these cells determined by IVIG treatment might parallel its down-regulation effect on NK cell cytotoxicity and, similarly to NK cells, the decrease in NKT-like cell numbers might not be essential for IVIG efficacy.

The current concept of RRF comprises both RM and RIF.[5] These two apparently distinct conditions might share common physiopathogenic mechanisms, at least in a selected subgroup of patients. An immunological alteration would potentially affect patients following either natural or artificial fertilization techniques. Scher et al.[32] have reported implantation failure rates after *IVF* up to 98% in women with previous RM, supporting this hypothesis. Most of the patients scheduled for IVIG in our series (92.5%) had past medical history of recurrent miscarriages. Taking these considerations into account, we have included in this study women with both RM and RIF and expanded NK/NKT-like cell subsets. No statistically significant differences in terms of immunological parameters between women from both groups were observed when analysed separately. As expected, women in the RM group showed lower miscarriage and higher live birth rates than the RIF group, in both patients with and without IVIG. Prior occult pregnancy losses that might have been missed in women with natural gestations could explain these minor differences observed.

Williams et al.[33] demonstrated that all decidual lymphocytes are present along the whole pregnancy. In particular, $CD56^+$ cell numbers in the decidua did not vary during the first and second trimesters of gestation, and they were present at considerable levels in the late third trimester samples analysed. In contrast to many similar studies,[24,25,28,34] we have administered IVIG every three to 4 weeks from the date of known pregnancy to 35-week gestation. The high live birth rate observed in our series might be related to the long-term duration of the IVIG treatment.[5] IVIG dose is another variable that makes it difficult to compare different studies. We have used 400 mg/kg of body weight during the first trimester of pregnancy and 200 mg/kg until 35 weeks of gestation. Finally, patient selection could importantly bias the results of many similar studies. In contrast to other studies, we selected the studied subjects from women with RRF without any known underlying disease, based on their baseline NK/NKT-like cell percentages; this selection criterion might account for the different results obtained in the current study compared with previously published results.[4,24–26]. No age-related exclusion criteria were applied for patient selection, and women from 27 to 44 years referred to our Unit were included in this study.

Four IVIG-treated Units and two patients without IVIG had positive autoantibody determinations. Although no autoimmune disease diagnosis had been made for any of these patients, these isolated immunological deviations were reported to be related to recurrent pregnancy loss.[6] We did not consider the autoantibody profile among the selection criteria for the studied cohort, and this might have biased our results. However, the striking differences between IVIG-treated and untreated patients in terms of pregnancy outcome underpin the importance of the presence of a previous immunological alteration for therapy success. An additive effect of IVIG therapy on autoantibody production could have accounted for pregnancy success registered in the IVIG-treated group. Other haematological alterations reported to associate with RRF, such as coagulation Factor XIII, plasminogen activator inhibitor-1 and methylenetetrahydrofolate reductase mutations,[35,36] were not systematically screened in the studied cohort, and thus, they could add additional confounding effects on the study results.

Carp et al.[37] argued that a selection criteria of <4 RM is problematic for studies on the effect of IVIG on pregnancy loss. However, several studies have

confirmed the effectiveness of IVIG in RM and RIF of probable immunological aetiology.[5,8,11] The emotional stress generated by recurrent pregnancy losses balances the costs of the IVIG therapy. Yet, the improvement of *IVF* success would positively impact on financial, medical and emotional expenses associated with it. In this context, ethical reasons determined us to offer this treatment option to all women with three or more pregnancy losses in early stages of gestation and NK/NKT-like cell expansion. Although our results might be biased by the probability of pregnancy success independently of the IVIG treatment, the high clinical pregnancy and live birth rates observed for the IVIG-treated patients suggest the usefulness of this approach for a clinical situation of such high psychological impact.

Overall, our results support the effectiveness and safety of IVIG for the treatment of RRF in patients with $CD56^+$ cells expansion. The exact mechanisms conditioning this positive clinical outcome remain to be determined.

Author's roles

Study concept and design: MM, JC, MCR, DOM, EFC and SSR. Acquisition of data: MM, JC, DA, DOM, JG, AGS, AA, RRM, VO, MRM, JMM, PC, EM, CE, NDP, LH, JD, and SSR. Analysis and interpretation of data: MM, AGS, AA, MRM, JMM, VO, PC, EM. Drafting of the manuscript: MM, MCR, AGS. Critical revision of the manuscript for important intellectual content: JC, DA, SSR. Statistical analysis: MM, SSR. Obtained funding: JG and EFC Fundación Tambre. Administrative, technical, or material support: MM, DA, SSR JV, LOQ. Study supervision: SSR, EFC.

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Disclosure statement

All authors disclose any actual or potential conflict of interest including financial, personal and other relationships with other people or organizations within 3 years of submitting the work that could inappropriately bias our work.

References

1 Branch DW, Gibson M, Silver RM: Recurrent miscarriage. *N Engl J Med* 2010; 363:1740–1747.
2 Rai R, Regan L: Recurrent miscarriage. *Lancet* 2006; 368:601–611.
3 Kwak-Kim J, Park JC, Ahn HK, Kim JW, Gilman-Sachs A: Immunological models of pregnancy loss. *Am J Reprod Immunol* 2010; 63:611–623.
4 Stephenson MD, William H, Kutteh WH, Purkiss S, Librach , Schultz P, Houlihan E, Liao C: Intravenous immunoglobulin and idiopathic secondary recurrent miscarriage: a multicentered randomized placebo-controlled trial. *Hum Reprod* 2010; 25:2203–2209.
5 Stricker RB, Steinleitner A, Winger EE: Intravenous immunoglobulin (IVIG) therapy for immunologic abortion. *Clin Appl Immunol Rev* 2002; 2:187–199.
6 Kwak-Kim J, Yang MK, Gilman-Sachs A: Recurrent pregnancy loss: a disease of inflammation and coagulation. *J Obstet Gynaecol Res* 2009; 35:6109–6622.
7 Perricone C, De Carolis C, Giacomelli R, Zaccari G, Cipriani P, Bizzi E, Perricone R: High levels of NK cells in the peripheral blood of patients affected with anti-phospholipid syndrome and recurrent spontaneous abortion: a potential new hypothesis. *Rheumatology* 2007; 46:1574–1578.
8 Clark DA, Coulam CB, Stricker RB: Is intravenous immunoglobulins (IVIG) efficacious in early pregnancy failure? A critical review and meta-analysis for patients who fail in vitro fertilization and embryo transfer (IVF) *J Assist Reprod Genet* 2006; 23:1–13.
9 Lachapelle MH, Miron P, Hemmings R, Roy DC: Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. Altered profile and pregnancy outcome. *J Immunol* 1996; 56:4027–4034.
10 Coulam CB, Goodman C: Increased pregnancy rates after IVF/ET with intravenous immunoglobulin treatment in women with elevated circulating C56 + cells. *Early Pregnancy* 2000; 4:90–99.
11 Heilmann L, Schorsch M, Hahn T: CD3-CD56+CD16+ Natural Killer cells and improvement of pregnancy outcome in IVF/ICSI failure after additional IVIG-treatment. *Am J Reprod Immunol* 2010; 63: 263–265.
12 van den Heuvel MJ, Peralta CG, Hatta K, Han VK, Clark DA: Decline in number of elevated blood CD3+CD56+ NKT cells in response to intravenous immunoglobulin treatment correlates with successful pregnancy. *Am J Reprod Immunol* 2007; 58:447–459.
13 Kopcow HD, Rosetti F, Leung Y, Allan DSJ, Kutok JL, Strominger JL: T cell apoptosis at the maternal–fetal interface in early human pregnancy, involvement of galectin-1. *Proc Natl Acad Sci* 2008; 25:105.
14 Boyson JE, Aktan I, Barkhuff DA, Chant A: NKT cells at the maternal-fetal interface. *Immunol Invest* 2008; 37:565–582.
15 Godfrey DI, MacDonald HR, Kronenberg N, Smyth MJ, Van KaerL: NKT cells: what's in a name? *Nat Rev Immunol* 2004; 4:231–237.
16 Uemura Y, Suzuki M, Liu TY, Narita Y, Hirata S, Ohyama H, Ishihara O, Matsushita S: Role of human non-invariant NKT lymphocytes in the maintenance of type 2 T helper environment during pregnancy. *Int Immunol* 2008; 20:405–412.

17 Oliver-Miñarro D, Gil J, Aguaron A, Rodríguez-Mahou M, Fernandez-Cruz E, Sánchez-Ramón S: NK cell expansion in obstetrical antiphospholipid syndrome: Guilty by association? *Eur J Obstet Gynecol Reprod Biol* 2009; 145:227.

18 Kwak-Kim J, Chung-Bang HS, Ntrivalas EI, Mangubat CP, Beaman KD, Beer AE, Gilman-Sachs A: Increased T helper 1 cytokine responses by circulating T cells are present in women with recurrent pregnancy losses and in infertile women with multiple implantation failure after IVF. *Human Reprod* 2003; 18:767–773.

19 Maddur MS, Vani J, Hegde P, Lacroix-Desmazes S, Kaveri SV, Bayry J: Inhibition of differentiation, amplification, and function of human T_H17 cells by intravenous immunoglobulin. *J Allergy Clin Immunol* 2011; 127:823–830.

20 De Groot AS, Moise L, McMurry JA, Wambre E, Van Overtvelt L, Moingeon P, Scott DS, Martin W: Activation of natural regulatory T cells by IgG Fc–derived peptide 'Tregitopes'. *Blood* 2008; 112:3303–3311.

21 Jacobi C, Claus M, Wildemann B, Wingert S, Korporal M, Römisch J, Meuer S, Watzl C, Giese T: Exposure of NK cells to intravenous immunoglobulin induces IFNγ release and degranulation but inhibits their cytotoxic activity. *Clin Immunol* 2009; 133:393–401.

22 Ruiz JE, Kwak JYH, Baum L, Gilman-Sachs A, Beaman KD, Kim YB, Beer AE: Effect of intravenous immunoglobulin G on natural killer cell cytotoxicity in vitro in women with recurrent spontaneous abortion. *J Reprod Immunol* 1996; 31:125–141.

23 Ata B, Tan SL, Shehata F, Holzer H, Buckett W: A systematic review of intravenous immunoglobulin for treatment of unexplained recurrent miscarriage. *Fertil Steril* 2011; 95:1080–1085.

24 Jablonowska B, Selbing A, Palfi M, Ernerudh J, Kjellberg S, Lindton B: Prevention of recurrent spontaneous abortion by intravenous immunoglobulin: a double-blind placebo-controlled study. *Hum Reprod* 1999; 14:838–841.

25 Perino A, Vassiliadis A, Vucetich A, Colacurci N, Menato G, Cignitti M, Semprini AE: Short-term therapy for recurrent abortion using intravenous immunoglobulins: results of a double-blind placebo-controlled Italian study. *Hum Reprod* 1997; 12:2388–2392.

26 Stephenson MD, Dreher K, Houlihan E, Wu V: Prevention of unexplained recurrent spontaneous abortion using intravenous immunoglobulin: a prospective, randomized, double-blinded, placebo-controlled trial. *Am J Reprod Immunol* 1998; 39:82–88.

27 Kwak JYH, Kwak FMY, Ainbinder SW, Ruiz AM, Beer AE: Elevated peripheral blood natural killer cells are effectively suppressed by immunoglobulin G infusions in women with recurrent spontaneous abortion. *Am J Reprod Immunol* 1996; 35:363–369.

28 Perricone R, Di Muzio G, Perricone C, Giacomelli R, De Nardo D, Fontana L, De Carolis C: High levels of peripheral blood NK cells in women suffering from recurrent spontaneous abortion are reverted from high-dose intravenous immunoglobulins. *Am J Reprod Immunol* 2006; 55:232–239.

29 Tha-In T, Bayry J, Metselaar HJ, Kaveri SV, Kwekkeboom J: Modulation of the cellular immune system by intravenous immunoglobulin. *Trend Immunol* 2008; 29:608–615.

30 Arruvito L, Sotelo AI, Billordo A, Fainboim L: A physiological role for inducible FOXP3 + TREG cells. Lessons from women with reproductive failure. *Clin Immunol* 2010; 136:432–441.

31 Liu Y-S, Wu L, Tong X-H, Wu L-M, He G-P, Zhou G-X, Luo L-H, Luan H-B: Study on the relationship between Th17 cells and unexplained recurrent spontaneous abortion. *Am J Reprod Immunol* 2011; 65:503–511.

32 Scher J, Salazar zC: Clinical experience with IVIg Rx in patients with prior failed ivf pregnancies: report of 30 consecutive patients. *Am J Reprod Immunol* 2000; 44:121–124.

33 Williams PJ, Searle RF, Robson SC, Innes BA, Bulmer JN: Decidual leucocyte populations in early to late gestation normal human pregnancy. *J Reprod Immunol* 2009; 82:24–31.

34 Winger EE, Reed JL, Ashoush S, Ahuja S, El-Toukhy T, Taranissi M: Treatment with adalimumab (Humira®) and intravenous immunoglobulin improves pregnancy rates in women undergoing IVF. *Am J Reprod Immunol* 2009; 61:113–120.

35 Goodman CS, Coulam CB, Jeyendran RS, Acosta VA, Roussev R: Which thrombophilic gene mutations are risk factors for recurrent pregnancy loss? *Am J Reprod Immunol* 2006; 56:230–236.

36 Coulam CB, Wallis D, Weinstein J, DasGupta DS, Jeyendran RS: Comparison of thrombophilic gene mutations among patients experiencing recurrent miscarriage and deep vein thrombosis. *Am J Reprod Immunol* 2008; 60:426–431.

37 Carp HJA, Toder V, Gazit E, Ahiron R, Torchinski A, Mashiach S, Shoenfeld Y: Further experience with intravenous immunoglobulin in women with recurrent miscarriage and a poor prognosis. *Am J Reprod Immunol* 2001; 46:268–273.