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ORIGINAL ARTICLE

Pre-Pregnancy Levels of Peripheral Natural Killer Cells as Markers for Immunomodulatory Treatment in Patients with Recurrent Miscarriage

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Abstract Immunological risk factors in patients with recurrent miscarriage (RM) are discussed controversially. Abnormalities of natural killer cells (NK) have been described in RM patients. Lipid infusions are known to modulate lymphocyte subsets. The aim of this study was to identify immune parameters that predict success of treatment with lipid infusions in RM patients with elevated NK. In sum, $n=341$ couples with RM were screened for established risk factors and peripheral lymphocyte subpopulations as well as uterine NK cells. We identified $n=136$ patients with ≥ 2 consecutive RM and elevated NK. So far, $n=40$ RM patients with NK disorders were treated with lipid infusions starting at positive pregnancy test, every 2 weeks until 12 + 0 weeks of gestation (GW) or miscarriage. The pre-pregnancy immune diagnostics in idiopathic RM (iRM) patients with ongoing pregnancy were compared to the group with miscarriages and healthy controls ($n=15$). Pre-pregnancy immune diagnostics differed significantly between the groups, with significant higher levels of peripheral NK (% and /µL) in iRM patients who miscarried again compared to controls ($p=0.0035$ and $p=0.0019$). Furthermore, iRM patients show lower percentages of $CD3^+$ lymphocytes than healthy controls ($p=0.0049$). In $n=22/40$ (55%) patients, pregnancy is ongoing > 12 + 0 GW. RM patients with very high pre-pregnancy peripheral NK (pNK) lymphocytes might not benefit from lipid infusions. Pre-pregnancy immunomodulatory treatment in RM patients might be helpful to lower pNK levels and establish an immune environment which is supportive for fetal development.

Keywords Recurrent miscarriage · Natural killer cells · Lipid infusion · Immune diagnostics

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Introduction

Recurrent miscarriage (RM) is a major unsolved complication in reproductive medicine and affects about 1–3% of couples during their reproductive years (Carrington et al. 2005; Shakhar et al. 2003).

Several risk factors have been established including anatomic, endocrine, and genetic abnormalities (Toth et al. 2010). With regard to immune changes, only screening for antiphospholipid antibodies is recommended in the guidelines (Toth et al. 2015). Despite intensive diagnostics, about 50% of the cases are classified as idiopathic RM (iRM). The care and treatment of these couples with unknown cause of RM are particularly challenging.

The involvement of the immune system in the rejection of the semi-allogeneic fetus and the pathogenesis of RM has been discussed for a long time. Recently, several immune parameters have been described that might differ between RM patients and controls (Kuon et al. 2015a, b). Uterine and peripheral natural killer cells (uNK, pNK) are now in the focus of debate as potential new immunologic markers and targets in RM (King et al. 2010; Moffett and Shreeve 2015; Sacks 2015).

Peripheral NK are mainly detected by flow cytometry and described as absolute numbers or percentage of total lymphocytes. Elevated pNK are associated with decreased uterine blood flow in early pregnancy, possibly leading to hypertension, preeclampsia, and fetal growth restriction (Koo et al. 2015; Lyall et al. 2013).

Uterine NK are located in the endometrium and are non-cytotoxic, pro-angiogenic, and represent the majority of lymphocytes at the feto-maternal interface in early pregnancy (King et al. 1998; Lash et al. 2006). The interaction and possible exchange between pNK and uNK are a matter of debate (Moffett and Shreeve 2015; Russell et al. 2013; Sacks 2015).

A recent review described elevated pNK numbers as well as pNK percentages of total lymphocytes in women with RM compared to controls, however no differences in uNK levels (Seshadri and Sunkara 2014). Changes in NK cell parameters have thus been used as targets of immunomodulatory therapy in RM patients. One of the treatment options to modulate NK cell imbalance is an intravenously applied fat emulsion containing soy bean oil, glycerin, and egg phospholipids, known as lipid infusions (Shreeve and Sadek 2012).

The exact mechanism of action of lipid infusions is unknown. However, some studies describe a suppression of NK cell cytotoxicity, NK cell number, as well as pro-inflammatory cytokines of TH1 cells (Granato et al. 2000; Meng et al. 2016). Lipid infusions contain fatty acids and their metabolites like PGD2/E2/I2/J2 are activators of the peroxisome proliferator-activated receptor-γ (PPAR-γ), which is associated with a suppression of cellular functions (Meng et al. 2016).

The goal of this study was to identify immune parameters that predict success of treatment with lipid infusions in patients with elevated NK. This helps to select patients, who benefit from lipid infusions.

Materials and Methods

In total, $n=341$ couples with RM were recruited between March 2013 and March 2016 (Fig. 1). Routine screening of non-pregnant RM patients (RM screening test) included diagnostics of (1) anatomical disorders by vaginal ultrasound and office hysteroscopy; (2) endocrine dysfunctions [polycystic ovary syndrome according to the Rotterdam criteria, hyperprolactinemia, hyperandrogenemia, insufficiency of the corpus-luteum and thyroidal dysfunctions (hypo-/hyperthyreoidism, thyroid autoantibodies)]; (3) autoimmune disorders (antinuclear antibodies >1:160, anticardiolipin antibodies (IgG, IgM), anti-β2-glycoprotein (IgG, IgM), or lupus anticoagulant); (4) deficiencies in coagulation factors (protein C, protein S, factor XII, or antithrombin); (5) inherited haemostatic changes (mutations in the factor V or prothrombin gene); and (6) parental chromosomal disorders (numerical aberrations).

A group of 15 healthy non-pregnant women, aged 20–40 years, with a history of ≥1 uncomplicated pregnancy

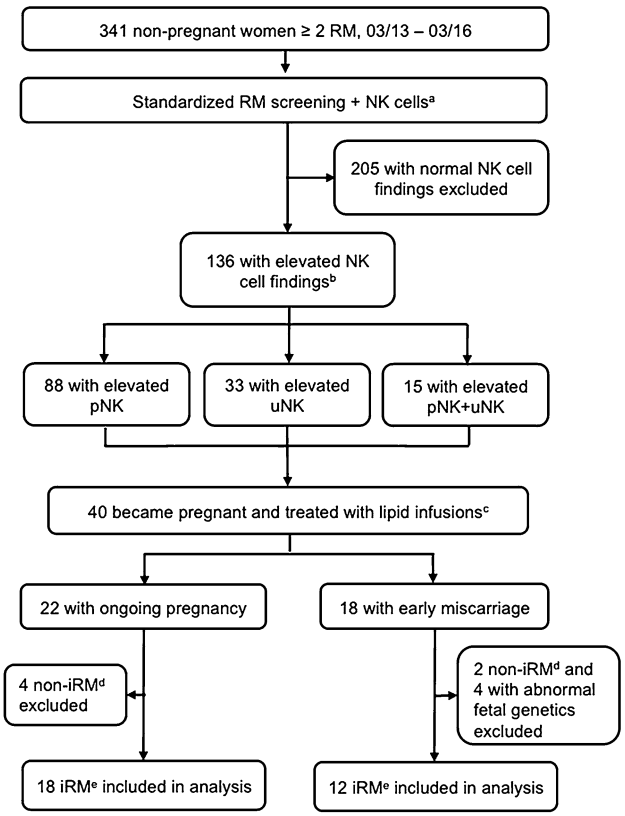

Fig. 1 Flowchart of the trial profile. $N=341$ non-pregnant women with a history of ≥2 RM were recruited. The standardized RM screening included (1) anatomical disorders; (2) endocrine dysfunctions; (3) autoimmune disorders; (4) deficiencies in coagulation factors; (5) inherited haemostatic changes; (6) parental chromosomal disorders. NK cell diagnostics included pNK and uNK testing (for details see "Materials and Methods" section). [a]pNK diagnostics in $n=341$ patients, uNK diagnostics in $n=252$ patients only; [b]elevated NK cell findings: absolute pNK > 280/μL and/or uNK > 300/mm^{2}; [c]treatment with lipid infusions: started with positive pregnancy test, applied every 2 weeks until 12 + 0 GW or miscarriage; [d]non-iRM: RM patients with detection of ≥1 established risk factor in RM screening; [e]iRM: RM patients without detection of an established risk factor in RM screening

and healthy term born babies, who had never received a blood transfusion, without regular medication or hormonal contraception served as controls. In the control group, there was no history of miscarriages or other obstetrical complications. Peripheral lymphocyte subpopulations as well as uNK (in patients only) were analyzed in the mid-luteal phase (details see below). Diagnostics were performed at least 3 months after the last pregnancy. Elevated NK cell findings were defined as absolute pNK >280/μL as it was described as the upper limit of the reference range in the Department of Transplantation-Immunology, University of Heidelberg and/or uNK >300/mm^2. We identified $n=136$ patients with ≥2 consecutive RM and elevated NK cell findings, $n=88$ women with elevated pNK only, $n=33$ with elevation in uNK only, and $n=15$ with elevation in both parameters. Out of these, $n=93$ had primary RM (women who had no live births) and $n=43$ secondary RM (women who had one or more previous live births followed by ≥2 consecutive RM). All patients with elevated NK cell findings were offered to attend our department after positive pregnancy test for therapy with lipid infusions. In the study period, $n=40$ patients became pregnant and were treated. To identify immune parameters that predict success of treatment, results of the pre-pregnancy immune diagnostics (see below) were compared between iRM patients that miscarried again, those with ongoing pregnancy and healthy controls. RM patients with fetal chromosomal disorders were not included in this analysis.

Characteristics of RM patients and controls are shown in Table 1. Signed informed consent was obtained from all participants, allowing analysis of all clinical and laboratory data mentioned in this paper. The Human Investigation Review Board of the Ruprecht-Karls University Heidelberg approved the study.

Table 1 Characteristics of RM patients treated with lipid infusions

	Miscarriage	Ongoing pregnancy	Controls
Study population	18[a] (45%)	22 (55%)	15 (100%)
Age[b]	35.9 ± 3.9	33.9 ± 3.8	33.07 ± 6
Gravidity[c]	4 (2–7)	3.5 (2–7)	1 (1–3)
Parity[c]	0 (0–1)	0 (0–3)	1 (1–3)
No. of miscarriages[c]	4 (2–5)	3 (2–4)	0 (0–0)
No. of iRM (%)	16 (89%)	18 (82%)	0 (0%)
No. of pRM (%)	12 (66%)	12 (55%)	0 (0%)
No of sRM (%)	6 (34%)	10 (45%)	0 (0%)
BMI[b]	24.1 ± 3.8	24.4 ± 4.6	21.9 ± 3.4
P4[b]	8.3 ± 5.3	10.6 ± 9.7	10.7 ± 5.1
AMH[b]	2.3 ± 1.3	2.1 ± 1.9	n.a.
TSH[b]	1.8 ± 0.6	1.7 ± 0.6	n.a.
Elevated NK cell findings[d]			
pNK only	9/18 (50%)	11/22 (50%)	2/15 (13%)
uNK only	7/18 (39%)	10/22 (45%)	n.a.
pNK and uNK	2/18 (11%)	1/22 (5%)	n.a.

Characteristics: study population (*n*), age (years), gravidity, parity, No. of miscarriages (number of miscarriages), No. of iRM (number of idiopathic recurrent miscarriage), No. of pRM, sRM (number of primary recurrent miscarriage, secondary recurrent miscarriage), BMI (body mass index, kg/m^2), P4 (progesterone levels, ng/mL) at time of immune diagnostics (luteal phase of the menstrual cycle), AMH (Anti-Muellerian hormone, ng/mL), and TSH (thyroid stimulating hormone, mU/L)

n.a. not assessed

[a] n=9 with genetic testing, $n=4$ aberrant karyotype

[b] Mean ± SD

[c] Median (min–max)

[d] Elevated NK cell findings: absolute pNK >280/μL, uNK >300/mm^2

Analysis of Peripheral Lymphocyte Subpopulations

Peripheral blood levels of lymphocyte subpopulations including $CD45^+CD3^-CD56^+CD16^+$ NK were determined using four-color fluorescence flow cytometry. In detail, $CD45^+CD3^+CD16^-CD56^-CD19^-$, $CD45^+CD3^-CD16^+CD56^+CD19^-$, $CD45^+CD3^-CD16^-CD56^-CD19^+$, $CD45^+CD3^+CD4^+CD8^-$, $CD45^+CD3^+CD4^-CD8^+$, $CD45^+CD3^+CD4^+DR^+$, $CD45^+CD3^+CD4^-DR^+$ (corresponding to $CD45^+CD3^+CD8^+DR^+$), $CD45^+CD3^+CD25^+$, and $CD4^+CD25^+FoxP3^+CD127^-$ lymphocyte subsets were defined using four-color fluorescence flow cytometry. IgG2a/fluorescein isothiocyanate, IgG2a/phycoerythrin, IgG2a/allophycocyanin, and IgG2a/peridinin-chlorophyll-protein complex antibodies served as isotype controls. All antibodies were purchased from Becton Dickinson (BD)/Pharmingen (Heidelberg, Germany). Ten microliters (μL) of a mixture of four different monoclonal antibodies conjugated with fluorescein isothiocyanate, phycoerythrin, allophycocyanin, or peridinin-chlorophyll-protein complex were added to 50 μL of heparinized whole blood and incubated for 15 min at room temperature. Erythrocytes were lysed with NH_4Cl for 15 min. In case of studying intracellular proteins (FoxP3), cell membranes were permeabilized using BD Perm/Wash buffer (BD Bioscience). Next, FoxP3 monoclonal antibody (BD Pharmingen) was added, cells were incubated for 30 min at room temperature and washed using phosphate buffered saline (PBS). A fluorescence-activated cell sorter Calibur flow cytometer (BD Biosciences, Heidelberg, Germany) was used to analyze lymphocyte subsets. Calibration of the flow cytometer was performed every day using CaliBRITE beads (BD Pharmingen, Heidelberg, Germany) to ensure optimal counting.

Detection of uNK Cells

In $n=252$ patients, a uterine biopsy was taken in the mid-luteal phase to analyze uterine $CD56^+$ NK by immunohistochemistry. Endometrial biopsies were fixed in 5% buffered formalin for at least 24 h and embedded in paraffin. Then, the samples were cut at 4 µm, mounted on SuperFrost/Plus slides (Menzel, Germany), and deparaffinized and rehydrated. Antibodies were diluted in Dako Antibody Diluent with Background Reducing Components (Dako #S3022, Germany). Antigen retrieval was accomplished using citrate buffer. Samples were incubated with Peroxidase Block from Dako EnVision + System-HRP (DAB)-Kit (Dako K4007, Germany) for 7 min to inhibit endogenous peroxidase activity and washed in Tris-buffered saline (TBS)-Tween20 (0.05%; PBS, pH 7.6). Next, samples were incubated with the primary mouse anti-human CD56 antibody (clone:123C3, isotype: IgG1, concentration: 1:100) for 1 h and for 30 min with the secondary antibody [labeled polymer-HRP anti-mouse, clone: DAK-GO1, isotype: IgG1, concentration: 1:100; Dako EnVision + System-HRP (DAB)-Kit, Germany] at room temperature. Between each step, all samples were washed profusely with TBS-Tween20 (0.05%). The peroxidase reaction was achieved with DAB (3,3′-diaminobenzidine; Dako EnVision + System-HRP (DAB)-Kit, Germany) and discontinued with water after 15 min. Hematoxylin staining was followed by mounting the cover slide with Histofluid. Two experienced biologists/physicians analyzed all samples independently using a Zeiss AxioPlan Microscope and the AxioVison 4.8 program. $CD56^+$ uNK were evaluated as absolute numbers per mm^2.

Treatment

Lipid infusion therapy (8 mL 20% Intralipid®, Fresenius Kabi, Graz, Austria in 250 mL Saline) was started with positive pregnancy test [mean 4 + 2 gestational weeks (GW)]. The therapy was applied every 2 weeks until 12 + 0 GW or miscarriage.

Statistics

Statistical analysis was performed using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla, CA, USA, http://www.graphpad.com. For the comparison of pre-pregnancy immune diagnostics in the three groups (iRM patients treated with lipid infusions and ongoing pregnancy, iRM patients treated with lipid infusions and miscarriage, healthy controls), one-way analysis of variance (ANOVA) followed by Holm–Sidak's multiple comparison s test was used. The Holm–Bonferroni test was used to correct for multiple comparisons to protect against identifying too many differences as significant.

Results

Study Population

Characteristics of RM patients are shown in Table 1. $N=33$ (85%) of the couples showed iRM, whereas in $n=6$ (15%) of the cases ≥1, established risk factor was found ($n=1$ with uterus subseptus (hysteroscopic resection was performed before pregnancy) and antiphospholipid syndrome; $n=1$ with TPO antibodies and hypothyreosis (TSH 3.75 mU/L) in the initial screening (treatment with L-thyroxine was initiated, TSH level before pregnancy was <1.5 mU/L); $n=3$ were heterozygous carriers of a factor V Leiden mutation and $n=1$ heterozygous carriers of the G20210A prothrombin mutation).

Treatment with Lipid Infusions and Outcome

In total, $n=40$ RM patients with elevated NK cell findings were treated with lipid infusions in the study period. Due to the presence of further risk factors, some patients received additional medications (Table 2). In 22/40 (55%) of the cases, pregnancy is ongoing >12 GW, and in 18/40 (45%) of the patients, an early miscarriage <12 GW occurred (mean GW at miscarriage: 8.4 ± 1.6). In $n=9$ of the cases, a genetic testing of the embryo was performed, which revealed an aberrant karyotype in $n=4$. None of the patients treated with lipid infusions showed side effects (e.g., allergic reactions).

Table 2 Treatments during pregnancy of RM patients

	Miscarriage	Ongoing pregnancy
Lipid infusions[a]	18/18 (100%)	22/22 (100%)
Progesterone[b]	18/18 (100%)	22/22 (100%)
LMWH[c]	8/18 (44%)	8/22 (36%)
ASS[d]	8/18 (44%)	9/22 (41%)
Corticosteroids[e]	4/18 (22%)	2/22 (9%)

[a]Intralipid (8 mL 20% in 250 mL Saline 0.9%)

[b]Vaginal progesterone (daily dose of 600 mg, until GW 12)

[c]Low molecular weight heparin (Dalteparin-Natrium 5000 I.E. once a day)

[d]Acetylsalicylic acid (100 mg/day)

[e]Daily dose of 5–20 mg, until sonographic detection of fetal heart beat; all treatments started with positive pregnancy test

Comparison of Pre-Pregnancy Immune Diagnostics in Treated iRM Patients

pNK and uNK levels did not differ significantly between iRM patients with miscarriage and iRM patients with ongoing pregnancy and no correlation between pNK and uNK in the pre-pregnancy immune diagnostics in treated iRM patients was observed. However, pre-pregnancy levels of pNK (% and /µL, $p=0.0035$, $p=0.0019$) differed significantly between iRM patients with ongoing pregnancy, iRM patients with miscarriage and healthy controls. Multiple comparison tests showed higher pre-pregnancy percentages and absolute levels /µL of $CD45^+CD3^-CD56^+CD16^+$ pNK percentages in iRM patients with miscarriage than controls (Holm–Sidak's multiple comparison test: patients with miscarriage vs. controls, pNK % $p=0.0027$, /µL $p=0.0013$; Fig. 2; Table 3).

In addition, there was a trend towards lower $CD3^+$ lymphocytes (%, $p=0.0049$) in RM patients compared to controls. Pre-pregnancy immune profile of iRM patients with

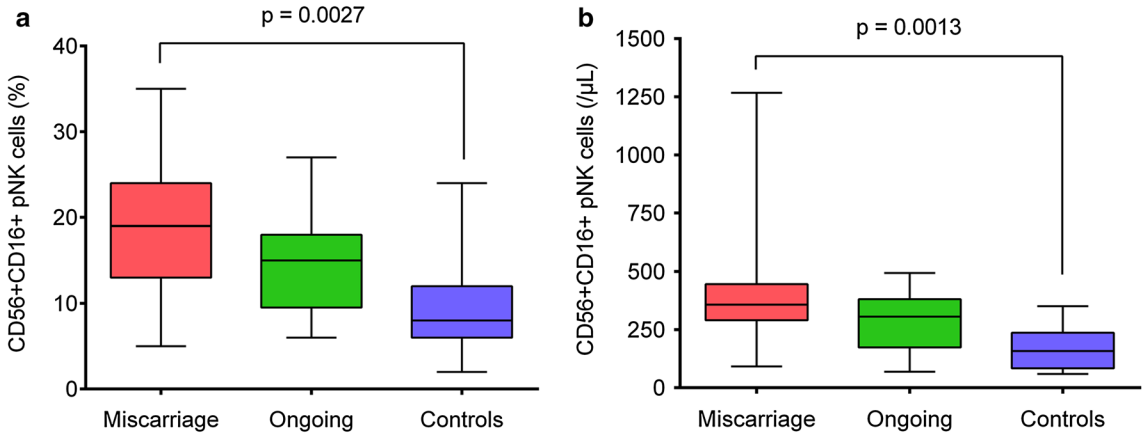

Fig. 2 Pre-pregnancy pNK **a** percentages and **b** /µL in iRM patients with miscarriage, ongoing pregnancy, and healthy controls. Pre-pregnancy $CD45^+CD3^-CD16^+CD56^+$ pNK **a** percentages of total lymphocytes and **b** /µL were significantly higher in patients that miscarried again compared to healthy controls. ANOVA, followed by Holm–Sidak's multiple comparison test. Whiskers show 5 and 95% percentiles. GraphPad Prism version 6.01 was used to create the figure

Table 3 Comparison of pre-pregnancy immune diagnostics in treated iRM patients and controls

	Miscarriage ($n=12$)	Ongoing pregnancy ($n=18$)	Healthy controls ($n=15$)	p
pNK (/µl)[a]	357.4 (290.2/445.6)	305.9 (173/380.6)	158.1 (83.7/236.4)	0.0019*
pNK (%)[a]	19 (13/24)	15.0 (9.5/18.0)	8 (6/12)	0.0035*
uNK (/mm^2)[b]	258 (77.75/380.3)	353 (185/484)	n.a	0.2535
$CD45^+$ (/µl)	2097 (1679/2418)	1912 (1449/2192)	1655 (1395/2258)	0.1878
$CD3^+$ (/µl)	1481(1091/1811)	1344 (1070/1517)	1258 (978.2/1891)	0.3995
$CD3^+$ (%)	70 (62/75)	73.0 (59.5/75.5)	79 (73/83)	0.0049
$CD8^+$ (/µl)	486.9 (398.4/543.2)	463.7 (301.7/612.5)	432.3 (307.8/729.5)	0.8253
$CD8^+$ (%)	23 (19/28)	24 (20.0/30.5)	26 (23/33)	0.1864
$CD4^+$ (/µl)	987.3 (667.2/1231)	800.3 (649.7/897.4)	733 (625.3/1017)	0.2492
$CD4^+$ (%)	43 (34/51)	42.0 (37.5/50.5)	48 (43/53)	0.1866
$CD19^+$ (/µl)	217.6 (201.5/251.6)	192.6 (152.3/250.7)	176.9 (130.2/236.4)	0.4085
$CD19^+$ (%)	12 (9/13)	13 (8.0/14.0)	11 (8/13)	0.3338
Treg (/µl)[c]	23.51 (16.2/31.39)	18.5 (11.37/22.59)	17.52 (8.47/30.13)	0.1554
Treg (%)[c]	2.64 (1.87/3)	2.0 (1.49/3.2)	2 (1.25/3.88)	0.9004

n.a. not assessed

[a]$CD45^+CD3^-CD56^+CD16^+$ pNK

[b]$CD56^+CD16^+$ uNK

[c]$CD4^+CD25^+FoxP3^+CD127^-$ T cells. Parameters shown as median (Q25/Q75). Asterisks indicating significant results. After adjusting the p value for multiple comparisons (Holm–Bonferroni test) a p value <0.0042 was considered significant

ongoing pregnancy showed no significant difference compared to healthy controls.

Discussion

This study aimed to identify immune parameters that predict success of treatment with lipid infusions in patients with elevated NK. There was no significant difference with regard to pNK and uNK levels between RM patients with ongoing pregnancy and patients with miscarriage ($p = 0.0652$, $p = 0.2535$), and therefore, pNK numbers were not a predictor of effectiveness of lipid infusions within our study. However, results of pre-pregnancy immune diagnostics differed between the groups, with significant higher levels of pNK in iRM patients who miscarried again compared to controls. RM patients with very high pre-pregnancy pNK lymphocytes might not benefit from lipid infusions and probably need additional (pre-pregnancy) medication.

In the analysis of pre-pregnancy, immune diagnostics RM patients that miscarried again despite lipid infusions showed signs of an active immune response already before the onset of pregnancy, which is indicated by highly elevated pNK. This group of iRM patients showed a mean pNK level of 474/µL, which was higher than mean + 2 standard deviations (SD) pNK/µL of healthy controls (mean + 2SD pNK of healthy controls: 340/µL).

In contrast, the immune profile of patients with ongoing pregnancy did not differ from healthy controls with at least one healthy pregnancy; therefore, we hypothesize that lipid infusions might help to keep the immune system in stable state and are a successful immunomodulatory treatment when the immune system has resources to adapt to the pregnancy. Regulatory T cells are induced when they are needed and so the lower regulatory T cells in these patients might indicate quiescence of the immune system.

Roussev et al. (2007) showed a suppression of NK cell cytotoxicity by intralipids which was equal efficacy than intravenous immunoglobulin (IVIg) and soluble HLA-G. Furthermore, in patients with abnormal NK activity results ($n = 50$), who received intravenous lipid solution (2–4 mL of 20% intralipid in 250 mL saline), a normalization of NK activity was found 1 week after treatment in 78% of cases and a suppressive effect lasted in 47 patients between 6 and 9 weeks (Roussev et al. 2008). Coulam and Acacio (2012) showed a live birth/ongoing pregnancy rate of 61% of women treated with lipid infusions compared to 56% with IVIg in a group of patients experiencing reproductive failure ($n = 200$, $n = 162$ with a history of recurrent implantation failure and $n = 38$ with recurrent pregnancy loss) and elevated NK cell activity. In a recently published prospective, randomized clinical trial RM patients ($n = 192$, ≥3 RM before GW 12) with an elevation of pNK ($CD56^+CD16^+$) percentage of total lymphocytes > 20% received a treatment with lipid infusion (250 mL 20% intralipids, the first application on the third day of the menstrual cycle, than every 2 weeks until pregnancy occurred and weekly during pregnancy until GW 12) or IVIg (25 g, first given on menstrual cycle day 8, 9 or 10, than once a month and weekly in pregnancy until GW) (Meng et al. 2016). The rate of successful pregnancy (defined as ongoing pregnancy > 12 GW, exclusion of miscarriages with abnormal chromosomes) was 92.1% in the lipid infusion and 88.2 in the IVIg group ($p = 0.415$) (Meng et al. 2016).

Safety concerns need to be considered when introducing new therapies especially in (early) pregnancy. We did not detect any maternal side effect or complications associated with this therapy. Therefore, we conclude that lipid infusions are safe and a well-tolerated immunomodulatory treatment during pregnancy. Still, analysis of potential harmful effects of lipid infusions in a large number of patients and evaluation of neonatal outcome in a long-term perspective is mandatory and part of our ongoing studies. Some patients received additional medications and a genetic testing of the embryo was not performed in all cases, which indicates the heterogeneity of the patients and a limitation of the study. Future studies, which aim to evaluate the efficacy of any immunmodulatory treatment including lipid infusions, need to put intense effort to select a homogenous study population to draw robust conclusions. Only well-designed clinical trials evaluating the effects of lipid infusions in RM patients, ideally prospective, randomized, double-blind and placebo-controlled will provide reliable results.

Future questions that need to be raised concerning lipid infusions include the comparison of different dosages, intervals between the applications, and start and duration of treatment during pregnancy. In addition, pre-pregnancy lipid infusions might be a treatment option for patients with elevated NK. Furthermore, reference ranges of pNK as well as uNK have yet not been established. Still it is not established, whether absolute numbers of pNK or pNK percentages of total lymphocytes need to be interpreted. In the literature, a pNK percentage > 18% was defined as clearly elevated (Beer et al. 1996). In addition, a pNK percentage ≥ 12% was described to be associated with poor reproductive outcome (Michou et al. 2003; Thum 2004). UNK diagnostic is not standardized with regard to methodology immunohistochemistry vs. flow cytometry (Kamoi et al. 2015; Tuckerman et al. 2004; Quenby et al. 1999), sample processing, and interpretation, as well as calculation of uNK e.g., number of uNK per mm^2 of tissue, uNK as a percentage of total stromal cells (Clifford et al. 1999; Quenby et al. 1999; Tuckerman et al. 2004). In consequence, the definition of elevated NK is a matter of debate (King et al.

2010; Michou et al. 2003; Moffett and Shreeve 2015; Sacks 2015).

For the analyses in this study, uNK/mm^2 were calculated, which is an established method in immunohistochemistry (Bologna-Molina et al. 2011). Thus far, it has not been demonstrated if the proportion to space or to other cells is more important or predictive. The proportion to other cells may have more variables, such as different cell types (stromal cells, endothelial cells, other immune cells, etc.), and neglects the potential influence of the extracellular matrix.

A recently published review shows higher pNK (% and /μL), but not uNK in RM patients compared to healthy controls (Seshadri and Sunkara 2014). In accordance with our results, no correlation between pNK and uNK is described (Laird et al. 2011). There are numerous hypotheses regarding the source of uNK particularly during the luteal phase, which include recruitment and trafficking of pNK into the uterus and conversion to uNK phenotype and function (Croy et al. 2006; Kitaya et al. 2005; Park et al. 2010; Seshadri and Sunkara 2014), transformation from in utero resident CD34$^+$ stem cells (Seshadri and Sunkara 2014; Sulke et al. 1985; Vacca et al. 2011) as well as differentiation from immature circulating progenitors (Male et al. 2010; Seshadri and Sunkara 2014; Tang et al. 2011). Peripheral immune markers that reflect intrauterine environment would allow clinicians to monitor the effect of immunomodulatory therapies without repetetive invasive uterine biopsies and even during pregnancy. Studies that monitor pNK and uNK in patients after treatment with lipid infusions will help to detect responsiveness of these immune cells and are part of our ongoing research. The current data do not provide sufficient evidence to conclude which diasgostic marker (pNK or uNK) has higher clinical relevance in patients with RM. More studies are needed to elucidate the interaction between pNK and uNK as well as to further clarify the pathophysiological role of NK in the context of RM.

In conclusion, RM patients with very high (in contrast to high) pre-pregnancy pNK might not benefit from lipid infusions. Pre-pregnancy immunomodulatory treatment in these patients might be helpful to lower pNK levels and establish an immune environment which is supportive for fetal development.

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Compliance with Ethical Standards

Conflict of Interest None.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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