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ORIGINAL ARTICLE

Proportion of Cytotoxic Peripheral Blood Natural Killer Cells and T-Cell Large Granular Lymphocytes in Recurrent Miscarriage and Repeated Implantation Failure: Case–Control Study and Meta-analysis

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Abstract

We aimed to compare the proportion of peripheral blood natural killer (NK) cells ($CD3^-CD56^+$) and T-cell large granular lymphocytes ($CD8^+CD57^+$) during preconception in a homogenous group of women with unexplained well-defined recurrent miscarriage (RM) and repeated implantation failure (RIF) vs healthy controls in relation to pregnancy outcomes. This case–control study followed by a literature review and meta-analysis was conducted in three university hospitals. Patients and controls were consecutively recruited from December 2015 to October 2017. In total, 115 women were included in the study: 54 with RM, 41 with RIF and 20 healthy controls with ≥ 2 term births. Percentages of $CD3^-CD56^+$ and $CD8^+CD57^+$ cells and sub-populations of $CD3^-CD56^+$ cells did not differ between cases and controls. The results for women with subsequent miscarriage did not differ from those with live births. The meta-analysis of the literature showed higher NK-cell proportions in RM [mean difference 3.47 (95% CI 2.94–4.00); $p < 0.001$] and RIF [mean difference 1.64 (95% CI 0.82–2.45); $p < 0.001$] than controls. However, the heterogeneity between the different studies was high. The proportion of peripheral blood $CD3^-CD56^+$ and $CD8^+CD57^+$ cells in the preconception period does not reflect the risk of implantation failure or miscarriage and should not be recommended indicators for the management of RM and RIF. Further prospective large studies are needed to develop a reliable peripheral blood marker of immune deregulation.

Keywords Natural killer cells · NK · T-cell large granular lymphocytes · T-LGL · Recurrent miscarriage · Recurrent implantation failure · RIF · Pregnancy outcome

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Introduction

Human reproduction is a complex process, and a certain proportion of early pregnancies fail to continue (Shepard and Fantel 1979). Most embryonic and foetal losses are observed at the per-implantation period and are sporadic, but some women experience repeated early pregnancy failures. Recurrent miscarriage (RM) is defined as a history of at least three consecutive spontaneous miscarriages before 12 weeks of gestation and occurs in about 1–3% of women (Mekinian et al. 2016). Recurrent implantation failure (RIF) is defined as the absence of pregnancy after at least three transfers of good-quality embryos after in vitro fecundation/intracytoplasmic sperm injection (IVF/ICSI) (Thornhill et al. 2005) and is observed in about 50% of couples undergoing assisted reproductive technology.

More than 50% of RM and RIF cases remain unexplained (i.e., without precise aetiologies after extensive exploration), and recent data showed an altered proportion of endometrial immune cells in the preconception period in up to 82% of cases (Chen et al. 2017; Lédée et al. 2016). Excessive activation of uterine natural killer (uNK) cells and T-helper 1 cell profile consistent with an inflammatory cytotoxic endometrial profile could affect the embryo–endometrium interaction and trophoblastic invasion during early pregnancy, and cause RM and RIF (Chen et al. 2017; Lédée et al. 2016). Nevertheless, endometrial cell analysis is still difficult in routine screening and the preconception data could be less valuable at the time of the pregnancy. Even though endometrial and blood lymphocytes seem different in their phenotypes and functions (Manaster and Mandelboim 2008; Moffett-King 2002), less invasive tools guiding the management of RM and RIF are needed.

Several studies have suggested increased numbers of peripheral blood NK cells in women with RM (King et al. 2010; Ntrivalas et al. 2005; Seshadri and Sunkara 2014; Shakhar et al. 2003), with less consensual data in RIF (Fukui et al. 2008; Ntrivalas et al. 2005; Sacks et al. 2012). However, the design of those studies was heterogeneous, with various definitions of RIF and RM. Several studies included only two, early, or not-consecutive miscarriages, and other aetiologies of RM and RIF were not always excluded. Various definitions of peripheral blood NK cells were considered in these studies, such as $CD56^+$, $CD16^+$ or $CD56^+CD16^+$ cells. In a recent meta-analysis, NK cells were evaluated in women with infertility, with conflictual data considering absolute numbers or percentages (Seshadri and Sunkara 2014). The population of lymphocytes strongly represented in the human endometrium and mostly affected by hormonal fluctuations is represented by uNK cells (Wira et al. 2015), but other immune cells also seem involved in endometrial maturation, embryo implantation and placentation. Among them, T lymphocytes represent about 40–50% of endometrial immune cells, and a decrease in number of $CD8^+$ T lymphocytes as well as altered activation status have been described in women with RM (Southcombe et al. 2017; Wira et al. 2015).

T-cell large granular lymphocytes (T-LGLs) are defined as post-thymic antigen-primed, constitutively activated $CD3^+CD8^+$ T lymphocytes characterised by the presence of cytotoxic granulations in their cytoplasm and the co-expression of CD57 ($CD3^+CD8^+CD57^+$) (Lamy et al. 2017; Zhang et al. 2016). $CD57^+$ expression is connected to the persistent immune stimulation of T cells, may result from multiple cell divisions and may predict replicative senescence of $CD8^+$ T cells (Strioga et al. 2011). Their presence can be detected after hematopoietic stem-cell, bone-marrow or solid-organ transplantation (Gill et al. 2012; Matsuda et al. 2017; Mohty et al. 2002), but their accumulation can be physiologically observed in healthy individuals and is associated with natural ageing (Strioga et al. 2011). Chronic viral infection (principally cytomegalovirus and HIV), acute physical stress, autoimmune diseases, chronic pulmonary disease, cancer, graft-versus-host disease, chronic alcoholism or immunosuppression has been proposed as possible mechanisms of their expansion (Dolstra et al. 1995; Mohty et al. 2001; Strioga et al. 2011). Their function and pathogenesis are not fully elucidated, but they seem to exert a suppressive effect on polyclonal T-cell activation and cytotoxic activity, B-cell differentiation and immunoglobulin production (Wang et al. 1994). Yet, the implication of T-LGLs in the pregnancy implantation process and in women with RM and RIF has not been analysed.

We aimed to compare the proportion of peripheral blood NK cells and T-LGLs in the preconception period in a homogenous group of women with unexplained, well-defined RM and RIF vs healthy controls and in relation to pregnancy outcomes. These data were supplemented with a systematic literature review and meta-analysis of all available studies of blood NK cells.

Patients and Methods

Participants

This case–control study followed by literature review and meta-analysis was conducted with the collaboration of the Department of Internal Medicine of Saint-Antoine Hospital (Paris), the Department of Gynaecology, Obstetrics and Reproductive Medicine of Tenon Hospital (Paris) and the Department of Gynaecology and Obstetrics of Trousseau Hospital (Paris). Patients and controls were consecutively recruited from December 2015 to October 2017.

The study groups consisted of 54 women with at least three consecutive miscarriages (RM) and 41 women with failure of at least three transfers of good quality embryos (RIF). We included 20 fertile women with a history of ≥ 2 term births without any obstetrical or maternal adverse event allowing for a normal term birth from Tenon and Saint Antoine hospitals as controls.

All patients and controls gave informed consent before enrolment and the study protocol was approved by the local ethics committee.

Clinical data collected in the preconception period were age, number of pregnancies, number of consecutive miscarriages, number of IVF/ICSI and embryo transfers, body mass index, tobacco and alcohol use, prior medical and surgical history, and medications during each pregnancy.

All patients were non-pregnant women aged 18–45 years with regular menstrual cycles without any immunomodulatory treatment and without aetiology found on usual

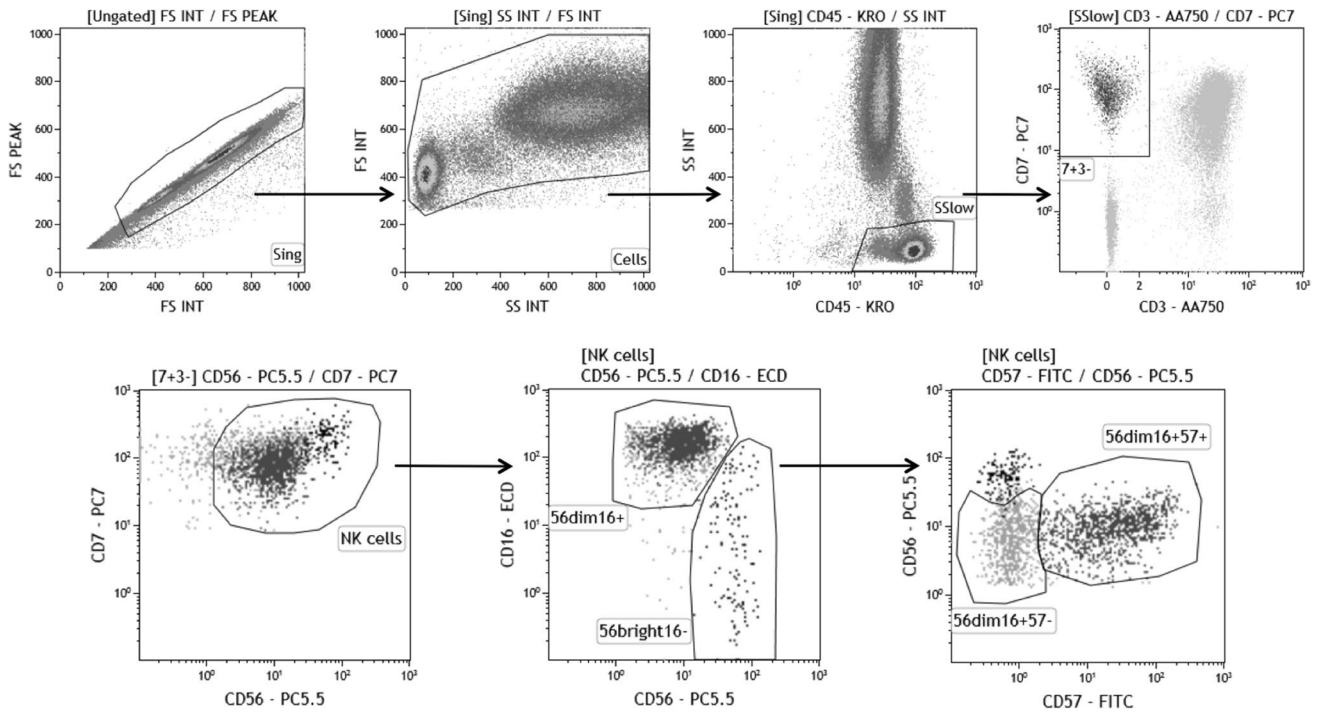

Fig. 1 Representative gating strategy used to select NK cells. The total leukocytes were selected using gating weak Side Scatter (SS) and $CD45^+$ positivity. Among the $CD3^-CD7^+$ cells, $CD56^+$ cells were defined as total NK cells. Two sub-populations of $CD56^+$ NK cells were classified with CD56 and CD16 ($CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$) and CD57 expression

screening for RM and RIF: parental karyotypes, electrophoresis of haemoglobin, fasting blood glucose, complete blood count, anti-Müllerian hormone level, thyroid-stimulating hormone level, homocysteine level, 1-25-OH vitamin D level, comprehensive thrombophilia screening (protein-S, protein-C, anti-thrombin III, activated protein-C resistance, prothrombin gene mutation, factor V Leiden positivity, anti-phospholipid antibodies), autoimmune tests (antinuclear, anti-dsDNA, anti-extractable nuclear antigen, and anti-thyroid [anti-thyroperoxidase and anti-thyroglobulin] antibodies), anti-transglutaminase antibodies and anatomical tests (hysteroscopy and/or pelvis ultrasonography). Women with autoimmune thyroiditis ($n=6$) were included in case of pregnancy failure despite normalised thyroid-stimulating hormone level and substitutive thyroid hormone levels during the previous pregnancy.

Flow Cytometry of Peripheral Blood NK Cells and T-LGLs

Surface and cytoplasmic marker analyses of peripheral blood samples were performed within 4 h of collection using fluorescence-activated cell sorting (FACS) according to the manufacturer's recommendation. For the surface markers, the suspension was incubated with antibodies for CD3-AA750, CD8-AA700, CD16-ECD, CD45-KRO, CD56-PC5.5, CD57-FITC and CD7-PC7 (Beckman Coulter) for 15 min in the dark. Flow cytometry involved use of the Navios cytometer with Kaluza software (both Beckman Coulter). NK cells were defined as $CD3^-CD7^+CD56^+$. CD56 intensity and CD16 expression allowed for distinguishing immature $CD3^-CD56^{bright}CD16^-$ and mature $CD3^-CD56^{dim}CD16^+$ NK cells. CD57 was evaluated in the $CD3^-CD56^{dim}CD16^+$ subset to define the activation state of mature NK cells (Fig. 1). T-LGLs were defined as $CD3^+CD8^+CD57^+$ (Fig. 2).

Literature Review and Meta-analysis

Search Strategy

Two investigators (KK and AM) searched MEDLINE via PubMed, Web of Science, congress programs and the Cochrane Library for English-language articles published up to January 2018 by assessing the following keywords in the title and abstract: early RMs, unexplained miscarriages, miscarriages, RMs, recurrent pregnancy loss, IVF, in vitro fertilisation, fertilisation-in vitro, intracytoplasmic sperm injection, sperm injection intracytoplasmic, assisted reproductive techniques, embryo transfer and embryo implantation, NK cells. All articles with sufficient data were included in the literature review.

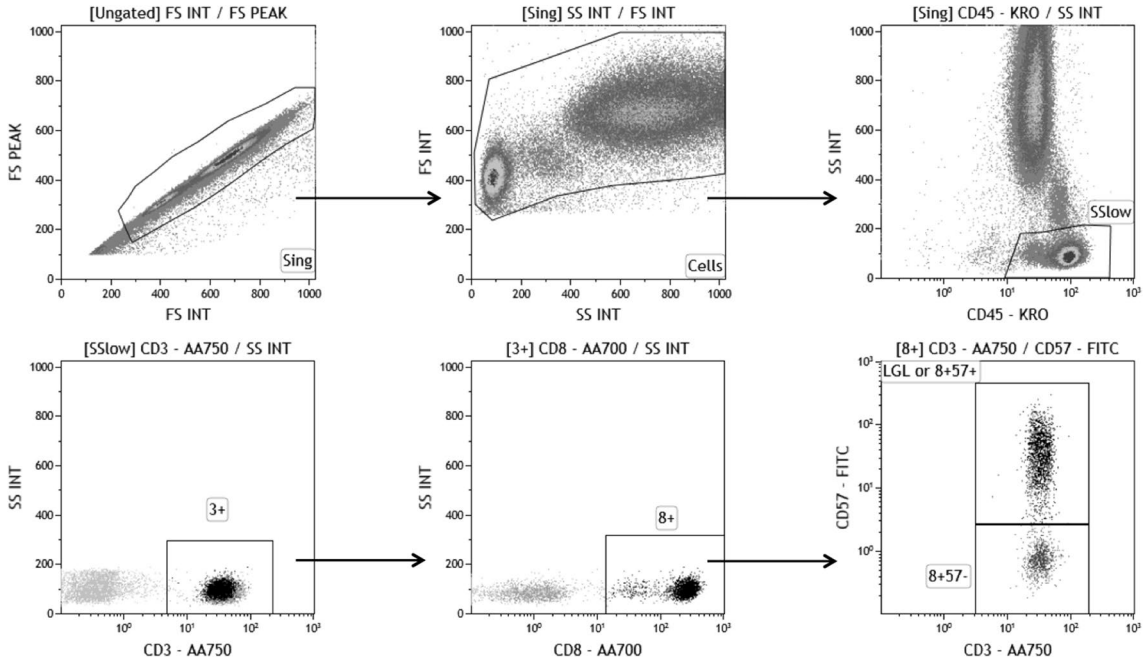

Fig. 2 Representative gating strategy used to characterise T-LGLs. The total leukocytes were selected by gating weak Side Scatter (SS) and $CD45^+$. Among the total lymphocytes, $CD3^+CD8^+$ cells were selected and $CD8^+CD57^+$ lymphocytes were defined as T-LGLs

Study Selection for the Meta-analysis

We included articles describing cases with (1) RM, (2) infertility, (3) age > 18 years, (4) a control group of healthy women, and (5) results of peripheral NK analysis expressed in percentages with mean and SD. Exclusion criteria were lack of sufficient data and evaluating NK-cell proportion during pregnancy. Inclusion and exclusion criteria for the meta-analysis were checked independently for each article by two reviewers (KK and AM). In cases of disagreement, articles were re-examined and discussed until consensus was achieved.

Statistical Analysis

Data are described with frequencies (%) for categorical variables and median (range) for continuous variables. Fisher's exact test was used to compare qualitative variables and the non-parametric Mann–Whitney/Wilcoxon or Kruskal–Wallis test for continuous variables. $P < 0.05$ was considered statistically significant. Statistical analyses involved use of GraphPad Prism v7.0 (GraphPad Software, San Diego, CA, USA).

The meta-analysis was performed according to the recommendations of the Cochrane Collaboration (Bero and Rennie 1995). Relative risks from individual studies were meta-analysed with fixed-effects and random-effects models as appropriate. The prevalence of NK cells in the different groups was analysed by the standardised mean difference. Heterogeneity of the exposure effects was evaluated graphically with forest plots and statistically with the I^2 test to quantify heterogeneity across studies. We explored the causes of heterogeneity by population features, exposure and study quality. Because of the differences in NK nomenclature, sub-analyses were performed according to sub-populations (i.e., $CD56^+/CD16^+/CD56^+CD16^+$, $CD56^+CD16^+$, $CD56^{dim}CD16^+$, or $CD56^{bright}$) in women with RM, infertility and healthy controls. Meta-analysis involved use of Review Manager (RevMan) v5.3 (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, 2014).

Results

Population Characteristics

From December 2015 and October 2017, 95 women were included: 54 with RM and 41 with RIF (Table 1). The median age of women was 36.3 years (range 25.0–45.2). In the control group of 20 women with a history of a median of two term live births (range 2–3) the median age was 36.0 years (32.0–43.0).

In total, 12 women (13%) had anti-Müllerian hormone level < 1.1 ng/ml: 3 had basal antral follicular count ≥ 5 and 5 had undergone IVF treatment for infertility. Eight women (8%) had an autoimmune disease, namely thyroiditis ($n = 4$), psoriasis ($n = 1$), rheumatoid polyarthritis ($n = 1$), coeliac disease ($n = 1$) and Crohn disease ($n = 1$);

Table 1 Characteristics of women with RM and RIF

	RM $n=54$	RIF $n=41$
Clinical characteristics		
Age (years), median (range)	36.3 (25.0–43.2)	36.3 (27.9–45.2)
Obesity (BMI > 30 kg/m^2), *n* (%)	8 (15)	6 (14)
Autoimmune disease, *n* (%)	4 (7)	6 (14)
Thyroiditis, *n* (%)	2 (4)	4 (10)
Endometriosis, *n* (%)	1 (2)	4 (10)
Obstetric history, median (range)		
No. of pregnancies	6.0 (3.0–16.0)	0.0 (0.0–7.0)
No. of live births	1.0 (0.0–3.0)	0.0 (0.0–1.0)
No. of embryos transferred	0.0 (0.0–2.0)	7.0 (3.0–17.0)
No. of spontaneous miscarriages	5.0 (3.0–15.0)	0.0 (0.0–3.0)
Laboratory data		
AMH level (ng/ml), median (range)	2.2 (0.1–7.8)	2.4 (0.001–9.8)
TSH titres (mUI/l), median (range)	1.8 (0.7–6.9)	2.0 (0.4–5.0)
1,25-OH vitamin D level (pg/ml), median (range)	45.2 (9.0–106.8)	36.7 (22.6–98.4)
C protein activity (%), median (range)	106.0 (68.0–205.0)	106.0 (76.0–133.0)
S protein activity (%), median (range)	87.0 (62.0–119.0)	89.0 (56.0–112.0)
Antithrombin III activity (%), median (range)	102.0 (80.0–130.0)	103.0 (74.0–118.0)
ANA level > 160[e], *n* (%)	10 (18)	11 (27)
Gamma globulin level (g/l), median (range)	12.0 (7.0–19.1)	12.2 (6.6–16.3)
CH50 activity (%), median (range)	72.0 (14.0–133.0)	65.0 (18.0–100.0)
Anti-TPO antibody positivity, *n* (%)	2 (4)	7 (17)
Anti-TG antibody positivity, *n* (%)	1 (2)	3 (7)

Results are shown as number (%) or median (range)

BMI body mass index, *AMH* anti-Müllerian hormone, *TSH* thyroid-stimulating hormone, *1,25-OH vitamin D* 1,25-dihydroxy-vitamin D, *ANA* antinuclear antibodies, *CH50* total haemolytic complement, *anti-TPO* anti-thyroid peroxidise, *anti-TG* anti-thyroid globulin, *RM* recurrent miscarriage, *RIF* repeated implantation failure

21 (23%) were positive for antinuclear antibodies [median level 1/160e (range 1/160–1/1280)], in 6% associated with the anti-thyroid antibodies and autoimmune thyroiditis and without any clinical signs, with antinuclear antibody specificity or associated antibodies suggestive of an autoimmune disease in the remaining cases.

The median number of pregnancies and live births was higher for women with RM than RIF [6.0 (3.0–16.0) vs 0.0 (0.0–7.0); $p < 0.0001$ and 1.0 (0.0–3.0) vs 0.0 (0.0–1.0); $p = 0.0001$] (Table 1). History of endometriosis was more frequent for women with RIF than RM [4 (10%) vs 1 (2%); $p = 0.05$], as was the incidence of anti-thyroid peroxidase antibodies [7 (17%) vs 2 (4%); $p = 0.004$].

The time interval after last abortion or implantation failure and the blood analysis was 9 months (1–36) and 5 months (2–26) in the RM and the RIF groups, respectively. A subsequent pregnancy appeared 2 months (0–6) after the blood analysis in 31 women in the RM group. In the RIF group, 15 women have had a subsequent embryo transfer in the 6 months (1–6).

Blood NK Cells

The median proportion of total $CD3^-CD56^+$ NK cells did not differ between women with RM or RIF and healthy controls [9.4% (2.5–31.6), 9.4% (4.3–29.3) and 10.6% (3.4–20.2); $p = 0.43$, respectively], nor did the median proportion of immature $CD3^-CD56^{bright}CD16^-$ NK cells differ [6.7% (0.0–19.6), 8.1% (0.0–23.0) and 5.8% (2.0–18.9); $p = 0.18$]. Similarly, the median proportion of mature $CD3^-CD56^{dim}CD16^+$ NK cells did not differ among groups [93.3% (80.4–100.0), 91.9% (77.0–100.0) and 94.3% (81.1–98.0), $p = 0.18$], nor did the median proportion of $CD3^-CD56^{dim}CD16^+CD57^+$ activated NK cells differ [48.6% (17.7–85.0), 49.6% (18.2–80.9) and 45.2% (28.2–77.2); $p = 0.75$].

For women with RM, 31/54 had a subsequent pregnancy within the 6 months after NK cell analysis: 12 had a RM and 19 a live birth. The median proportion of $CD3^-CD56^+$ NK cells did not differ between the miscarriage and live-birth groups [9.8% (3.2–31.6) vs 9.4% (4.1–15.6); $p = 0.7$]. Similarly, the median proportion of NK

sub-populations—immature $CD3^-CD56^{bright}CD16^-$, mature $CD3^-CD56^{dim}CD16^+$ and $CD3^-CD56^{dim}CD16^+CD57^+$ activated NK cells—did not differ between the two groups. The number of patients under steroids or intralipid infusions for the subsequent pregnancy did not differ between the two groups (data not shown).

For women with RIF, 28/41 had subsequent embryo transfers within 6 months after NK cell analysis; 15 women became pregnant: 4 had a miscarriage and 11 a live birth. The median proportion of $CD3^-CD56^+$ NK cells did not differ between women who conceived and did not conceive [7.8% (5.2–26.6) vs 9.6% (4.4–18.8); $p=0.39$], nor did the median proportion of NK sub-populations—immature $CD3^-CD56^{bright}CD16^-$, mature $CD3^-CD56^{dim}CD16^+$ and $CD3^-CD56^{dim}CD16^+CD57^+$ activated NK cells—differ. The median proportion of $CD3^-CD56^+$ NK cells did not differ between women who had a miscarriage and a live birth [10.9% (5.7–26.6) vs 7.8% (5.2–15.1); $p=0.34$], nor did the median proportion of NK sub-populations—immature $CD3^-CD56^{bright}CD16^-$, mature $CD3^-CD56^{dim}CD16^+$ and $CD3^-CD56^{dim}CD16^+CD57^+$ activated NK cells—differ.

Blood T-LGL Lymphocytes

The median proportion of $CD3^+CD8^+CD57^+$ (T-LGL) lymphocytes did not differ between women with RM or RIF and healthy controls [5.4% (0.4–14.4) and 4.3% (0.0–18.4) vs 3.8% (0.3–9.7); $p=0.22$] (Table 2). However, the median proportion of $CD3^+CD8^+CD57^+$ was < 10% for all healthy controls but ≥ 10% for 11 women with RM (20%) and 4 women with RIF (10%) ($p=0.03$ and $p=0.3$ for RM women and RIF women vs healthy controls).

For women with RM, the median proportion of $CD3^+CD8^+CD57^+$ cells did not differ between those with subsequent RM and a live birth [6.0% (0.7–12.5) vs 5.7% (0.4–14.4); $p=0.8$].

For women with RIF, the median proportion of $CD3^+CD8^+CD57^+$ cells did not differ between those with and without conception [3.3% (0.0–10.7) vs 3.9% (0.4–15.8); $p=0.4$]. Similarly, the median proportion of $CD3^+CD8^+CD57^+$ cells did not differ between women with a miscarriage and a live birth [2.3% (1.2–4.3) vs 3.3% (0.0–10.7); $p=0.6$].

Meta-analysis

The literature review of 22 studies evaluating NK cells in RM and infertile populations is presented in Tables 3 and 4.

The literature search yielded 16 citations for the RM population (Chao et al. 1995; Emmer et al. 2000; Fukui et al. 2008; Ghafourian et al. 2014; Hosseini et al. 2014; Karami et al. 2012; King et al. 2010; Kuon et al. 2017; Michou et al. 2003; Ntrivalas et al. 2005; Prado-Drayer et al. 2008; Shakhar et al. 2003; Souza et al. 2002; Triggianese et al. 2016; Wang et al. 2008; Zhu et al. 2017). Only ten studies had relevant data and were included in the meta-analysis. The definition of RM was heterogeneous, with four studies including patients with ≥ 2 consecutive spontaneous miscarriages and seven including those with ≥ 3 consecutive spontaneous miscarriages. The NK percentage was defined as $CD56^+$ cells in four studies, $CD56^+$ or $CD16^+$ cells in three and $CD56^+CD16^+$ cells in four. The $CD56^{dim}CD16^+$ and $CD56^{brigh}$ sub-populations were evaluated in three and in two studies, respectively.

The meta-analysis of 11 studies (including our results) reporting the proportion of NK cells defined as $CD56^+$ and/or $CD16^+$ in women with RM showed increased proportion of NK cells compared with controls [mean difference 3.47 (95% confidence interval (CI) 2.94–4.00); $p<0.001$] (Fig. 3). The heterogeneity between the studies was high (Fig. 4), with I^2 of 89% ($p<0.0001$). The proportion of $CD56^+CD16^+$ NK cells was significantly higher for women with RM than healthy controls [mean difference 2.72 (95%

Table 2 Proportion of $CD3^-CD56^+$ NK cells and $CD3^+CD8^+CD57^+$ T-LGLs and their sub-populations in women with RM and RIF

NK cell and T-LGL populations	RM or RIF $n=95$	RM $n=54$	RIF $n=41$	Controls $n=20$
Total NK cells: $CD3^-CD56^+/CD45^+$	9.4 (2.5–31.6)	9.4 (2.5–31.6)	9.4 (4.3–29.3)	10.6 (3.4–20.2)
$CD56^{bright}CD16^-/CD56^+$	7.6 (0.0–23.0)	6.7 (0.0–19.6)	8.1 (0.0–23.0)	5.8 (2.0–18.9)
$CD56^{dim}CD16^+/CD56^+$	92.4 (77.0–100.0)	93.3 (80.4–100.0)	91.9 (77.0–100.0)	94.3 (81.1–98.0)
$CD56^{dim}CD16^+CD57^+/CD56^+$	48.6 (17.7–85.0)	48.6 (17.7–85.0)	49.6 (18.2–80.9)	45.2 (28.2–77.2)
$CD56^{dim}CD16^+CD57^-/CD56^+$	42.0 (13.2–73.5)	43.6 (13.2–68.3)	41.6 (15.0–73.5)	48.0 (18.9–65.2)
T-LGL: $CD3^+CD8^+CD57^+/CD45^+$	4.4 (0.0–18.4)	5.4 (0.4–14.4)	4.3 (0.0–18.4)	3.8 (0.3–9.7)
$CD3^+CD8^+CD57^-/CD45^+$	17.0 (0.0–28.5)	15.8 (6.2–25.5)*	17.6 (0.0–28.5)	18.9 (12.8–27.6)*

Results are shown as median (range) percentage among analysed cell populations as indicated

RM recurrent miscarriage, *RIF* repeated implantation failure, *Dim* diminished, *NK* natural killer cells, *T-LGL* T-cell large granular lymphocytes

*$p<0.05$

Table 3 Literature review of studies analysing NK cells in women with RM

References	NK cells	RM			Healthy fertile controls	
		Type	*n*	%	*n*	%
Shakhar et al. (2003)	CD56 or CD16	≥3, unexplained	67	13.17 ± 0.93	13	8.6 ± 1.57
King et al. (2010)	CD56	≥3	104	11.4 ± 4.87	33	8.8 ± 5.37
	$CD56^{dim}$ % of NK			94.7 ± 3.85		92.5 ± 6.41
Chao et al. (1995)	CD56 or CD16	≥3, unexplained	10	19.8 ± 12.6	21	13.5 ± 7.1
Emmer et al. (2000)	CD56	≥2	142	13.2 (10.4–16.0)	37	13.53 (10.8–16.3)
	$CD56^{+}CD16^{+}$			11.6 (9.0–14.2)		12.0 (9.6–14.4)
Souza et al. (2002)	CD56 or CD16	–	9	169 cells (127–178)	9	230 cells (111–273)
Prado-Drayer et al. (2008)	$CD56^{+}CD16^{+}$	≥2	18	13.9 ± 7.1	10	6.0 ± 2.9
	$CD56^{dim}CD16^{+}$ % of lymphocytes			6.7 ± 5.9		0.5 ± 0.7
Wang et al. (2008)	CD56	≥3	85	20.9 ± 6.7	27	20.4 ± 6.3
	$CD56^{+}CD16^{+}$ % of lymphocytes			16.5 ± 6.0		16.6 ± 6.0
Hosseini et al. (2014)	$CD56^{+}CD16^{+}$ % of NK	≥2	15	46.0 ± 19.1	15	38.6 ± 19.2
Triggianese et al. (2016)	$CD56^{+}CD16^{+}$	≥2	145	15.8 ± 6.4	45	11.2 ± 4.7
Zhu et al. (2017)	CD56 or CD16	≥3	31	13.0 ± 6.2	37	11.0 ± 4.8
	$CD56^{bright}$ % of lymphocytes			0.71 ± 0.33		0.68 ± 0.51
	$CD56^{dim}CD16^{+}$ % of lymphocytes			11.0 ± 5.8		8.6 ± 4.4
Kuon et al. (2017)	$CD56^{+}CD16^{+}$	≥3	147	12.0 ± 4.9	42	10.6 ± 4.2
Ntrivalas et al. (2005)	CD56	≥3	15	7.09 ± 1.64	7	6.31 ± 1.7
	$CD56^{dim}CD16^{+}$ % of lymphocytes			5.64 ± 1.9		5.37 ± 1.44
Fukui et al. (2008)	CD56	≥2	25	10.8 (9.33–16.10)	15	12.66 (11.8–15.87)
	$CD56^{bright}$ % of lymphocytes			0.56 (0.43–0.69)		0.52 (0.38–0.61)
	$CD56^{dim}$ % of lymphocytes			10.01 (9.33–16.10)		12.27 (10.86–15.25)
Michou et al. (2003)	CD56 or CD16	2–5	25	217 cells ± 138.8	11	131.0 ± 63.1
Karami et al. (2012)	$CD56^{bright}$ % of lymphocytes	≥2	23	0.61 (0.1–1.5)	36	0.62 (0.12–0.92)
	$CD56^{dim}$ % of lymphocytes			4.0 (8.29–23.81)		5.37 (2.17–7.82)
Ghafourian et al. (2014)	CD56	≥2, unexplained	23	13.1 (9.24–24.24)	36	5.98 (2.42–8.69)

Data are shown as median (range) or mean ± SD percentage

Dim diminished, *NK* natural killer cells, *RM* recurrent miscarriage

CI 1.72–3.71); $p < 0.001$], and the sub-population of NK cells characterised as $CD56^{dim}CD16^{+}$ was higher [mean difference 1.30 (95% CI 0.26–2.35); $p = 0.01$]. The proportion of $CD56^{bright}$ NK cells did not differ between the groups [mean difference 0.03 (95% CI −0.09 to 0.14); $p = 0.66$] (Fig. 3).

The literature search yielded 11 citations for the infertile population (Fornari et al. 2002; Fukui et al. 2008; Ghafourian et al. 2014; Karami et al. 2012; Mardanian et al. 2015; McGrath et al. 2009; Michou et al. 2003; Ntrivalas et al. 2005; Sacks et al. 2012; Santillán et al. 2015; Vujisić et al. 2004). Only five studies had relevant data and were included in the meta-analysis. One study included women with unexplained infertility; in one study RIF was defined as failure of ≥2 in vitro fertilisation treatments and in two studies as the failure of ≥3 embryo transfers; and one study did not specify the definition of implantation failure. The NK-cell populations were defined as $CD56^{+}$ in four studies and $CD56^{+}$ or $CD16^{+}$ in another study. The meta-analysis was not performed for NK sub-populations in this group of patients because of few available data. The proportion of peripheral blood NK cells was higher for women with RIF than controls [mean difference 1.64 (95% CI 0.82–2.45); $p < 0.001$] (Fig. 3). The heterogeneity between the different studies was high (Fig. 4), with I^2 of 95% ($p < 0.0001$).

Discussion

Repetitive early pregnancy failures involve an increasing number of couples, with no reason found in half of the cases. Some of the cases can be explained by chromosomal abnormalities in relation to advanced age of the woman. Nevertheless, the implantation and placentation processes can be compared to the mechanisms of allogeneic graft, which makes the theory of defective tolerance plausible. Immunological abnormalities are found in more than three quarters of endometrial biopsies of patients with RM and RIF but the

Table 4 Literature review of studies analysing NK cells in women with RIF

References	NK cells	RIF			Healthy fertile controls	
		Type	*n*	%	*n*	%
Ntrivalas et al. (2005)	CD56	≥3 ET	15	3.97 ± 0.56	7	6.31 ± 1.7
	$CD56^{dim}CD16^{+}$ % of leukocytes			3.35 ± 0.59		5.37 ± 1.44
Fukui et al. (2008)	CD56	≥2 IVF with ≥4 ET	20	8.77 (7.23–13.06)	15	12.66 (11.8–15.87)
	$CD56^{bright}$ % of leukocytes			0.37 (0.33–0.57)		0.52 (0.38–0.61)
	$CD56^{dim}$ % of leukocytes			8.32 (6.87–13.75)		12.27 (10.86–15.25)
Michou et al. (2003)	CD56 or CD16	≥3 IVF	33	272.2 cells ± 115.7	11	131.0 ± 63.1
Karami et al. (2012)	$CD56^{bright}$ % of leukocytes	≥IVF	20	0.65 (0.35–0.95)	36	0.62 (0.12–0.92)
	$CD56^{dim}$ % of leukocytes			14.08 (8.07–22)		5.37 (2.17–7.82)
Ghafourian et al. (2014)	CD56	≥2 IVF	20	14.01 (8.5–22.87)	36	5.98 (2.42–8.69)
Sacks et al. (2012)	CD56	≥ET	171	11.3 ± 4.9	33	8.73 ± 5.3
	$CD56^{dim}$ % of NK			94.46 ± 3.78		92.57 ± 6.34
Fornari et al. (2002)	CD56 or CD16	Unexplained infertility	18	428 cells ± 42	8	118 cells ± 24
McGrath et al. (2009)	CD56	Unexplained infertility	15	13.5 (8.2–19.6)	8	14.2 (4.9–20.9)
Mardanian et al. (2015)	$CD56^{dim}CD16^{+}$ % of leukocytes	Infertility	34	13.62 ± 4.63	16	4.51 ± 0.98
	$CD56^{bright}CD16^{+}$			0.77 ± 0.22		0.71 ± 0.28
Santillán et al. (2015)	CD56 or CD16	≥3 ET	30	13.4 ± 1.2	35	8.4 ± 0.7
Vujisić et al. (2004)	–	≥3 IVF	18	5.5 (3.2–7.8)	NA	7.7 (2.4–15.1)

Data are shown as median (range) or mean ± SD percentage

Dim diminished, *ET* embryo transfer, *IVF* in vitro fecundation, *NK* natural killer cells, *RIF* recurrent implantation failure, *NA* not accessible

biopsy is not easily available and is time-consuming (Lédée et al. 2016). Less invasive diagnostic tools such as blood immune analysis are needed to guide the management of unexplained infertility; among them, the proportion of NK cells and other cytotoxic cells can be reliable blood markers. Here, we found that NK cell and T-LGL proportions are not sufficient markers to presume an immune origin in women with RM and RIF. In this homogeneous series of patients without any aetiological explanation for pregnancy failure despite extensive biological exploration, the NK-cell proportion did not differ from that in healthy controls.

Our observations support previous results for RM patients (Chao et al. 1995; Ntrivalas et al. 2005; Wang et al. 2008; Zhu et al. 2017) and an infertile population (McGrath et al. 2009). However, several studies reported an increased NK-cell proportion in RM patients (King et al. 2010; Ghafourian et al. 2014; Prado-Drayer et al. 2008; Shakhar et al. 2003; Triggianese et al. 2016) and in infertile populations (Ghafourian et al. 2014; Ntrivalas et al. 2005; Santillán et al. 2015), whereas one study (Sacks et al. 2012) found a decreased proportion of NK cells in an infertile population compared with the control group. The apparent discrepancies in the results can be explained by the non-consensual definition of NK cells in those studies. Moreover, RM and RIF definitions vary according to studies: some studies included patients with two recurrent miscarriages, not consecutive miscarriages and even not unexplained miscarriages. Another explanation could be a monthly variation of immune blood cells within the menstrual regulation. Indeed, endometrial immune-cell concentrations fluctuate during the menstrual cycle under the action of sex steroid hormones, and their proliferation and activation depend on locally secreted factors (Wira et al. 2015). For infertile women, McGrath et al. (2009) did not observe a difference in the peripheral blood NK population when the phase of menstrual cycle was ignored. However, during the secretory phase, the authors observed a higher proportion of circulating NK cells in infertile women. Prado-Drayer et al. (2008) evaluated the proportion of NK cells during the secretory phase (D17–D26 of the cycle) and found an increased proportion in the RM population. The daily fluctuations of peripheral blood NK concentrations have also been reported (Petitto et al. 1992). Moreover, the time interval between the last abortion or implantation failure and blood analysis might have influenced NK peripheral blood results.

Furthermore, the role of peripheral blood and endometrial lymphocytes is distinct. The function of blood lymphocytes is mainly connected to the immune defence response, and their cytotoxic activity is regulated by many extrinsic and intrinsic factors, such as infection, anxiety and stress (Benschop et al. 1998).

The production of peripheral blood and endometrial immune cells is heterogeneous. NK cells in peripheral organs might originate from hematopoietic stem cells present in target organs, whereas circulating blood cells originate mostly from the bone marrow (Daussy et al. 2014).

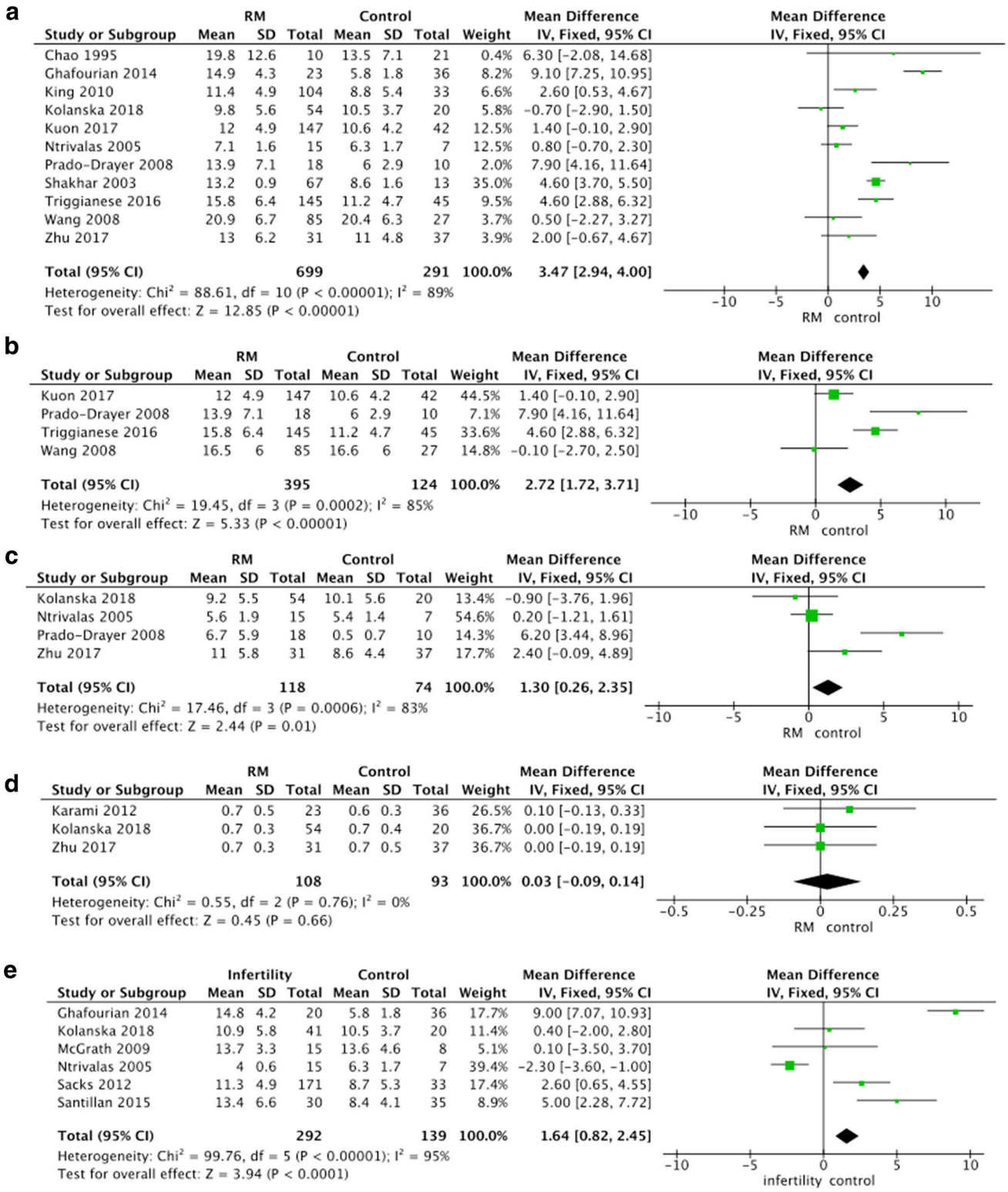

Fig. 3 Meta-analysis of the literature studies of NK cell percentages in women with RM and RIF and healthy fertile controls, including our data. Total NK cells (**a**), $CD56^{+}CD16^{+}$ NK cells (**b**), $CD56^{dim}CD16^{+}$ NK cell sub-population (**c**) and $CD56^{bright}$ NK cell sub-population (**d**) in RM women vs healthy fertile controls and total NK cells in infertile women vs healthy controls (**e**). Data are mean ± SD proportion of total leukocytes

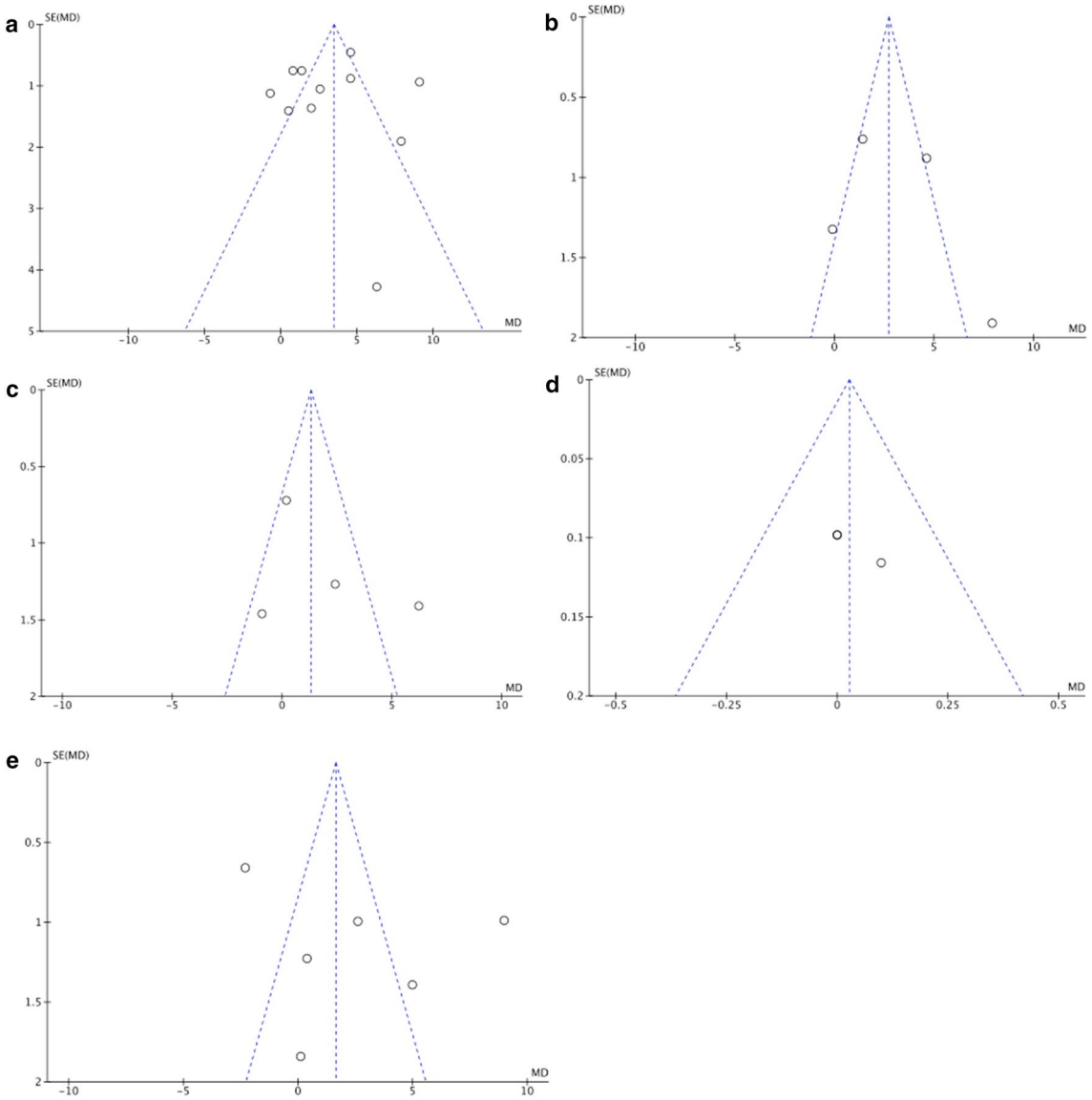

Fig. 4 Forest plots showing the heterogeneity of literature studies of NK-cell proportions in women with RM and RIF and healthy controls, including our data. Total NK cells (**a**), $CD56^{+}CD16^{+}$ NK cells (**b**), $CD56^{dim}CD16^{+}$ NK cell sub-population (**c**) and $CD56^{bright}$ NK cell sub-population (**d**) in women with RM vs healthy controls and total NK cells in infertile women vs healthy controls (**e**). Data are mean ± SD proportion of total leukocytes

Moreover, the phenotypes of peripheral blood and endometrial NK cells differ. More than 90% of the endometrial NK population is characterised by the immature phenotype, $CD56^{bright}CD16^{-}$, whereas the most dominant sub-population in the blood is the mature phenotype, $CD56^{dim}CD16^{+}$ (Mekinian et al. 2016; Moffett-King 2002). Therefore, extrapolating the results of peripheral blood immune cell analysis is difficult for predicting the immunological status of the endometrium.

Our meta-analysis, similar to the previous meta-analysis (Seshadri and Sunkara 2014), showed increased NK-cell proportion in women with RM and with infertility; but in the high level of heterogeneity, various study population selections and NK-cell definitions preclude from definitely concluding on the value of NK-cell analysis in women with RM and RIF. In particular, two studies (Ghafourian et al. 2014; Prado-Drayer et al. 2008) reported remarkably low proportions of NK cells in healthy controls, which strongly influence the results of the meta-analysis.

Women with high NK-cell activity in the preconception period may have low rates of subsequent pregnancy success (Aoki et al. 1995). In the present study, the proportions of NK cells and T-LGLs did not differ between

women with a consecutive miscarriage and live birth or with implantation failure and successful pregnancy.

Another interesting point is the value of peripheral blood NK-cell proportions to monitor the immunomodulatory treatment. Steroid treatment of patients with unexplained RM and RIF presenting high preconception number of endometrial NK cells significantly reduces the number (Quenby et al. 2005). Progesterone promotes the anti-cytotoxic cytokine profile induced by the NK-cell synthesis of progesterone-induced blocking factor (Raghupathy et al. 2009). Intravenous immunoglobulin administration decreases peripheral blood proportion of NK cells (Moraru et al. 2012), with discordant effects on the chances of pregnancy (Christiansen et al. 2015; Moraru et al. 2012; Stephenson et al. 2010). Intralipid administration modifies the peripheral blood NK-cell activity in vitro (Roussev et al. 2007) and in vivo (Roussev et al. 2008). Even if steroids and intralipid treatments were used in our patients, no difference was shown between patients with and without a live birth; but more specific studies are needed.

The major strength of the study is the large number of patients included in the analysis from a homogeneous population of women with RM and RIF. The patients were recruited in three strictly collaborating departments with a pluridisciplinary consultation meeting and an exhaustive exploration of RM and RIF. Recurrent miscarriage and repeated implantation failure populations were considered separately to limit the bias related to factors specific to each condition. The blood samples were analysed without consideration of menstrual cycle phase and the long recruitment period, which could explain some variation in NK-cell proportions. Only relative proportions of NK and T-LGL cells were analysed, which presents a limitation for the meta-analysis.

In conclusion, peripheral blood $CD3^{-}CD56^{+}$ and $CD8^{+}CD57^{+}$ cell levels are not associated with implantation failure or miscarriage and should not be recommended indicators for the management of RM and RIF. Further prospective large studies are needed to develop a reliable peripheral blood marker of the immune deregulation potentially involved in RIF and RM.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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.

Informed consent Informed consent was obtained from all individual participants included in the study.

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