ORIGINAL ARTICLE

Ovulation stimulation protocols utilizing GnRH-antagonist/hCG, promote cytotoxic cell populations, predominant in patients with embryo implantation complications

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Abstract

OBJECTIVE: Gonadotropin-releasing hormone (GnRH) antagonist combined with the human chorionic gonadotropin hormone (hCG) is commonly used in assisted reproduction techniques (ARTs) to induce controlled ovarian hyperstimulation (COH) and to synchronize oocyte maturation. While hCG is known to have immunomodulatory properties, we aimed to assess its effect on immunological changes, with respect to HLA-G binding receptors and embryo implantation success.
DESIGN: The study involved 103 subjects, including patients undergoing COH protocols (n=66), divided on the basis of the pair's fertility disorder (FD) causes (female FD, n=29; male FD, n=37), and age matched healthy women (n=37). The relative distribution of T cell (CD3+/CD4+, CD3+/CD8+) and NK cell (CD56^{bright}/CD16–, CD56^{dim}/CD16+) populations was evaluated together with HLA-G ligands KIR2DL4 and LILRB1 expression by flow cytometry in the peripheral blood of all subjects, as well as in patient follicular fluids.
RESULTS: Both groups of patients exhibited a significant decrease of their CD4/CD8 index, a down-modulation of LILRB1-positive CD8 T cells, and increased KIR2DL4-positive NK cell distribution, when compared to the healthy donors. We attribute these changes to the COH protocol, since the only significant change between the patient groups was in the number of cytotoxic CD56^{dim} NK cells (elevated in the female FD group). Patients with male FD causes, having an above-average CD4/CD8 index (≥3.17) and below-average KIR2DL4+/CD56^{bright} NK cell levels(≤13.3%), exhibited higher embryo implantation rates.

CONCLUSION: The GnRH antagonist/hCG protocol promotes CD3+/CD8+ and KIR2DL4+ NK cell levels, more abundant in subjects with lower implantation rates, and thus decreases the embryotransfer success in otherwise fertile women.

INTRODUCTION

The continuously growing rates of sterility of women of reproductive age in almost all developed countries represents a serious socio-economic problem (Caputo *et al.* 2008). Therefore, the development of new diagnostic and predictive tools/parameters, as well as treatment options for fertility disorders, is of great importance. Assisted reproductive technologies (ARTs) have represented a major step forward in this field (Bower & Hansen 2005).

The prerequisites of prosperous infertility treatment are the availability of multiple fertilizable oocytes of high quality (Arslan *et al.* 2005) and successful subsequent embryo implantation. Controlled ovarian hyperstimulation (COH) is an essential step to trigger oocyte maturation in an appropriate number of follicles to increase the rate of success (Arslan *et al.* 2005). However, lower implantation rates per embryo then those in natural cycles is a major limiting factor of ARTs (~30%, CDC 2010 and ESHRE 2009), (Hoozemans *et al.* 2004).

Various modifications to the hyperstimulation protocols have been established. The combination of GnRH agonist pituitary suppression and exogenous gonadotropins in ART protocols has resulted in significant beneficial effects in the past (Meldrum *et al.* 1989; Muasher 1992), while the subsequent introduction of GnRH antagonists protocols have prevented premature luteinizing hormone (LH) surges (Albano *et al.* 1997). The antagonist protocol, starting on day 6 or 7 of the menstrual cycle (fixed regimen) is now most common due to its simplicity, reduced use of gonadotropin (Arslan *et al.* 2005), and its better implantation rates (Kolibianakis *et al.* 2003).

Human chorionic gonadotropin influences many immune-cell subpopulations (Giuliani *et al.* 1998), with well described effects on dendritic cells and macrophages (Abu Alshamat *et al.* 2012; Wan *et al.* 2008). Since an immune response may play a crucial role in fertility and implantation, there is a growing interest in the examination of local innate and adaptive immunity (Chaouat *et al.* 2007a; Chaouat *et al.* 2007b).

"Immune tolerance" to the fetus requires that uNK cells do not engage towards a rejection/cytotoxic pathway. Non-classical HLA-G (human leukocyte antigen G) molecule expression is associated with placentation and protection of the allogeneic fetus from the maternal immune system (Hunt & Langat 2009; Hunt *et al.* 2005). Trophoblast mHLA-G (membrane bound HLA-G) and sHLA-G (soluble HLA-G) have been shown to provoke the deletion of activated T cells, as well as to inhibit NK cell mediated cytotoxicity (Apps *et al.* 2008; Carosella 2011; Le Bouteiller & Tabiasco 2006). HLA-G is present in human embryonic stem cells, human oocytes, and pre-implantation embryos throughout different developmental stages (Carosella 2011; Verloes *et al.* 2011).

Embryonic, as well as trophoblast HLA-G and incidentally trophoblast HLA-C, cannot exert such a set of tolerogenic functions in the absence of the proper receptors on maternal cells (Hiby *et al.* 2010). Among HLA-G ligands recently attracting the most interest are the killer cell immunoglobulin-like receptor KIR2DL4 (Middleton & Gonzelez 2010) and leukocyte immunoglobulin-like receptors LILRB1 (also known as ILT-2, CD85j) and LILRB2 (also known as ILT-4, CD85d). LILRBs are expressed by T and B lymphocytes, as well as by uterine and peripheral NK cells and mononuclear phagocytes, and generally act by interfering with activating signals (Hunt & Langat 2009). KIR2DL4, mainly restricted to CD56^{bright} NK cells, exhibits the structural characteristics of both activating and inhibitory KIR (Faure & Long 2002; Kikuchi-Maki *et al.* 2003; Rajagopalan *et al.* 2001), possessing both a charged transmembrane arginine residue and a single cytoplasmic ITIM. KIR2DL4 are found on NK cells (Goodridge *et al.* 2003; Lopez-Botet *et al.* 2000) and also under certain circumstances on T cells (Tilburgs *et al.* 2009).

The majority of NK cells present in the pre/peri implantation decidua, pregnant uterus, and follicular fluid have the CD56^{bright}/CD16– phenotype and are endowed with immunoregulatory properties, and the production of angiogenic factors and cytokines (Bulmer *et al.* 1991; Fainaru *et al.* 2010; Loke & King 1995). On the other hand, peripheral NK cells comprise mainly the cytotoxic CD56^{dim}/CD16+ subpopulation (Moffett-King 2002; Saito *et al.* 2008; Trowsdale & Moffett 2008).

To elucidate the effect of the GnRH-antagonist/hCG COH protocol, we analyzed the response of NK and T cell subpopulations and the expression of their KIR2DL4 and LILRB1 receptors in patients undergoing ART, and compared their values with those of healthy donors.

MATERIALS AND METHODS

Cohort of patients and healthy donors

The analysis comprised 103 subjects. Healthy donors (n=37, age=35.5±4.1) and age matched (*p*≥0.05) women undergoing the GnRH-antagonist/hCG COH protocol (n=66, age=33.6±0.9), which were further divided into two groups on the basis of the cause of the pair's fertility disorder (FD) (female FD, n=29; male FD, n=37). Diagnosed male FD patients represented healthy, fertile women; while the diagnosed female FD group represented infertile women.

Healthy donor peripheral blood samples were provided by the Blood Transfusion Service of Thomayer

Hospital in Prague, Czech Republic, with informed consent of all donors. All patient samples used in this study were collected and processed with informed consent of the women undertaking the infertility treatment course at the Institute for the Care of Mother and Child in Prague, Czech Republic.

Peripheral blood samples (2–4 ml) were taken on the day of oocyte collection; and the total volume of follicular fluid (FF) varied according to the number of mature follicles (5–50 ml).

Controlled ovarian hyperstimulation protocol

Only women with the gonadotropin releasing hormone (GnRH) antagonist/hCG protocol (most common process) were enrolled for this study. Briefly, 0.25 mg of antagonist was administered at day 7 of the menstrual cycle (fixed regimen) and given daily until the day of hCG administration (6500 IUs of hCG were given to all women subcutaneously 34–36 hr before peripheral blood and oocyte collection, in order to coincide with oocyte maturation). Precise ultrasound folliculometry was performed rather than Estradiol (E2) levels measurement for COH response prediction.

Isolation of follicular fluid cells (FFC)

Centrifuged (200×g, 4 °C, 10 min) follicular fluids were subjected to a 30 sec incubation with 0.15 M ACK solution (ammonium chloride with potassium: Sigma, St. Louis, MO, USA) to lyse any potential contaminating red blood cells (RBC). Afterwards, the cells were twice washed with 10 ml of ice-cold PBS (200×g, 4 °C, 10 min) and resuspended in 100 μl of PBS for further analysis (see section *Statistical analysis*).

Isolation of peripheral blood mononuclear cells (PBMC)

Blood samples of patients and healthy donors were transferred into heparin-supplemented H-MEMd media (blood:media=1:3, Institute of Molecular Genetics (IMG), ASCR, Prague, Czech Republic), layered by 10 ml onto 3 ml of Ficoll-Telebrix density gradient (1.077; Sigma and Leciva, Prague, Czech Republic) and centrifuged (400×g, 22 °C, 45 min). PBMC were collected, washed twice (300×g, 22 °C, 5 min) in H-MEMd media (IMG, ASCR), counted, resuspended in PBS, and prepared for flow cytometry (see section *Flow cytometry*).

Flow cytometry

PBMC or FFC in PBS were seeded on 96-well microtiter plates by 5×10^5 cells per well, and stained with antibodies according to the standard manufacturer protocol. A multifluorescent (9 channels; Supplementary Figure 1) staining protocol was devised to minimize sample volume requirements.

All analyzed cells were propidium iodide (PI) negative (live cells), singlet cells with standard morphology (Supplementary Figure 2, B and D), and CD45 positive (2D1, Per-CP; leukocyte common antigen) to exclude contamination by other cell types (Supplementary Figure 2, A and C). CD3 (UCHT1, Pacific Blue), CD4 (S3.5, APC-Alexa750), and CD8 (3B5, Pacific Orange) markers were used for the evaluation of T cells (Supplementary Figure 2, E and F); and CD56 (CMSSB, PE-Cy7) and CD16 (LNK16, Alexa700) were used to distinguish cytokine producing (CD56^{bright}/CD16–) and cytotoxic (CD56^{dim}/CD16+) NK cell populations (Supplementary Figure 2, H and I). The HLA-G ligands, KIR2DL4 (181703, PE) and LILRB1 (292305, APC), were assessed on NK and T cells, respectively (Supplementary Figure 2, G and J).

Monoclonal antibodies were purchased from either Becton-Dickinson (Franklin Lakes, NJ, USA), Life Technologies (Carlsbad, CA, USA), eBioscience (San Diego, CA, USA), R&D Systems (Minneapolis, MN, USA), AbD Serotec (Raleigh, NC, USA), or Exbio Praha (Vestec, Czech Republic). PBMCs (5×10^5 cells/well) were stained with the antibody mixture for 30 min on ice, washed, and measured on a BD LSR II instrument (Becton-Dickinson); and the data analysis was performed using FlowJo 7.6.5 software (Tree Star, Ashland, OR, USA).
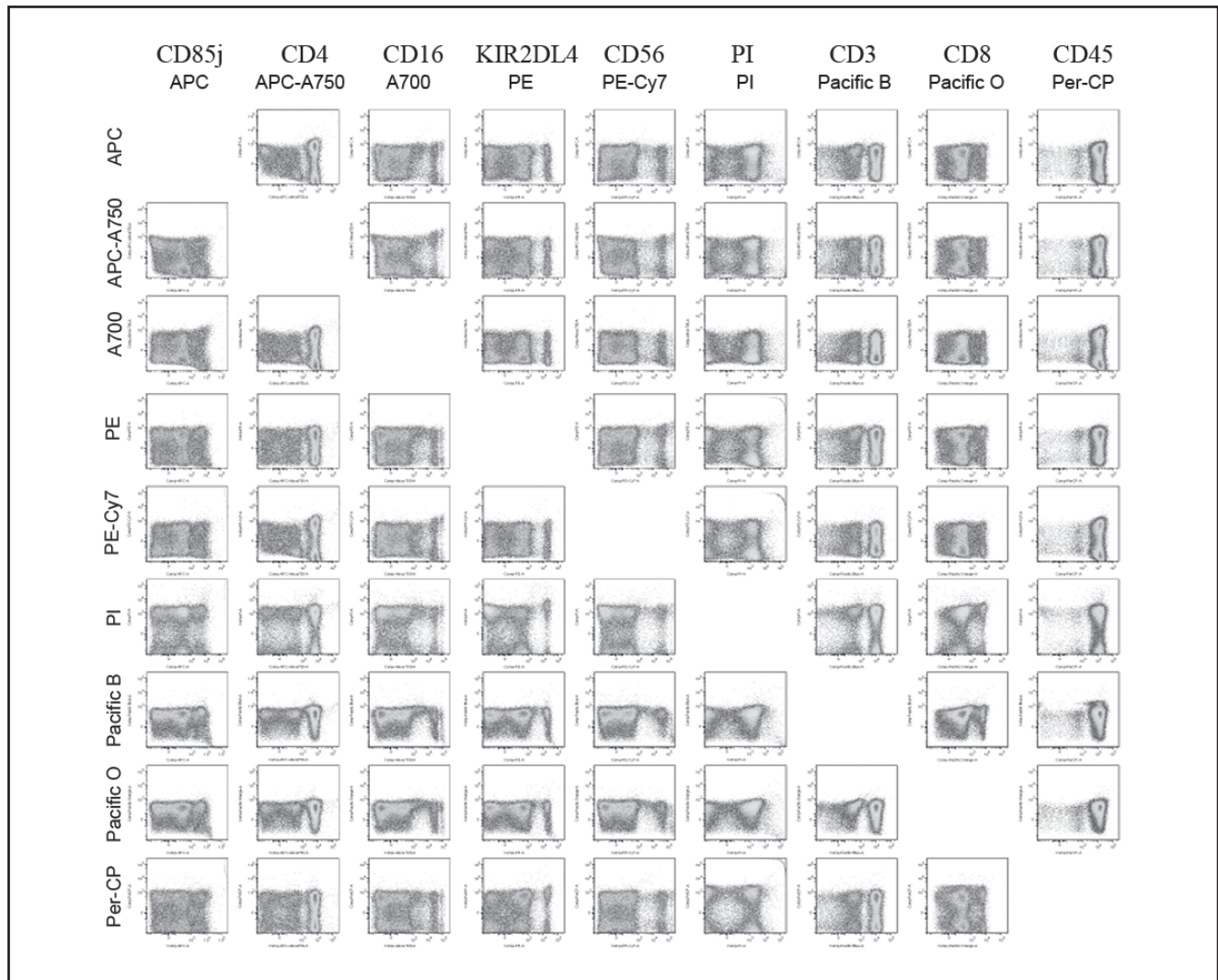
Statistical analysis

The values presented in the figures are plotted as means with 95% confidence intervals. Due to the wide and mostly non-Gaussian distribution of human cytometry values (D'Agostino and Pearson omnibus normality test not passed), we utilized a Mann-Whitney U-test (MWU, nonparametric) instead of the more common Student's T-test to compare the two groups of samples. One-way ANOVA (Kruskal-Wallis test, not assuming Gaussian distribution) with a Dunn's post-test (95% confidence intervals) was used to establish significant differences between three or more groups. Values of $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***) were considered statistically significant as calculated and plotted by Prism 5 statistics software (GraphPad Software, La Jolla, CA, USA).

RESULTS

Patient age influences implantation success rate, but not immune cell distribution

Since age is a known detrimental factor for the outcome of ART, we intended to determine its effect on the studied cell populations. We thus divided the patient samples according to their age into groups of below and above the average value (33.2±3.8 years; Figure 1, A), and compared these groups on the basis of implantation success rate and relative distribution of T and NK cells subpopulations in male and female caused FD. Increasing age correlates with a measurable decrease in implantation success in female FD from 37.5% to 7.7%, relative to male FD (Figure 1, B; from 31.3% to 33.3%), but does not influence the proportion of the studied cell

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Suppl. Fig. 1. Spectral overlap compensation matrix of a 9-channel staining protocol. Columns represent single-stain controls of given fluorochromes, while rows represent their spectral overlap into all remaining channels after applied arithmetic compensation. There is no visible fluorescence overlap in the entire panel.
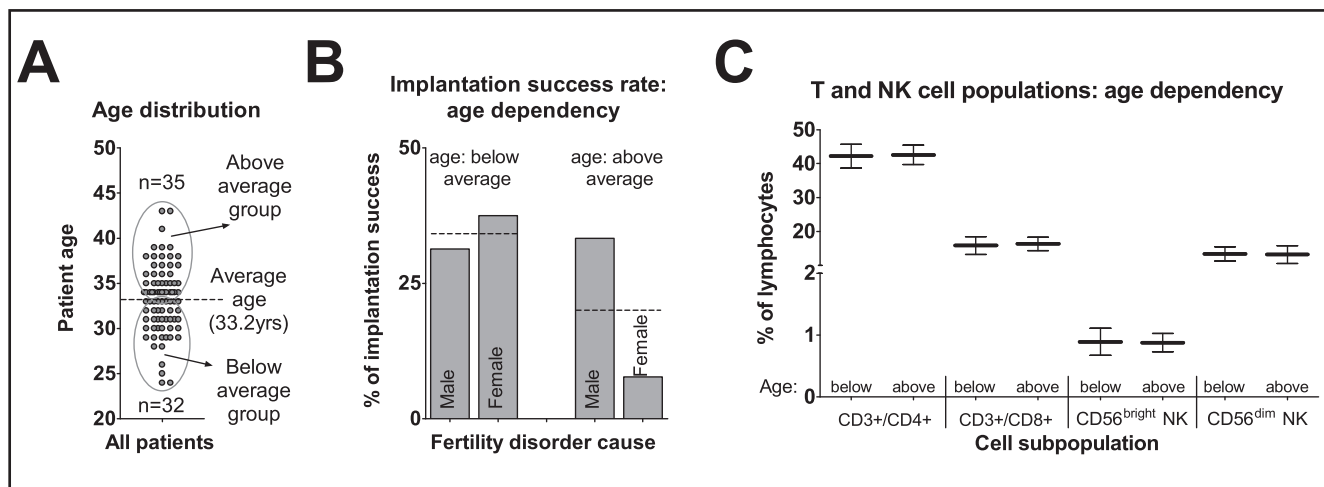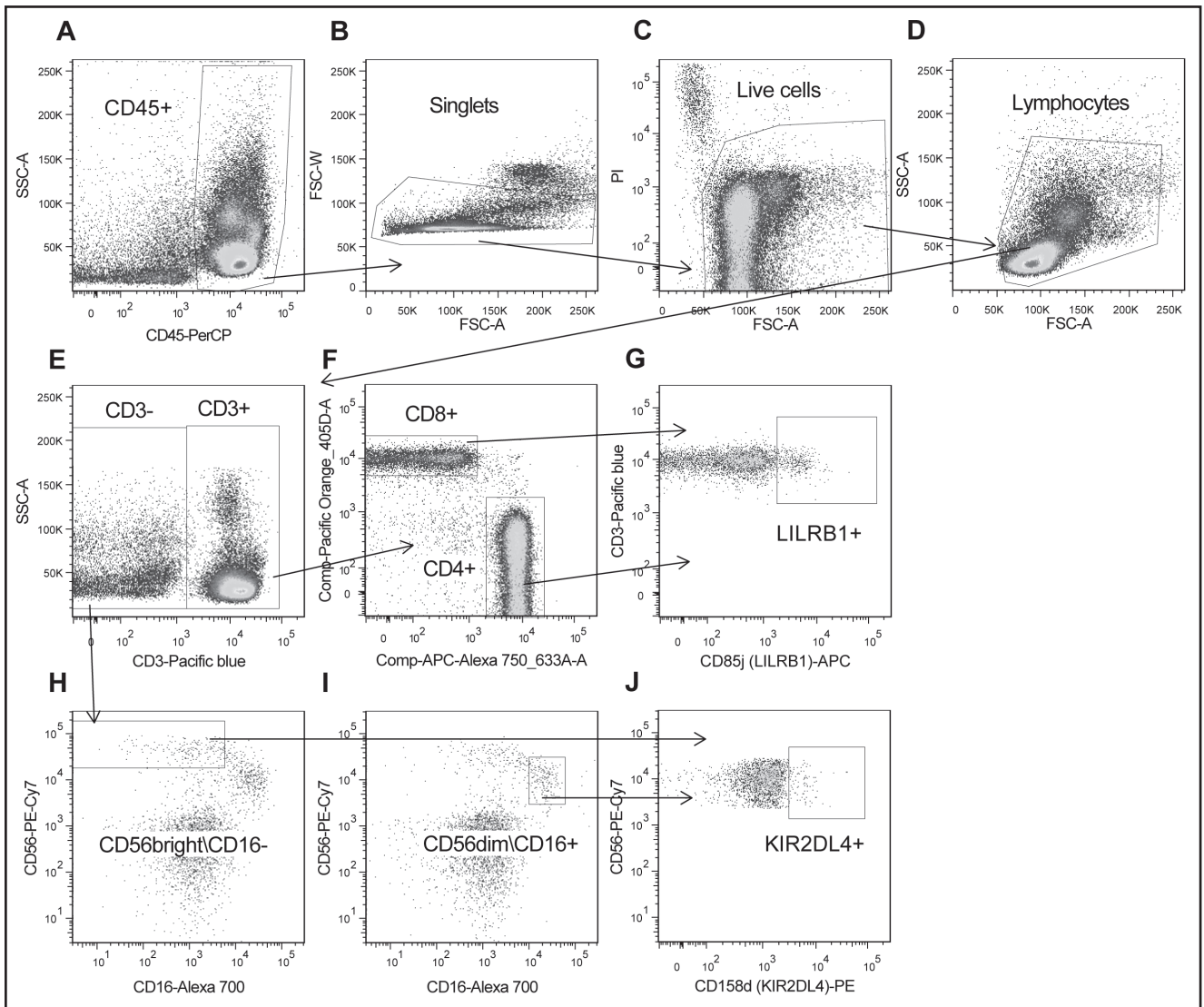

Fig. 1. Age impact on implantation success rate and immune cell populations. Patient samples were sorted into two groups according to their age at the time of ART initiation, into above and below average age (33.2±3.8 years; dashed line) (A). The evaluation of overall implantation success rates is taken from the below and above average aged male and female FD diagnosed groups. Each column represents the percentage of implantation success taken from all samples in a given group, where the dashed line represents the average of all patients (B). T and NK cell subpopulations in peripheral blood are compared on the same basis (C). Values are plotted as means with 95% confidence intervals, where Mann-Whitney p values below 0.05 were considered significant: $p \leq 0.05$ (*); $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

Suppl. Fig. 2. FACS data gating strategy. Gating was performed sequentially: first, CD45+ cells were selected for analysis in order to discard non-lymphocyte cells (**A**). Then doublets (**B**) and dead cells (**C**) were discarded and standard cell morphology was selected (**D**). CD3+ cells (**E**) were further divided into CD4/CD8 subpopulations (**F**) and their LILRB1 expression was analyzed (**G**). CD3- cells were divided according to their CD56/CD16 expression into CD56^{bright}/CD16- (**H**) and CD56^{dim}/CD16+ (**I**) NK cell populations - their KIR2DL4 expression was further analyzed in **J**.

populations in either PBMC (Figure 1, C) or follicular fluids (data not shown). Similarly, the LILRB1- and KIR2DL4-positive cell levels are not dependent on age either in PBMC or FFC (data not shown).

The GnRH antagonist/hCG stimulation protocol severely influences the immune cell population proportion in PMBC

To distinguish the immunological impacts evoked by the GnRH antagonist/hCG ovulation stimulation protocol from those detected in female infertility, we compared the obtained cytometry data with that of patients with male caused FD and of healthy women, in relation to embryo implantation success (Figure 2). Both male and female FD patient groups had significantly

decreased CD4+ and upregulated CD8+ T cells (a lowered CD4/CD8 index), when compared to healthy donors (Figure 2, A; HD CD4/CD8 index=5.7±1.1; all patients CD4/CD8 index=3.2±0.4; $p<0.001$). LILRB1 expressing CD8+ T cell levels are also less abundant in both groups of patient PBMC samples (Figure 2, C). Cytokine producing CD56^{bright} NK cell levels remain unchanged, while cytolytic CD56^{dim} NK cells are upregulated significantly only in the female FD group (Figure 2, B). The relative distribution of KIR2DL4+ NK cell populations is significantly enhanced in both male and female FD patients (Figure 2, D). Thus, we could add the investigation of this phenotype to the COH treatment protocol.

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A low CD4/CD8 index and augmented CD56^dim proportion in patient PMBC is accompanied by a decreased implantation success rate in male caused FD

The T cell ratio was significantly changed by controlled ovarian hyperstimulation (CD4/CD8 index: healthy donor (HD)=5.7±1.1; patient=3.2±0.4; $p<0.001$). To determine whether this change may have any detrimental effect on ART outcome, we divided the patient samples into two groups according to their CD4/CD8 index in PBMC, into the above-average (≥3.2) and below-average (≤3.2) ratios depicted in Figure 3, A. While the female FD group retains its implantation rate at the same level (~24%), male FD diagnosed patients have embryotransfer success repressed at below-average CD4/CD8 index values from 42.9% to 26.1% (Figure 3, B). Concurrently with the decreased CD4/CD8 index, CD56^dim NK cells are augmented in PBMC (Figure 3, C) and lessened in their levels in follicular fluid (from 7.5±1.5 to 5.0±1.7; $p=0.0491$) at above-average CD4/CD8 ratios. In addition, CD56^dim NK cells are favored only in the PBMC of the female FD group, with a high CD4/CD8 index (male FD=2.5±4.3%; female FD=18.7±3.4%; $p=0.042$). This may explain why high CD4/CD8 index bearing female FD subjects do not exhibit increased implantation rates as does the male FD group (Figure 3, B). No significant differences were observed in the LILRB1- and KIR2DL4-positive T and NK cell levels, respectively; in any of the patient follicular fluid samples (data not shown), despite the inherently low CD4/CD8 index, when compared to PBMC (FFC=2.6±0.4; PBMC=3.2±0.4; $p=0.001$).
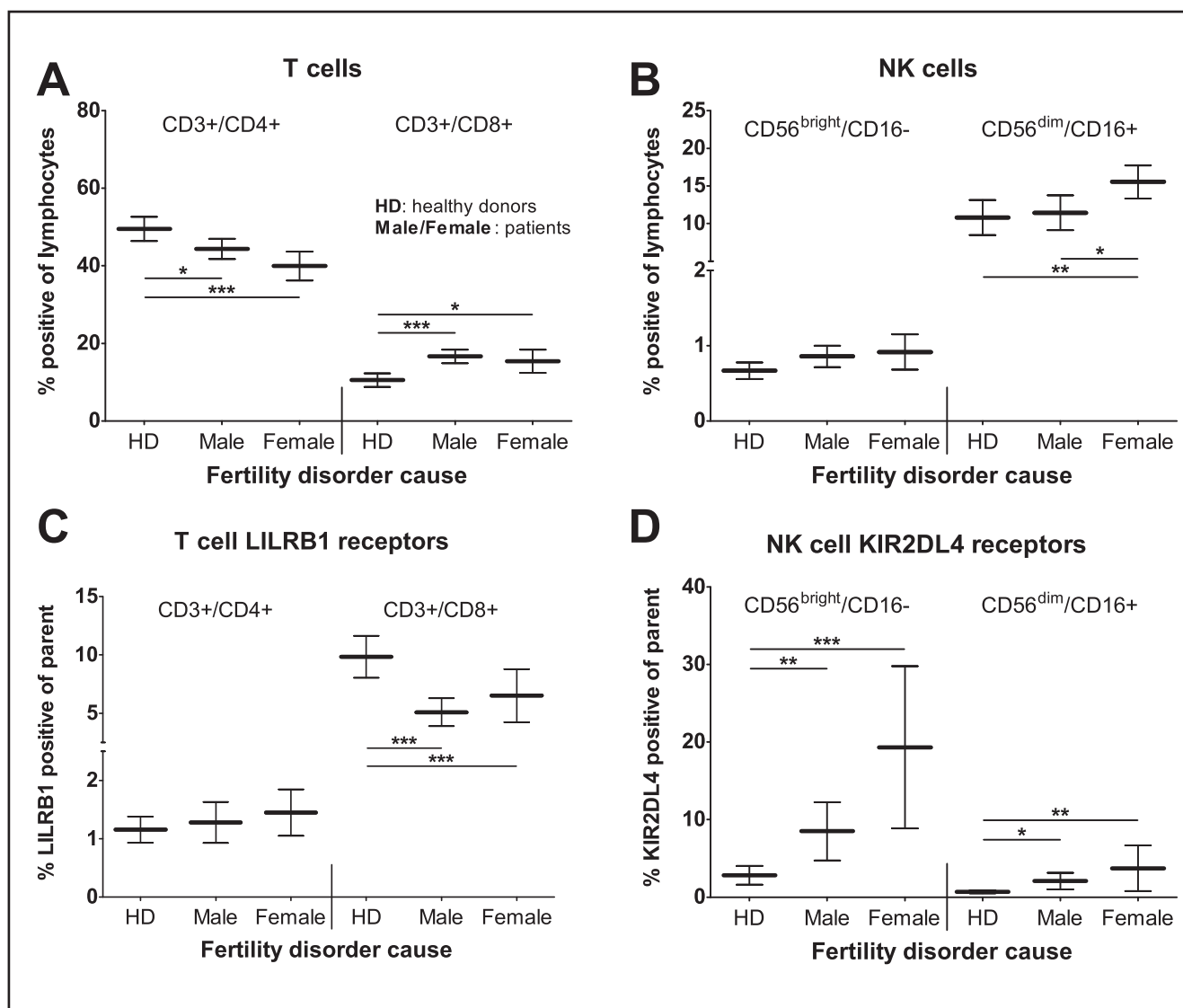

Fig. 2. Relative distribution of T and NK cell subpopulations in healthy donors and ART patients. The CD4+ and CD8+ T cell out of CD3+ lymphocytes (A) and CD56^bright and CD56^dim NK cell percentage of all lymphocyte (B) subpopulations and their respective LILRB1 (C) and KIR2DL4 (D) HLA-G ligands were compared between healthy donors (HD) and the male and female FD diagnosed patients. Values in C and D are a percentage counted from the respective parent population, as described in the figures. The data are plotted as means with 95% confidence intervals, where Mann-Whitney p-values below 0.05 were considered significant: $p≤0.05$ (*); $p≤0.01$ (**) and $p≤0.001$ (***).

High KIR2DL4-positive CD56^{bright} NK cell levels in PBMC are accompanied by higher KIR2DL4+/CD56^{dim} counts and decreased male FD implantation success rates

Since KIR2DL4-positive CD56^{bright} NK cell levels were significantly elevated in the ART patients, we divided their samples also on the basis of the prevalence of this population into above-average (≥13.3% KIR2DL4+) and below-average (≤13.3% KIR2DL4+) groups (Figure 4, A). Higher KIR2DL4+ levels on CD56^{bright} NK cells were accompanied only by higher KIR2DL4 levels on CD56^{dim} NK counterparts (from 1.3±0.4% to 4.9±2.8%; $p≤0.001$), and also by severely

decreased implantation rates in the male FD group (from 37.9% to 12.5%) (Figure 4, B). CD56^{dim} NK cell levels are again favored by the female FD group with below-average KIR2DL4+CD56^{bright} levels (male FD=11.9±2.8%; female FD=16.5±2.3%; $p=0.005$). This may also explain why infertile women with below-average KIR2DL4+CD56^{bright} NK cells do not increase implantation rates (~24%) as in the case of the male FD group with below-average KIR2DL4+CD56^{bright} cells (Figure 4, B). No significant differences between these groups were observed in follicular fluid sample values (data not shown).
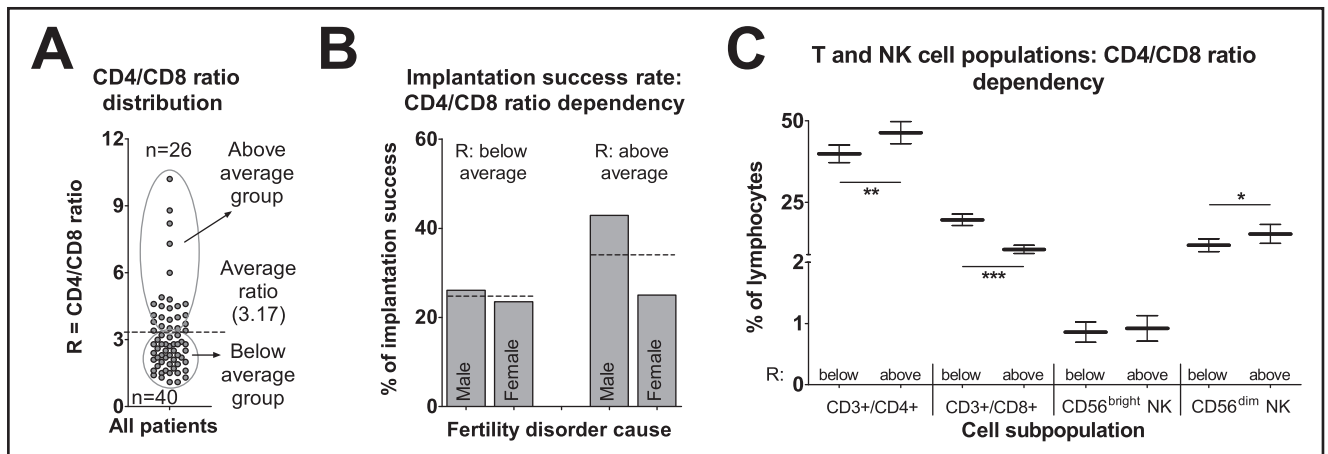

Fig. 3. Distribution of ART patient peripheral blood samples according to their CD4/CD8 index. Patient samples were distributed according to their CD4/CD8 T cell ratio and sorted into two groups of either below or above-average (average CD4/CD8 index=3.2±0.4; dashed line) values (A). The overall implantation success rates were compared on the basis of this division in the male and female FD diagnosed groups. Each column represents the percentage of implantation success, where the dashed line represents the average implantation success rate of all subjects, and the columns show the success rate proportion in male and female caused FD (B). The T and NK cells subset proportions in PBMC were analyzed relative to the CD4/CD8 index. Values are plotted as means with 95% confidence intervals, where Mann-Whitney p-values below 0.05 were considered significant: $p≤0.05$ (*); $p≤0.01$ (**) and $p≤0.001$ (***). The significant changes in CD4 and CD8 T cell levels cannot be taken into consideration, since their levels were used to divide the patient samples.
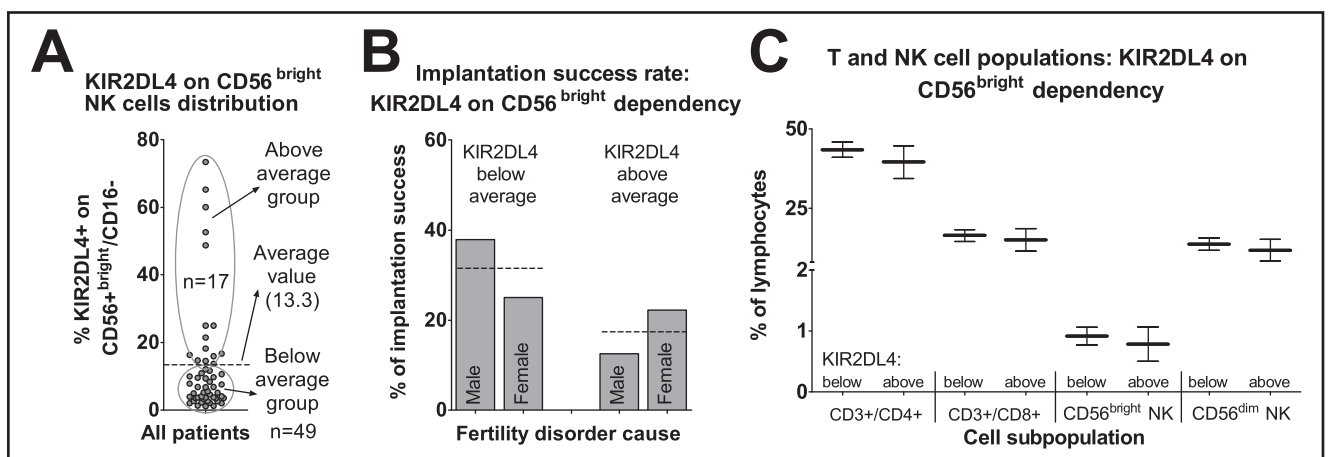

Fig. 4. Distribution of samples according to the proportion of KIR2DL4-positive CD56^{bright} NK cells in ART patient peripheral blood. Patient samples were distributed according to the levels of KIR2DL4-positive CD56^{bright} NK cells in PBMC and divided into two groups with below or above-average values (average = 13.3±5.1) (A). The values represent the percentage of the KIR2DL4-positive subset out of the CD56^{bright}/CD16- NK cells. The overall implantation success rates were compared between below and above-average KIR2DL4+CD56^{bright}/CD16- NK cells of the male and female FD diagnosed patient groups. The columns represent the percentage of implantation success rate taken from male and female FD diagnosed patients, and the dashed line represents the average of all samples (B). The T and NK cell subpopulations levels in the peripheral blood were then compared (C). Values are plotted as means with 95% confidence intervals, where Mann-Whitney p-values below 0.05 were considered significant: $p≤0.05$ (*); $p≤0.01$ (**) and $p≤0.001$ (***).

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DISCUSSION

Human chorionic gonadotropin is known to influence the composition of the immune system in general (Giuliani *et al.* 1998) with well documented effects on macrophages (Abu Alshamat *et al.* 2012; Wan *et al.* 2009) and dendritic cells (Wan *et al.* 2008) in particular. Our results show its effect on T cells, NK cells, and on their HLA-G binding receptors LILRB1 and KIR2DL4.

KIR2DL4 was described to activate cytokine production but only weakly activate cytotoxicity (Kikuchi-Maki *et al.* 2003; Miah *et al.* 2008; Rajagopalan *et al.* 2001). CD56^{bright} NK cells are known to produce TNF-α and IFN-γ (Saito *et al.* 1993); and CD56^{dim} NK cells produce IFN-γ (De Maria *et al.* 2011), which in high doses have detrimental effect on embryo implantation (Chaouat *et al.* 2007a). Thus, the increased levels of KIR2DL4 positive CD56^{bright} and CD56^{dim} NK cells induced by controlled ovarian hyperstimulation may contribute to ART failure by causing very early (immediate) post implantation embryo rejection ("occult loss"). Our study documented lower implantation rates in patients with above-average KIR2DL4-positive CD56^{bright} levels. Moreover, female FD patients with increased CD56^{dim} cell proportions, with below-average KIR2DL4, had comparable implantation rates to those of the above-average KIR2DL4 group.

LILRB1, as an inhibitory receptor among T cells, causes impaired signaling through TCR and decreased IL-2 and IFN-γ production (Brown *et al.* 2004; Moysey *et al.* 2010). Here, the GnRH-antagonist/hCG stimulation protocol decreased the levels of peripheral LILRB1-positive CD8+ T cells despite their increased overall levels, promoting cytotoxic T cells with lower inhibition potency. As shown in this study, patients with a lower CD4/CD8 index in PBMC exhibited lower implantation rates. Female FD diagnosed patients with an above-average CD4/CD8 index, on the other hand, exhibited comparable implantation rates to those of the below-average group; which could be caused by higher CD56^{dim} NK cell levels, when compared to the male FD group. Previously higher levels of peripheral CD56^{dim} NK cells were observed in patients with recurrent spontaneous abortions (Karami *et al.* 2012) or repeated IVF cycles failure (Sacks *et al.* 2012). In agreement with this published data, the COH protocol used in our study increases the levels of CD56^{dim} NK cells only in female FD diagnosed patients. In addition, higher implantation rates, accompanied by a higher CD4/CD8 index or lower KIR2DL4 levels, seen in male FD, were not observed in the female FD patients. Thus, we can deduce that an increased proportion of CD56^{dim} NK cells play the major role in embryotransfer success. Nevertheless, the female FD patients who underwent ART were further handicapped by the COH process itself. In healthy, fertile women (male FD group) cell populations associated with decreased implantation rates were promoted by the COH.

Follicular fluid T and NK cells described previously revealed no significant differences in their relative distribution or in the expression of HLA-G ligands, when successful and failed embryotransfer groups were compared in patients with idiopathic infertility or endometriosis (Lachapelle *et al.* 1996; Lukassen *et al.* 2003). The results of this study proved that the levels of these particular populations and their receptors in the follicular fluids have little or no prediction value for the actual embryotransfer outcome.

CONCLUSION

We show here that the GnRH-antagonist/hCG COH protocol promotes cell subpopulations that are undesirable for the outcome of embryotransfer, and further hinder the success rate of implantation for fertile, as well as infertile women. We thus propose the review and revision of this process, and that it most likely be widened to include other COH protocols to identify and eliminate their potential detrimental effects on embryo implantation. Our study identified a decreased CD4/CD8 index and higher populations of CD56^{dim} NK cells, together with the dysregulation of HLA-G ligands (KIR2DL and LILRB1). Thus, we also deem the above-studied cell populations and their receptors useful as diagnostic and prediction markers in future studies and clinical trials.

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