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IVF-ET妊娠母胎界面免疫微环境变化特点的实验研究*

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【摘要】 目的 探究体外受精-胚胎移植（*in vitro* fertilization-embryo transfer, IVF-ET)妊娠母胎界面蜕膜免疫细胞中自然杀伤（natural killer, NK）细胞与γδT细胞的功能变化特征。**方法** 收取32例接受IVF-ET及12例自然受孕产妇的蜕膜组织，分别纳入IVF-ET组与NP（natural pregnancy, NP）组，取部分组织经石蜡包埋用于HE染色及免疫荧光染色检测；其余组织经消化、Percoll梯度离心分离出蜕膜免疫细胞（decidual immunocytes, DICs）；流式细胞术检测NP组和IVF-ET组中蜕膜NK细胞与γδT细胞的数量及其细胞表面活化标志CD69和NKG2D水平，同时检测功能性细胞因子IFN-γ、TNF-α、IL-17A、IL-10以及毒性颗粒granzyme B、perforin、granulysin的表达水平，比较和分析其相关免疫学指标的变化特点。**结果** 组织切片经HE染色后可见蜕膜组织的典型结构，并显示有较多淋巴细胞富集；免疫荧光染色结果显示：IVF-ET组中蜕膜NK（decidual NK, dNK）细胞占有核细胞总数比例低于NP组（$P<0.05$）；DICs经流式细胞术分析后显示：与NP组相比，IVF-ET组dNK细胞占淋巴细胞总数百分比降低（$P<0.05$）；IVF-ET组dNK细胞的IL-10与perforin表达水平下调（$P<0.05$）；而两组蜕膜γδT（decidual γδT, dγδT）细胞的数量差异无统计学意义，IVF-ET组的IL-10、IL-17A以及perforin表达下调（$P<0.05$）；相关细胞功能指标IFN-γ、TNF-α、granzyme B及granulysin的表达差异无统计学意义（$P>0.05$）。**结论** IVF-ET产妇的dNK细胞数量以及dNK、dγδT细胞的部分功能性细胞因子分泌有所下降，提示其母胎界面免疫微环境发生了一定变化，这些改变对妊娠结局的确切影响还有待进一步研究证实。

【关键词】 自然杀伤细胞 γδT细胞 母胎界面 免疫微环境 体外受精-胚胎移植

Experimental Study on the Characteristic Changes of the Immunological Microenvironment at the Maternal-Fetal Interface in IVF-ET Pregnancy

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【Abstract】 Objective To investigate the characteristic functional changes of the decidual natural killer (NK) cells and γδ T cells, two immunocytes in the decidua, at the maternal-fetal interface in *in vitro* fertilization-embryo transfer (IVF-ET) pregnancy. **Methods** Decidual samples were collected from 12 women of natural pregnancy (NP) and 32 women of IVF-ET pregnancy, who were enrolled in the NP group and the IVF-ET group, respectively. Then part of the decidual samples were paraffin-embedded for HE staining and immunofluorescence staining, while the rest of the samples were digested and Percoll was used for isolating decidual immunocytes (DICs) by gradient centrifugation. Flow cytometry was used to determine the cell counts of decidual NK cells and γδ T cells and the expression levels of their surface activation markers, CD69 and NKG2D in the NP and the IVF-ET groups. In addition, the expression levels of IFN-γ, TNF-α, IL-17A, and IL-10, the intracellular cytokines, and granzyme B, perforin, and granulysin, the cytolytic granules, were measured. The characteristic changes in the relevant immunological indicators were compared and analyzed. **Results** HE staining of the tissue specimens showed that the typical structure of decidua was observed, and that lymphocytes were enriched in the decidua. Immunofluorescence staining showed that the percentage of decidual NK (dNK) cells in nucleated cells of the IVF-ET group was significantly lower than that of the NP group ($P<0.05$). Flow cytometry analysis of DICs showed that, compared with those of the NP group, the percentage of dNK cells of the IVF-ET group was decreased ($P<0.05$) and the expression levels of IL-10 and perforin were significantly decreased in the IVF-ET group ($P<0.05$). However, there was no significant difference in the decidual γδ T (dγδT) cell count between the two groups. The expression of IL-10, IL-17A, and perforin was downregulated in the IVF-ET group ($P<0.05$). There was no significant difference in the expression of IFN-γ, TNF-α, granzyme B, and granulysin, the cellular function indicators ($P>0.05$). **Conclusion** The dNK cell count and the secretion of some intracellular cytokines of dNK and dγδT cells of

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women of IVF-ET pregnancy decreased to some degree, which suggests that certain changes may have taken place in the immunological microenvironment at the maternal-fetal interface. The specific effect of these changes on pregnancy outcomes needs further investigation.

【**Key words**】 Natural killer cells　γδ T cells　Maternal-fetal interface　Immunological microenvironment　*in vitro* fertilization-embryo transfer

体外受精-胚胎移植 (*in vitro* fertilization-embryo transfer, IVF-ET)是生殖医学领域最伟大的成就之一[1], 然而越来越多的研究表明IVF-ET后妊娠面临着更高风险的妊娠期疾病和不良妊娠结局, 如子痫前期(pre-eclampsia, PE)、妊娠期糖尿病(gestational diabetes mellitus, GDM)、早产(preterm birth, PTB)及低出生体重儿(low birth weight, LBW)[2-3], 但造成这种不良妊娠结局风险增加的原因及其机制尚不清楚。

母胎界面免疫微环境由滋养层细胞、蜕膜基质细胞与自然杀伤(natural killer, NK)细胞、T细胞、树突状细胞, 巨噬细胞等众多免疫细胞[4]及其分泌的细胞因子共同组成, 这些免疫细胞共同参与抵抗病原体入侵和介导母胎免疫耐受, 以维持妊娠的正常进行[5-6]。其中, 蜕膜NK(decidual NK, dNK)细胞被认为是母胎界面免疫细胞最重要的组成成分, 其表型主要为$CD56^{Bright}CD16^{-}$, 在妊娠早期, dNK细胞占蜕膜免疫细胞总数的70%[7]。已有研究证实dNK细胞除了发挥抗感染、介导母胎耐受作用外, 还参与促进子宫螺旋动脉重塑、胎盘形成以及胎儿的生长发育[8]。此外, 蜕膜上还存在一类较为特殊的、T细胞受体(T cell receptor, TCR)由γ链和δ链组成的蜕膜γδT(decidual γδT, dγδT)细胞[9]。本实验室前期的研究已证实dγδT细胞较外周血中的γδT细胞表现出更强的细胞因子分泌, 以及促进滋养层细胞迁移、侵袭、成管的能力[10]。现有研究表明dNK细胞与dγδT细胞的功能紊乱参与了复发性流产(recurrent pregnancy loss, RPL)、反复移植失败(recurrent implantation failure, RIF)以及子痫前期、早产等产科常见并发症的发生[8,11], 但IVF-ET后妊娠母胎界面dNK和dγδT细胞的功能是否存在变化有待研究。

本研究收集自然怀孕与IVF-ET产妇蜕膜标本, 观察dNK细胞和dγδT细胞数量, 以及相关细胞功能指标表达水平的变化, 探讨IVF-ET对母胎界面免疫微环境的影响以及为IVF-ET妊娠合并较高风险产科并发症这一临床问题寻求新的解决方案。

1　资料与方法

1.1　研究对象

将经IVF-ET后成功妊娠并于2021年6月–2022年1月在四川大学华西第二医院接受剖宫产手术的产妇蜕膜组织标本纳入实验组(IVF-ET组, n=32), 将产妇年龄、身体质量指数、孕期相匹配的自然受孕的产妇标本纳入正常对照组(NP组, n=12)。排除标准为: ①标本来源产妇患有免疫缺陷疾病; ②产妇或者配偶染色体异常, 如地中海贫血, 基因连锁缺陷等; ③产妇患有细菌, 真菌, 病毒感染性疾病; ④产妇生殖系统生理结构异常, 如子宫畸形; ⑤产妇内分泌激素水平异常。所有人体组织标本均按照四川大学华西第二医院操作规范在生物安全柜中进行操作与无害化处理。本研究已获四川大学华西第二医院伦理委员会审核批准[2020年(029)号]。

1.2　试剂与仪器

超净工作台购自美国Baker公司; 荧光显微镜购自日本Olympus公司; Ficoll淋巴细胞分离液购自天津灏洋生物制品有限责任公司; Percoll分离液购自瑞士Pharmacia公司; Zombie Aqua™ Fixable Viability Kit、布雷菲德菌素A以及莫能霉素购自美国BioLegend公司; 抗人神经细胞黏附分子1(neural cell adhesion molecule 1, NCAM1)抗体(1∶2000)购于美国Abcam公司; DAPI染液(1∶500)购自北京索莱宝科技有限公司; 细胞破膜试剂、山羊抗兔IgG(1∶4000)与CY5(1∶400)购自美国Thermo公司; 细胞刺激所用的佛波酯及离子霉素购于美国Sigma公司; 流式细胞仪购于美国BD Biosciences公司。本研究所使用流式抗体详细信息见表1。

1.3　蜕膜免疫细胞（decidual immunocytes, DICs）的分离

将取得的蜕膜标本放入适量含双抗的PBS缓冲液中, 保持低温并于30 min内运送至实验室, PBS缓冲液清洗之后用手术剪剪下适当大小组织用于石蜡包埋; 其余组织剪成1 ~ 2 mm^3的组织块, 加入1 mg胶原酶Ⅳ和150U DNase Ⅰ并补充无血清培养基至总体积为10 mL, 于37 ℃恒温培养振荡器75 r/min消化40 min; 加入2 mL胎牛血清用于终止消化, 加入适量缓冲液至悬液并用医用纱布过滤至另一培养板; 用巴氏吸管分别吸至100 μm、70 μm滤网依次过滤, 将过滤后悬液500×g离心10 min, 弃去上清, 将下层细胞用完全培养基重悬; 并按照说明书配制20%、40%、60% Percoll试剂, 加入重悬液离心后吸取的40% ~ 60%白膜层细胞即为DICs。

表 1 抗体信息

Table 1 List of antibodies

Antibody	Fluorochrome	Clone	Dilution	Company
Anti-CD3	FITC	UCHT1	1∶100	BioLegend
Anti-CD45	APC-cy7	HI30	1∶100	BioLegend
Anti-γδTCR	PE	B1	1∶100	BioLegend
Anti-CD56	PE-cy7	HCD56	1∶100	B&D
Anti-CD69	FITC	FN50	1∶100	BioLegend
Anti-CD16	BV421	3GB	1∶100	BioLegend
Anti-NKG2D	BV605	1D11	1∶100	BioLegend
Anti-granzyme B	BV421	QA18A28	1∶50	BioLegend
Anti-perforin	FITC	dG9	1∶50	BioLegend
Anti-granulysin	APC	DH2	1∶50	BioLegend
Anti-TNF-α	APC	MAb11	1∶50	BioLegend
Anti-IFN-γ	FITC	B27	1∶50	BioLegend
Anti-IL-17A	BV605	BL168	1∶50	BioLegend
Anti-IL-10	BV421	JES3-9D7	1∶50	BioLegend

1.4 HE染色和免疫荧光染色分析

按照文献报道[12]进行切片HE染色，最终细胞核被苏木精染成鲜明蓝色，细胞质被伊红染成粉红色。荧光染色时首先用二甲苯和乙醇脱蜡切片，然后用乙二胺四乙酸(EDTA, pH9.0)修复抗原。切片与修复液在高压锅炉中高温加热1.5 min后用冷水冷却使温度降至室温，再将切片放入3%H_2O_2中，室温封闭30 min后蒸馏水洗涤三次，10%山羊血清阻断非特异性结合位点30 min，加入50 μL抗人NCAM1抗体，4 ℃孵育过夜。第2天，切片用TBST洗涤3次，加入50 μL山羊抗兔IgG，37 ℃孵育45 min后洗涤3次，弃去残留液体。每张切片加入50 μL CY5，室温孵育10 min，随即用TBST清洗三次后将TBST弃掉。每张切片加入50 μL DAPI工作液，染色5 min后再用TBST 清洗两次。组织切片在倒置荧光显微镜上进行检测，dNK细胞被NCAM1抗体所染呈现红色，细胞核被DAPI染成蓝色，每张片子随机选取3个视野，并用imageJ软件计算双阳性细胞占有核细胞百分比。

1.5 免疫细胞表面标记、细胞因子及毒性颗粒指标检测

将分离出的DICs用PBS缓冲液清洗两遍并计数，取1×10^6个细胞重悬于1 mL PBS中，吸取100 μL用于抗体染色。在染色之前加入死细胞染色抗体2 μL室温孵育15 min，清洗之后加入5 μL Fc受体封闭剂，封闭10 min。再按照说明书指示浓度加入流式抗体并孵育30 min，用含3% FBS的PBS缓冲液清洗两次离心弃上清，400 μL PBS重悬细胞用于上机检测。毒性颗粒指标则在表面指标染色后进行30 min的破膜处理，再加入毒性颗粒抗体，孵育30 min，缓冲液清洗两遍离心弃上清，加入400 μL缓冲液重悬待上机。胞内细胞因子的检测需在加入抗体之前加入佛波酯、离子霉素、莫能霉素以及布雷菲德菌素A，置于37 ℃培养4 h，后续步骤同毒性颗粒指标检测。DICs经流式上机检测后采用 FlowJo软件进行分析，结合CD45、CD3、CD56、γδTCR指标进行NK细胞与γδT细胞圈门，相关指标的表达水平最终以阳性细胞百分比呈现。

1.6 统计学方法

服从正态分布的数据用$\bar{x}\pm s$表示，不服从正态分布则采用中位数(四分位数)表示。对于两组之间指标比较，根据其是否服从正态分布，选择t检验或非参数检验，P<0.05为差异有统计学意义。

2 结果

2.1 一般资料分析

由表2可见，在研究组与对照组各项数据比较中，无论是产妇的年龄、孕期、体质量指数，还是胎儿的体质

表 2 IVF-ET组和NP组基线特点

Table 2 Baseline characteristics of the IVF-ET group and the NP group

Baseline characteristic	NP (n=12)	IVF-ET (n=32)	P
Maternal age/yr.	33 (31, 35.75)	33 (29.25, 33)	0.4049
Gestational age/week	38.68±0.76	37.55±1.77	0.0752
BMI/(kg/m^2)	27.45±4.07	27.05±3.18	0.7361
Gravidity	2 (1, 3.75)	2 (2, 4)	0.2057
Parity	0.50±0.67	0.28±0.58	0.2386
Routine blood tests			
NEU/%	74.33±6.36	72.69±7.48	0.5055
LYM/%	18.38±6.14	19.21±6.27	0.6965
MON/%	6.30 (5.75, 6.75)	6.90 (6.00, 8.45)	0.3113
WBC/(×10^9 L^{-1})	8.05 (6.30, 9.25)	7.90 (6.43, 9.25)	0.9410
NEU/(×10^7 L^{-1})	6.31 (4.60, 6.95)	6.08 (4.24, 6.86)	0.9017
LYM/(×10^7 L^{-1})	0.54 (0.39, 0.59)	0.52 (0.45, 0.59)	0.9741
MON/(×10^7 L^{-1})	1.41±0.53	1.45±0.36	0.8198
Birth body mass/g	3 134.0±442.9	2 834.0±534.9	0.0932
Birth height/cm	49.00±1.95	48.43±2.15	0.4472
Infant sex (M/F)/case	5/7	17/15	ND
Newborn Apgar score			
1 min	10	10	ND
5 min	10	10	ND
10 min	10	10	ND

BMI: body mass index; NEU: neutrophils; LYM: lymphocytes; MON: monocytes; WBC: white blood cells; M/F: male/female; ND: not done.

量、身长、Apgar评分等指标, 差异均无统计学意义。

2.2　蜕膜HE染色及免疫荧光染色结果

组织切片经HE染色结果显示其具有蜕膜典型结构, 且有较多淋巴细胞富集(图1A中白色箭头所指); 对蜕膜组织进行免疫荧光染色后的结果显示NP组dNK细胞在所有有核细胞中的占比高于IVF-ET组〔(13.28±1.20)% vs. (6.56±1.37)%, P=0.006〕(图1B和图1C)。

2.3　dNK细胞与dγδT细胞数量及表型检测

流式结果呈现IVF-ET组中的dNK细胞占淋巴细胞总数的比例低于NP组〔(17.10±7.02)% vs. (22.41±8.50)%, P=0.04〕(图2A); 两组样本中细胞毒性更强的CD16$^+$细胞的比例组间差异无统计学意义(图2B); NP组与IVF-ET组的dγδT细胞的数量无明显差异(P>0.05)(图2C)。dNK、dγδT细胞表面活化指标CD69与NKG2D的测定结果显示, IVF-ET组中dγδT细胞NKG2D表达低于对照组〔(69.09±16.14)% vs. (79.39±7.42)%, P=0.04〕, 但两组dNK和dγδT细胞CD69水平差异均无统计学意义(图2D和图2E)。

2.4　dNK细胞与dγδT细胞的功能性因子表达水平检测

促炎性因子IFN-γ、TNF-α、IL-17A以及抑炎性分子IL-10的表达水平的测定结果显示: IVF-ET组中dNK细胞IL-10的表达水平低于NP组〔(2.26±1.36)% vs.(3.35±1.79)%, P=0.03〕(图3A和图3B); IVF-ET组dγδT细胞中IL-17A〔(4.48±4.70)% vs.(9.71±7.50)%, P=0.008〕与IL-10〔(4.01±3.71)% vs. (8.86±9.13)%, P=0.01〕表达也低于NP组(图3C和图3D), 但两组dNK、dγδT细胞的IFN-γ、

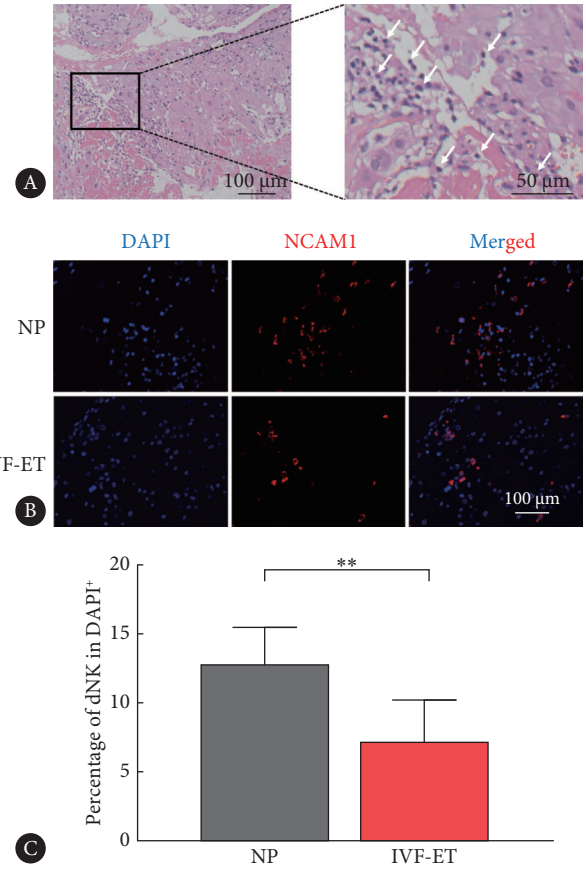

图 1　蜕膜组织HE染色以及dNK细胞免疫荧光染色分析

Fig 1　HE staining of decidual tissue and immunofluorescence staining analysis of dNK cells

A: HE staining showed enrichment of lymphocytes in the decidua; B: the distribution of dNK cells in the decidua from the NP and IVF-ET groups, as shown by immunofluorescence assay (scale bars=100 μm); C: percentage of dNK cells in all DAPI$^+$ nuclei in NP and IVF-ET decidua in each field, as quantified by ImageJ (n=5). ** P<0.01.

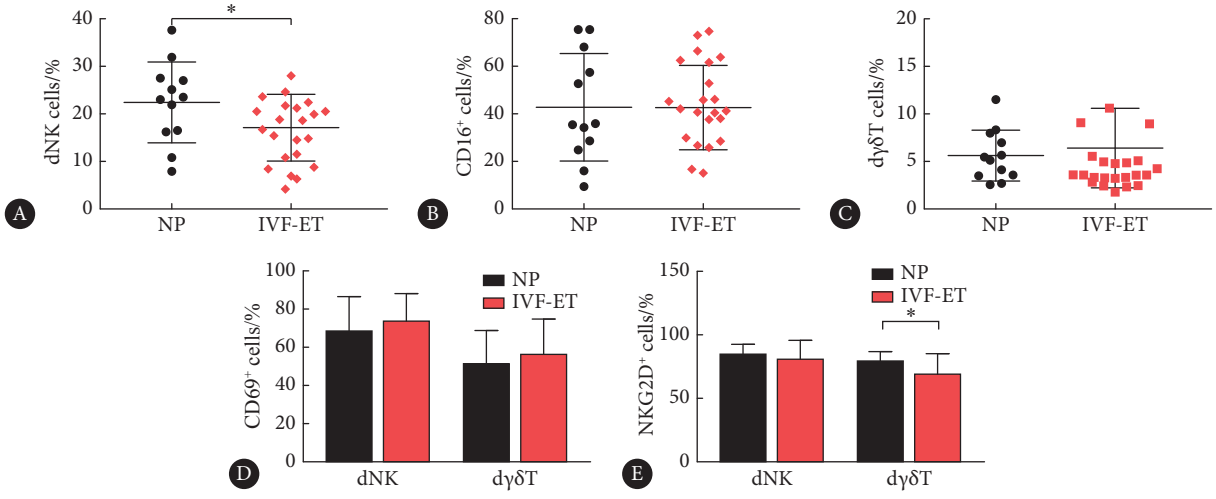

图 2　dNK细胞和dγδT细胞数量和表型测定

Fig 2　Determining the cell counts and phenotypes of dNK and dγδT cells

A: Percentage of NK cells was compared between the NP and the IVF-ET groups; B-C: percentage of CD16$^+$ NK cells and γδ T cells were compared between the NP and the IVF-ET groups; D-E: surface activation receptor CD69, NKG2D expression of dNK and dγδT cells were determined by flow cytometry. * P<0.05.

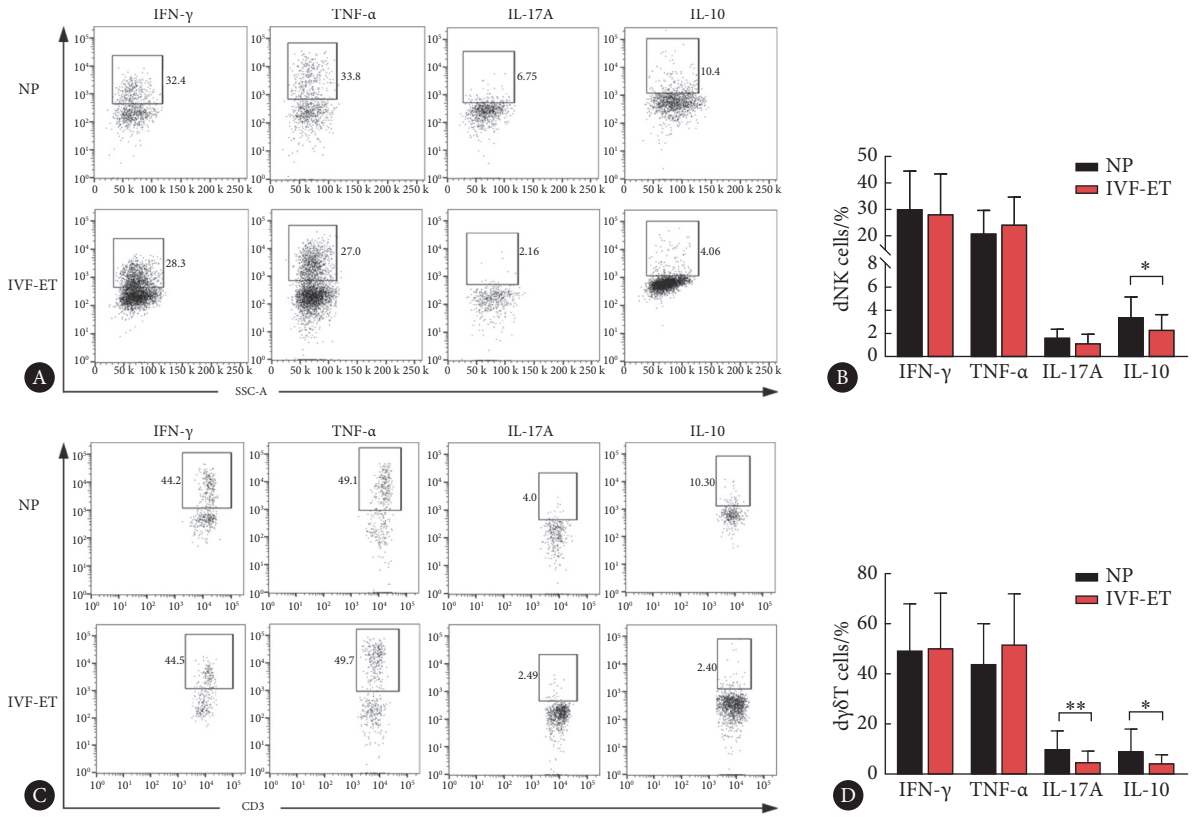

图 3 dNK细胞与dγδT细胞胞内细胞因子表达水平测定

Fig 3 Determining the expression level of intracellular cytokines of dNK and dγδT cells

A-B: representative flow cytometry and statistical analysis of the expression level of IFN-γ, TNF-α, IL-17A, and IL-10 on dNK cells; C-D: representative flow cytometry and statistical analysis of the expression level of IFN-γ, TNF-α, IL-17A, and IL-10 in dγδT cells. * P<0.05, ** P<0.01.

TNF-α表达水平差异均无统计学意义。

2.5 dNK细胞与dγδT细胞毒性颗粒表达水平检测

相比于NP组, IVF-ET组dNK细胞perforin的表达下调〔(72.38±16.34)% vs. (55.92±17.87)%, P=0.008〕(图4A和图4B); Granzyme B的表达也出现了降低〔(76.23±10.06)% vs. (67.12±19.42)%〕, 但差异无统计学意义; IVF-ET组dγδT细胞perforin表达也低于NP组〔(31.23±16.26)% vs. (42.83±16.69)%, P=0.04〕(图4C和图4D); 两组dNK、dγδT细胞Granulysin表达水平差异均无统计学意义。

3 讨论

在IVF-ET技术已相当成熟的今天, 如何进一步提高妊娠率、降低不良妊娠结局的风险依然是临床医生和科研工作者不断追求的目标。相较于自然受孕, IVF-ET后妊娠为何具有更高产科综合征风险尚不明确, 有研究利用全基因组芯片对IVF胎盘基因表达谱进行了分析, 发现其与自然受孕在胎盘重要功能有关基因的表达上具有差异, 提示辅助生殖技术可能对胎盘的形成和功能存在一定影响[13]; 此外, IVF-ET因为缺乏精浆和黄体的参与[14-15], 这会造成子宫内膜环境和功能发生相应改变, 这也被认为是IVF-ET妊娠不良结局风险增加的原因之一。本研究从母胎界面免疫微环境的角度出发, 分析在接受IVF-ET后, 母胎界面重要免疫细胞群是否存在数量及功能的改变。

母胎界面免疫微环境在维持妊娠、胎儿生长发育, 以及分娩过程中都发挥了重要作用, 且随着孕期的变化, 各类免疫细胞的比例与功能亦发生着动态变化[5]。作为NK细胞与T细胞表面活化标志, CD69、NKG2D与其他活化性受体NKp44、NKp46以及抑制性受体NKG2A、KIR2DL1等参与了炎症因子的分泌、细胞毒性颗粒的释放[16], 本研究还检测了几类重要的炎症指标, 如促炎因子IFN-γ、TNF-α、IL-17A与抑炎性因子IL-10; 此外, 为了分析dNK细胞、dγδT的细胞毒效应功能, 本研究还关注了granzyme B、perforin、granulysin的表达, 以检测dNK, dγδT细胞的免疫学功能变化。结果显示 IVF-ET后产妇dNK细胞的数量降低, 以及IL-10和perforin表达水平降低; dγδT细胞NKG2D水平, 以及IL-10、IL-17A及perforin的表达下调。dNK细胞数量和细胞毒性的下降这一现象在宫内生长受限(fetal growth restriction, FGR)、

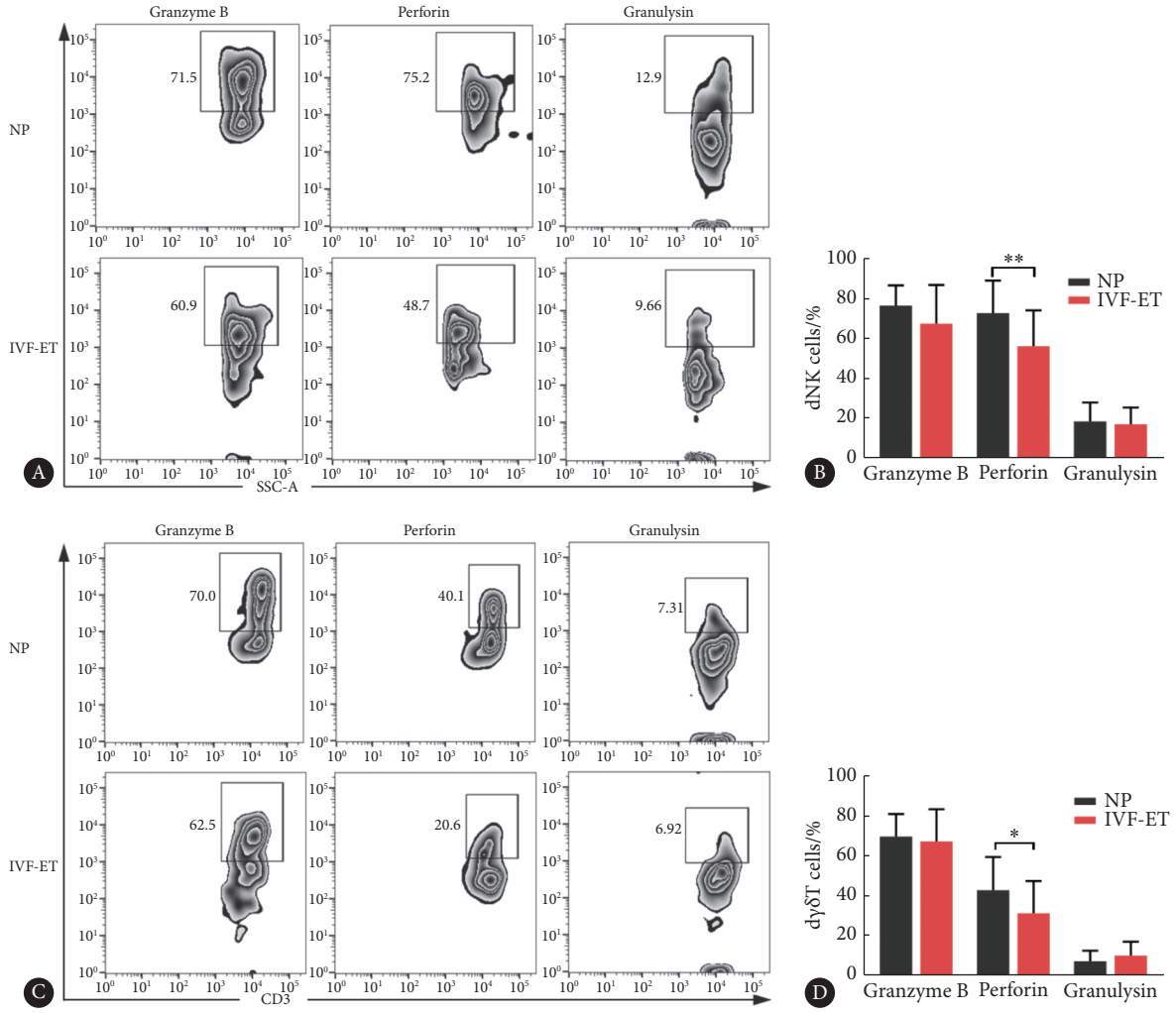

图 4 dNK细胞与γδT细胞的毒性颗粒表达水平测定

Fig 4 Determining the expression level of cytolytic granules of dNK and dγδT cells

A-B: representative flow cytometry and statistical analysis of the expression level of granzyme B, perforin, and granulysin in dNK cells; C-D: representative flow cytometry and statistical analysis of the expression level of granzyme B, perforin, and granulysin in dγδT cells. * P<0.05, ** P<0.01.

子痫和小于胎龄儿(small for gestational age, SGA)等不良妊娠结局也曾报道[17-19]。结合dNK细胞数量以及两类免疫细胞功能性因子分泌水平的改变,提示IVF-ET后妊娠母胎界面免疫微环境发生了相应改变,笔者认为此种变化极有可能是引起IVF-ET妊娠不良妊娠结局风险升高的重要原因。

对于母胎界面免疫微环境异常免疫状态的纠正已取得些许成果,如宫腔灌注人绒毛膜促性腺激素(human chorionic gonadotropin, HCG)可增加子宫NK (uterine NK, uNK)细胞数量但降低其细胞毒性[20]、利用糖皮质激素、粒细胞集落刺激因子等免疫制剂的使用可降低uNK细胞异常活化[21-22];也有学者[23]发现维生素D可诱导dγδT细胞向抗炎性表型分化,以期改善细胞因子分泌失衡以提高妊娠率和妊娠维持。当前基于母胎界面微环境的免疫细胞靶向干预的动物实验陆续开展,如有研究[24]发现体外可诱导和补充CD49a$^+$NK细胞可促进胎儿生长,以及FU等[25]提出小鼠静脉注射体外诱导的子宫样NK细胞也可发挥促进胎儿发育的作用,这些研究为治疗RPL、FGR以及降低IVF-ET妊娠不良妊娠结局的风险提供了新的思路。相信这类以免疫细胞为出发点的靶向调控未来能够进一步提高优生率,切实服务于临床患者。

本研究仍然有着一些局限,如未能控制纳入研究的产妇接受的胚胎移植方案,且纳入研究的样本数量也较少,探究IVF-ET妊娠对母胎界面微环境的具体影响还需进一步扩大研究队列、增加检测指标以及更为精细的分组。

综上,IVF-ET后妊娠产妇的母胎界面dNK细胞与dγδT细胞部分功能性免疫学指标较对照组有明显差异,提示其母胎界面免疫微环境发生了一定变化,但这些改变对母体和胎儿的直接或间接影响还有待后续进一步研究证实。

* * *

利益冲突 所有作者均声明不存在利益冲突

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