

AHR介导的FOXP3组蛋白乙酰化在系统性红斑狼疮 Tfr细胞分化中的作用

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[摘要] 目的: 系统性红斑狼疮(systemic lupus erythematosus, SLE)是一种以自身抗体异常生成、免疫失衡及多系统受累为主要特征的慢性自身免疫性疾病。本研究旨在探究芳香烃受体(aryl hydrocarbon receptor, AHR)激活对SLE患者滤泡调节性T(T follicular regulatory, Tfr)细胞分化和叉头框蛋白P3(forkhead box protein P3, FOXP3)表达的影响, 并从表观遗传学角度阐明其内在分子机制。方法: 选取2025年6月至12月于中南大学湘雅三医院皮肤科及风湿免疫科就诊的SLE患者18例及健康体检者18例, 并招募健康志愿者3例收集外周血完成机制部分研究。采用密度梯度离心法分离外周血单个核细胞, 分选CD4⁺ T细胞。采用流式细胞术检测Tfr细胞比例(Tfr%)及FOXP3、AHR的表达水平, 并分析其与疾病活动度的相关性。体外分离健康人CD4⁺ T细胞, 分为Control组[二甲基亚砷(dimethyl sulfoxide, DMSO)处理]、ITE组{AHR激动剂2-(1'-H-吲哚-3'-羰基)噻唑-4-羧酸甲酯[2-(1'-H-Indol-3'-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester, ITE]处理}、A-485组(P300抑制剂A-485处理)及ITE+A-485组(ITE、A-485共处理), 采用细胞计数试剂盒-8(cell counting kit-8, CCK-8)法、流式细胞术、蛋白质印迹法及实时反转录聚合酶链反应(polymerase chain reaction, PCR)检测细胞增殖活性、Tfr%及相应T细胞中FOXP3、AHR蛋白与细胞毒性T淋巴细胞相关蛋白4(cytotoxic T-lymphocyte associated protein 4, CTLA-4)及白细胞介素10(interleukin-10, IL-10)的mRNA表达水平。将AHR、P300过表达质粒共转染至健康人CD4⁺ T细胞中, 采用免疫共沉淀(co-immunoprecipitation, Co-IP)验证AHR与P300的蛋白质相互作用。采用染色质免疫共沉淀(chromatin immunoprecipitation, ChIP)-PCR验证AHR、P300在FOXP3启动子区结合位点, ChIP-定量PCR(quantitative PCR, qPCR)检测各组健康人CD4⁺ T细胞AHR、P300在FOXP3启动子区域的结合水平及组蛋白乙酰化水平。收集SLE患者外周血CD4⁺ T细胞, 经CD3/CD28 T细胞激活磁珠活化后, 分别给予DMSO与ITE处理。采用流式细胞术检测SLE患者CD4⁺ T细胞中Tfr%、FOXP3表达水平, 采用ChIP-qPCR检测AHR、P300在FOXP3启动子区域的结合水平及组蛋白乙酰化水平。结果: 与健康对照组相比, SLE患者外周血CD4⁺ T细胞中Tfr%显著降低($P<0.001$), 且与SLE疾病活动指数(systemic lupus erythematosus disease activity index, SLEDAI)呈负相关($r=-0.803$, $P<0.001$); Tfr细胞中FOXP3和AHR蛋白表达水平均显著下调(均 $P<0.01$), 且二者呈正相关($r=0.596$, $P<0.05$)。CCK-8法结果显示ITE在0~349.27 nmol/L浓度范围内、A-485在0~93.20 nmol/L浓度范围内对CD4⁺ T细胞增殖活性无明显影响(均 $P>0.05$)。体外实验结果显示, 与Control组相比, ITE组Tfr%、AHR、FOXP3蛋白, 以及CTLA-4、IL-10的mRNA表达水平均显著上调(均 $P<0.05$)。Co-IP实验证实, AHR与P300在CD4⁺ T细胞中存在蛋白质相互作用。ChIP-PCR结果显示, AHR在FOXP3启动子区域-78 bp至-263 bp及-431 bp至-596 bp区段存在结合位点, P300可结合至FOXP3启动子区域-78 bp至-263 bp区段。ChIP-qPCR结果显示, 与Control组相比, ITE组FOXP3启动子区域组蛋白H3尾部赖氨酸残基(H3K9ac、H3K14ac)的乙酰化水平及AHR、

收稿日期(Date of reception): 2026-04-27

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基金项目(Foundation item): 湖南省自然科学基金(2023JJ20090); 湖南省科技创新计划(2023RC3086); 湖南省卫生健康委员会科研计划项目(202304129002); 中南大学湘雅三医院创新驱动计划(2023CXQD074); 中南大学汇智育才项目(YX202101)。This work was supported by the Natural Science Foundation of Hunan Province (2023JJ20090), the Science and Technology Innovation Program of Hunan Province (2023RC3086), the Research Programme Project of Health Commission of Hunan Province (202304129002), the Innovation-Driven Project of the Third Xiangya Hospital of Central South University (2023CXQD074), and the Wisdom Accumulation and Talent Cultivation Project of Central South University (YX202101), China.

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P300结合水平均显著升高(均 $P<0.05$), A-485组 *FOXP3* 启动子区域 H3K9ac、H3K14ac 乙酰化水平及 P300 结合水平均显著降低(均 $P<0.05$), AHR 结合水平无明显变化($P>0.05$)。功能挽救实验结果显示, 与 ITE 组相比, ITE+A-485 组 *FOXP3* 蛋白表达水平及 Tfr% 均显著降低(均 $P<0.05$), *FOXP3* 启动子区域 H3K9ac、H3K14ac 乙酰化水平及 P300 结合水平均显著降低(均 $P<0.05$), AHR 结合水平无明显变化($P>0.05$); 与 A-485 组相比, ITE+A-485 组 *FOXP3* 启动子区 AHR 结合水平及 *FOXP3* 蛋白表达水平均显著上调(均 $P<0.05$), 但低于 ITE 组, *FOXP3* 启动子区 H3K9ac、H3K14ac 乙酰化水平及 P300 结合水平均无明显变化(均 $P>0.05$)。在 SLE 患者 $CD4^+$ T 细胞中验证发现, ITE 处理后, Tfr% 升高、*FOXP3* 表达上调, *FOXP3* 启动子区乙酰化水平、AHR 及 P300 结合水平均升高(均 $P<0.05$), 与上述结果一致。结论: SLE 患者外周血 $CD4^+$ T 细胞中 Tfr% 降低及 *FOXP3* 表达下降可能与 AHR 表达不足有关。AHR 激活可促进 *FOXP3* 表达并诱导 Tfr 细胞分化, 其作用可能与 AHR 募集 P300 至 *FOXP3* 启动子区域并增强局部组蛋白乙酰化有关。

[关键词] 系统性红斑狼疮; 滤泡调节性T细胞; 芳香烃受体; 叉头框蛋白P3; P300蛋白; 组蛋白乙酰化

Role of AHR-mediated histone acetylation at *FOXP3* in Tfr cell differentiation in systemic lupus erythematosus

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ABSTRACT

Objective: Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by abnormal autoantibody production, immune dysregulation, and multisystem involvement. This study aims to investigate the effects of aryl hydrocarbon receptor (AHR) activation on the differentiation of T follicular regulatory (Tfr) cells and the expression of forkhead box protein P3 (*FOXP3*) in patients with SLE, and to elucidate the underlying molecular mechanisms from an epigenetic perspective.

Methods: Eighteen patients with SLE who attended the Departments of Dermatology and Rheumatology and Immunology of The Third Xiangya Hospital, Central South University, between June and December 2025, and 18 healthy individuals undergoing routine health examinations were enrolled. In addition, 3 healthy volunteers were recruited for peripheral blood collection for mechanistic experiments. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation, and $CD4^+$ T cells were sorted. Flow cytometry was used to determine the proportion of Tfr cells (Tfr%), as well as *FOXP3* and AHR expression, and their correlations with disease activity were analyzed. $CD4^+$ T cells isolated from healthy individuals were divided into a Control group [treated with dimethyl sulfoxide (DMSO)], an ITE group [treated with the AHR agonist 2-(1'H-indol-3'-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester (ITE)], an A-485 group (treated with the P300 inhibitor A-485), and an ITE+A-485 group (co-treated with ITE and A-485). Cell counting kit-8 (CCK-8) assay, flow cytometry, Western blotting and real-time reverse transcription polymerase chain reaction (PCR) were performed to assess cell proliferation activity, Tfr%, the protein expression of *FOXP3* and AHR, and the mRNA expression levels of cytotoxic T-lymphocyte associated protein 4 (*CTLA-4*) and interleukin-10 (*IL-10*) in the corresponding T cells. AHR and P300 overexpression plasmids were co-transfected into $CD4^+$ T cells from healthy individuals, and co-immunoprecipitation (Co-

IP) was conducted to verify the protein-protein interaction between AHR and P300. Chromatin immunoprecipitation (ChIP)-PCR was used to identify the binding sites of AHR and P300 within the *FOXP3* promoter region, while ChIP-quantitative PCR (qPCR) was performed to quantify AHR and P300 binding to the *FOXP3* promoter and histone acetylation levels in CD4⁺ T cells from healthy individuals in each group. Peripheral blood CD4⁺ T cells were also collected from patients with SLE, activated using CD3/CD28 T-cell activation beads, and treated with either DMSO or ITE. Flow cytometry was used to detect Tfr% and FOXP3 expression in CD4⁺ T cells from patients with SLE, and ChIP-qPCR was used to detect AHR and P300 binding to the *FOXP3* promoter and histone acetylation levels.

Results: Compared with healthy controls, patients with SLE had a significantly lower Tfr% among peripheral blood CD4⁺ T cells ($P<0.001$), which was negatively correlated with the systemic lupus erythematosus disease activity index (SLEDAI) ($r=-0.803$, $P<0.001$). FOXP3 and AHR protein expression levels in Tfr cells were also significantly downregulated (both $P<0.01$), and the two were positively correlated ($r=0.596$, $P<0.05$). CCK-8 assays showed that ITE at concentrations of 0 to 349.27 nmol/L and A-485 at concentrations of 0 to 93.20 nmol/L had no significant effects on CD4⁺ T-cell proliferation activity (both $P>0.05$). In vitro experiments showed that, compared with the Control group, the ITE group had significantly increased Tfr%, AHR and *FOXP3* protein expression, and *CTLA-4* and *IL-10* mRNA expression levels (all $P<0.05$). Co-IP confirmed a protein-protein interaction between AHR and P300 in CD4⁺ T cells. ChIP-PCR showed that AHR had binding sites within the -78 to -263 bp and -431 to -596 bp regions of the *FOXP3* promoter, whereas P300 bound to the -78 to -263 bp region of the *FOXP3* promoter. ChIP-qPCR showed that, compared with the Control group, the ITE group exhibited significantly increased H3K9ac and H3K14ac acetylation within the *FOXP3* promoter region, together with increased binding of AHR and P300 (all $P<0.05$). In contrast, the A-485 group showed significantly decreased H3K9ac and H3K14ac acetylation and P300 binding within the *FOXP3* promoter region (all $P<0.05$), whereas AHR binding showed no significant change ($P>0.05$). In the functional rescue experiment, compared with the ITE group, the ITE+A-485 group showed significantly decreased FOXP3 protein expression and Tfr% (both $P<0.05$), as well as significantly decreased H3K9ac and H3K14ac acetylation and P300 binding within the *FOXP3* promoter region (all $P<0.05$), while AHR binding showed no significant change ($P>0.05$). Compared with the A-485 group, the ITE+A-485 group showed significantly increased AHR binding to the *FOXP3* promoter and increased FOXP3 protein expression (both $P<0.05$), although both remained lower than those in the ITE group. No significant differences were observed in H3K9ac and H3K14ac acetylation or P300 binding within the *FOXP3* promoter region (all $P>0.05$). Validation experiments in CD4⁺ T cells from patients with SLE showed that ITE treatment increased Tfr%, FOXP3 expression, histone acetylation within the *FOXP3* promoter region, and AHR and P300 binding (all $P<0.05$), consistent with the above findings.

Conclusion: The reduced Tfr% and decreased FOXP3 expression in peripheral blood CD4⁺ T cells from patients with SLE may be associated with insufficient AHR expression. AHR activation may promote FOXP3 expression and induce Tfr cell differentiation, potentially through recruitment of P300 to the *FOXP3* promoter region and enhancement of local histone acetylation.

KEY WORDS systemic lupus erythematosus; T follicular regulatory cells; aryl hydrocarbon receptor; forkhead box protein P3; P300 protein; histone acetylation

系统性红斑狼疮(systemic lupus erythematosus, SLE)是一种累及多系统的慢性自身免疫性疾病,以产生多种自身抗体和免疫复合物沉积为主要特征,可导致皮肤、关节、肾、血液系统及神经系统等多器官损害^[1]。SLE好发于育龄期女性,发病率高,给社会和家庭带来沉重的疾病负担。近年来研究^[2-3]表明,CD4⁺ T细胞的异常活跃、生发中心反应及滤泡相关T细胞异常与SLE疾病活动、自身抗体生成及靶器官损伤密切相关。

滤泡辅助性T(T follicular helper, Tfh)细胞是一类效应性T辅助细胞,主要位于生发中心,可促进B细胞活化和抗体应答^[4]。滤泡调节性T(T follicular regulatory, Tfr)细胞是高表达叉头框蛋白P3(forkhead box protein P3, FOXP3)的调节性T细胞(regulatory T cell, Treg)的一种特殊亚群,通过表达细胞毒性T淋巴细胞相关蛋白4(cytotoxic T-lymphocyte associated protein 4, CTLA-4)、白细胞介素(interleukin, IL)-10等抑制性分子限制Tfh细胞与B细胞的相互作用,从而抑制自身抗体产生,维持生发中心反应稳态^[5-6]。Tfr细胞数量减少或功能缺陷可导致生发中心反应过度增强、自身抗体产生增加,进而促进自身免疫病的发生、发展^[7]。研究^[8]发现,与健康对照组相比, SLE患者循环中Tfr细胞频率降低, Tfr细胞与Tfh细胞比值(Tfr/Tfh)下降,且与疾病活动性密切相关;低剂量IL-2治疗可通过上调Tfr细胞比例(Tfr%)、恢复Tfr/Tfh平衡而改善SLE患者病情^[9]。因此,阐明SLE患者Tfr细胞异常的分子机制对于理解SLE发病机制及寻找新的治疗靶点具有重要意义。

FOXP3是维持Treg细胞分化和功能稳定的核心转录因子^[10],也是Tfr细胞区别于Tfh细胞的标志性分子,其表达水平直接决定Tfr细胞的免疫抑制能力。FOXP3功能丧失可导致多种自身免疫性疾病, FOXP3表达下调被认为是SLE患者Tfr细胞功能异常的重要原因^[11]。FOXP3的表达受多种机制调控,其中表观遗传修饰具有重要作用。组蛋白乙酰转移酶P300/CBP可通过乙酰化组蛋白H3尾部赖氨酸残基(如H3K9ac、H3K14ac),促进FOXP3启动子区的染色质开放,上调FOXP3的转录表达,对Treg细胞的稳定性至关重要^[12]。Treg细胞特异性敲除P300可导

致FOXP3表达下调和致死性自身免疫病^[13]。上述研究提示,组蛋白乙酰化修饰异常可能是导致SLE患者FOXP3表达下调及Tfr细胞异常的重要机制之一。

芳香烃受体(aryl hydrocarbon receptor, AHR)是一类配体依赖性转录因子,在T细胞、B细胞、树突状细胞等多种免疫细胞中广泛表达,在维持免疫稳态中发挥重要作用^[14]。近年来, AHR在SLE中的作用逐渐受到关注。Shinde等^[15]发现凋亡相关信号触发的AHR活性对维持SLE免疫耐受至关重要; AHR激动剂tapinarof可通过抑制Tfh细胞分化,减轻SLE小鼠肾损伤^[16]; I型干扰素通过抑制AHR信号通路驱动C-X-C基序趋化因子配体13(C-X-C motif chemokine ligand 13, CXCL13)阳性Tfh细胞在SLE中的异常扩增,而激活AHR可逆转该病理过程^[17]。上述结果提示, AHR参与调控Tfh细胞的分化与功能,但对Tfr细胞的调节作用目前未见相关报道。

本研究假设AHR可能通过招募P300至FOXP3启动子区域,促进组蛋白乙酰化修饰,从而上调FOXP3的转录表达,进而调控Tfr细胞的分化和功能。本研究以SLE患者外周血CD4⁺ T细胞为研究对象,对比健康对照,分析Tfr%及Tfr细胞内FOXP3、AHR的表达变化,并进一步从表观遗传学角度探讨AHR对FOXP3表达及Tfr细胞分化的影响及其可能分子机制,以期对SLE发病机制及治疗提供新的理论依据。

1 对象与方法

1.1 伦理声明

本研究已获得中南大学湘雅三医院伦理委员会批准(审批号: 2022-S736),所有研究对象均签署知情同意书。

1.2 对象

收集2025年6月至12月中南大学湘雅三医院皮肤科及风湿免疫科确诊的SLE患者18例(SLE组),其中男2例,女16例,年龄为30~45(37.06±4.66)岁(表1)。SLE的诊断依据2019年欧洲抗风湿病联盟/美国风湿病学会SLE诊断标准^[18]。排除标准: 1)合并其他自身免疫性疾病; 2)合并恶性肿瘤或存在肿瘤病史;

3)入组前1个月内存在明确的感染性疾病;4)入组前3个月内存在免疫球蛋白冲击治疗、生物制剂治疗史;5)存在严重的基础疾病;6)处于妊娠或哺乳期的女性。收集患者的一般临床资料并采用SLE疾病活动指数(systemic lupus erythematosus disease activity index, SLEDAI)评价其疾病活动度。于中南大学湘雅三医院健康管理中心选取年龄、性别相匹配的健康者18例(健康对照组),其中男3例,女15例,年龄为31~45(37.83±4.31)岁。SLE组与健康对照组的性别构成及年龄差异均无统计学意义(均 $P>0.05$)。同时招募健康志愿者3例,收集外周血完成机制部分研究。

表1 18例SLE患者特征

Table 1 Characteristics of the 18 patients with SLE

Number	Gender	Age/Years	SLEDAI
1	F	36	3
2	F	41	7
3	F	32	0
4	F	45	5
5	F	34	2
6	F	39	8
7	F	31	1
8	F	43	4
9	F	40	6
10	F	33	3
11	M	38	7
12	F	44	2
13	F	30	0
14	F	35	5
15	F	42	8
16	M	37	4
17	F	33	6
18	F	34	7

F: Female; M: Male; SLEDAI: Systemic lupus erythematosus disease activity index.

1.3 主要材料与试剂

人淋巴细胞分离液购自美国Cytiva公司。人CD4⁺T细胞分选磁珠、LS分选柱均购自德国美天旎公司。RPMI 1640培养基购自美国Gibco公司。胎牛血清(fetal bovine serum, FBS)购自以色列BioInd公司。CD3/CD28 T细胞激活磁珠单抗购自北京百普赛斯科技股份有限公司。TRIzol试剂盒购自美国Invitrogen公司。RNA反转录试剂盒、SYBR绿色荧

光定量试剂盒均购自湖南艾科瑞生物工程有限公司。细胞计数试剂盒-8(cell counting kit-8, CCK-8)购自日本Dojindo公司。二喹啉甲酸(bicinchoninic acid, BCA)蛋白定量试剂盒购自美国Thermo Fisher Scientific公司。FITC-CD4抗体购自美国BD Biosciences公司。Zombie NIR™ Fixable Viability Kit、PE-FOXP3抗体、藻红蛋白(phycoerythrin, PE)-Cy7-C-X-C趋化因子受体5(C-X-C motif chemokine receptor 5, CXCR5)抗体、别藻蓝蛋白(allophycocyanin, APC)-程序性死亡受体1(programmed cell death protein 1, PD-1)抗体、PE标记抗AHR抗体均购自美国Biolegend公司。FOXP3抗体、 β -actin抗体、P300抗体、AHR抗体、组蛋白H3均购自美国Cell Signaling Technology公司。免疫共沉淀(co-immunoprecipitation, Co-IP)试剂盒购自美国Thermo Fisher Scientific公司。染色质免疫沉淀(chromatin immunoprecipitation, ChIP)试剂盒购自美国Epigentek公司。AHR激动剂2-(1'H-吲哚-3'-羧基)噻唑-4-羧酸甲酯[2-(1'H-Indol-3'-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester, ITE]、P300抑制剂A-485均购自美国MedChemExpress公司。重组人IL-2、IL-6、IL-12、IL-21、转化生长因子- β (transforming growth factor beta, TGF- β)均购自美国PeproTech公司。PCR引物由北京擎科科技股份有限公司合成。

1.4 方法

1.4.1 外周血单个核细胞分离与CD4⁺T细胞分选

采集研究对象外周静脉血5 mL,肝素抗凝,采用Ficoll密度梯度离心法分离外周血单个核细胞(peripheral blood mononuclear cells, PBMC),将血标本用磷酸盐缓冲液(phosphate buffered solution, PBS)等倍稀释后,缓慢加于外周血淋巴细胞分离液上层,2 000×g离心30 min,小心吸取云雾层细胞,于4℃、300×g离心10 min获得PBMC。采用人CD4⁺T细胞分选磁珠、LS分选柱及磁力架从PBMC中分选CD4⁺T细胞。

1.4.2 细胞培养与诱导分化

将分选获得的CD4⁺T细胞用含10% FBS、100 U/mL青霉素和100 μ g/mL链霉素的RPMI 1640培养基重悬,以 2×10^5 /孔接种于96孔板或以 1×10^6 /孔接种于6孔板。每孔加入CD3/CD28 T细胞激活磁珠(磁珠用量为5 μ L/ 1×10^6 个细胞)、IL-6(20 ng/mL)、IL-12(10 ng/mL)、IL-21(20 ng/mL)和TGF- β (5 ng/mL),于37℃、5% CO₂培养箱中培养72 h后,加入IL-2(10 ng/mL)诱导Tfr细胞分化。

1.4.3 流式细胞术

采用流式细胞术检测 Tfr 细胞比例(Tfr%)及细胞内 FOXP3、AHR 蛋白的表达水平。收集培养后的细胞, PBS 洗涤后调整细胞密度为 $1 \times 10^6/\text{mL}$ 。取 100 μL 细胞悬液, 依次加入下列荧光素标记抗体: FITC-CD4、PE-Cy7-CXCR5、APC-PD-1, 室温下避光孵育 20 min。用 PBS 洗涤后, 加入 Zombie NIR™ Viability 染料, 室温下避光孵育 15 min。再次 PBS 洗涤后, 使用 FOXP3/转录因子染色缓冲液套装进行固定破膜处理, 于 4 $^{\circ}\text{C}$ 下避光孵育 30~60 min, 洗涤后

加入 PE-FOXP3 抗体, 于 4 $^{\circ}\text{C}$ 下避光孵育 30 min。PBS 洗涤后重悬, 使用流式细胞仪检测, 采用 FlowJo 10.0 软件分析数据。将 $\text{CD4}^+\text{CXCR5}^+\text{PD-1}^+\text{FOXP3}^+$ 细胞群定义为 Tfr 细胞(图 1)。采用中位荧光强度(median fluorescence intensity, MFI)定量 Tfr 细胞内 FOXP3 或 AHR 蛋白表达水平: 在 Tfr 细胞门内, 读取 PE-FOXP3 或 PE-AHR 通道的 MFI 值。每次实验设同型对照作为背景荧光参照, 以样本 MFI 与同型对照 MFI 的比值表示目的蛋白的相对表达水平。

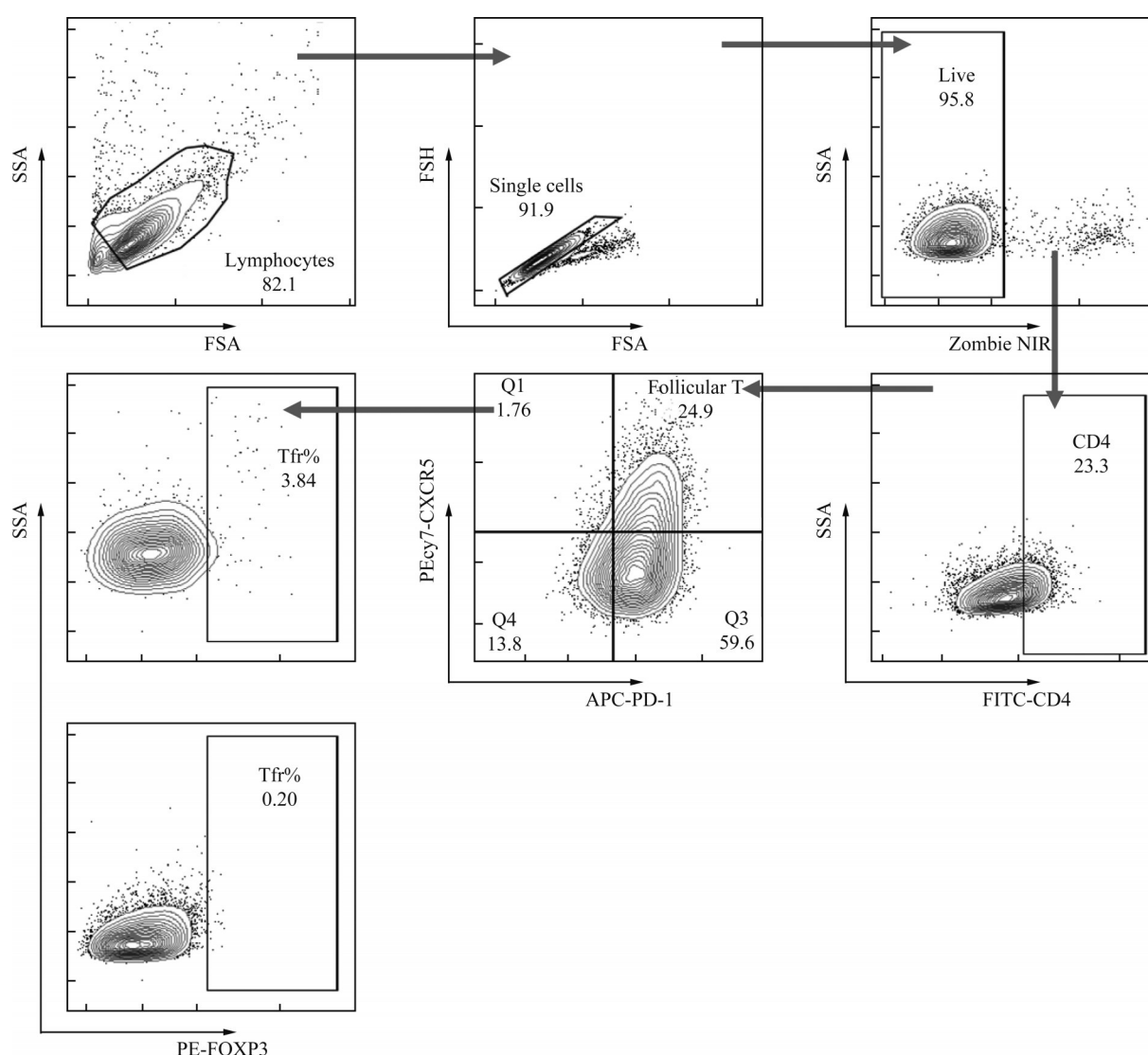


图1 流式细胞术检测 SLE 患者与健康对照 Tfr 细胞的圈门策略

Figure 1 Gating strategy for identifying Tfr cells by multiparameter flow cytometry

Lymphocytes were first gated based on FSA versus SSA. Next, singlets were selected using FSA versus FSH, then dead cells were excluded by Zombie NIR™ viability dye. After identifying CD4^+ T cells, $\text{PD-1}^+\text{CXCR5}^+$ follicular T cells were gated within the CD4^+ T cell population, and FOXP3^+ Tfr cells were subsequently discriminated. FOXP3: Forkhead box protein 3; SLE: Systemic lupus erythematosus; Tfr: T follicular regulatory; CXCR5: C-X-C motif chemokine receptor 5; PD-1: Programmed cell death protein 1; FSA: Forward scatter area; SSA: Side scatter area; FSH: Forward scatter height; APC: Allophycocyanin; PE: Phycoerythrin.

1.4.4 CCK-8法

采用CCK-8法检测不同浓度ITE(0、17.46、34.93、87.32、174.64、349.27 nmol/L)及A-485(0、9.32、18.64、46.60、93.20、184.40 nmol/L)对CD4⁺T细胞增殖活性的影响。将活化后的CD4⁺T细胞接种于96孔板,分别加入上述浓度的ITE或A-485,对照组加入等体积二甲基亚砜(dimethyl sulfoxide, DMSO)溶液,培养48 h后每孔加入10 μ L CCK-8溶液,继续孵育1 h。采用酶标仪测定450 nm波长处各孔吸光度值,确定对细胞增殖无显著影响的药物浓度并将其作为后续实验处理浓度。

1.4.5 药物处理与分组

将磁珠分选的健康人CD4⁺T细胞按 1×10^6 /孔接种于6孔板,按1.4.2所述的诱导条件诱导T_H17细胞分化,同时加入对应药物处理。根据实验目的将细胞分为4组:1)对照组(Control组),加入等体积DMSO溶液(终浓度 $\leq 0.1\%$);2)ITE组,加入AHR激动剂ITE(终浓度为349.27 nmol/L);3)A-485组,加入P300抑制剂A-485(终浓度为93.20 nmol/L);4)ITE+A-485组,同时加入ITE(终浓度为349.27 nmol/L)和P300抑制剂A-485(终浓度为93.20 nmol/L)。各组细胞于37 $^{\circ}$ C、5% CO₂培养箱中培养。培养第3天更换完全培养基(含10% FBS、100 U/mL青霉素、100 μ g/mL链霉素),并重新补加相应诱导条件及药物(DMSO、ITE、A-485或ITE+A-485),继续培养至第5~7天,根据后续实验安排,于不同时间点收集细胞。此外,将SLE患者的CD4⁺T细胞使用CD3/CD28T细胞激活磁珠活化后,分为SLE+DMSO组(加入等体积DMSO)及SLE+ITE组(加入对应浓度的ITE),培养完成后进行后续相关指标检测。

1.4.6 蛋白质印迹法

采用蛋白质印迹法(Western blotting, WB)检测蛋白质表达水平。收集各组细胞,采用细胞裂解液(含1%苯甲基磺酰氟和蛋白酶抑制剂cocktail)提取细胞总蛋白,采用核蛋白提取试剂盒提取核蛋白,用于检测AHR在细胞核中的表达水平。BCA法测定蛋白质浓度。取等量蛋白质样品经10%十二烷基硫酸钠(sodium dodecyl sulfate, SDS)-聚丙烯酰胺凝胶电泳分离,采用湿转法将其转至聚偏氟乙烯膜,室温下封闭1 h,分别加入AHR抗体(1:1 000)、FOXP3抗体(1:1 000)、P300抗体(1:1 000)、 β -actin抗体(1:2 000)及组蛋白H3抗体(1:2 000),于4 $^{\circ}$ C下孵育过夜。经含Tween 20的Tris缓冲盐水洗滌后,加入辣根过氧化物酶标记的二抗(1:5 000),室温下孵育1 h。采用电化学发光法显色,用ImageJ软件分析条带灰度值,以目的蛋白与内参蛋白灰度值的比值表示目的蛋白的

相对表达量。

1.4.7 实时反转录聚合酶链反应

采用TRIzol法提取细胞总RNA,用NanoDrop 2000微量分光光度计测定RNA浓度和纯度。按RNA反转录试剂盒说明书将RNA反转录为互补DNA(complementary DNA, cDNA)。以cDNA为模板,采用SYBR绿色荧光定量试剂盒进行实时反转录聚合酶链反应(real-time reverse transcription PCR, real-time RT-PCR)。以 β -actin为内参基因,采用 $2^{-\Delta\Delta C_t}$ 法计算mRNA的相对表达量。FOXP3正向引物为5'-GTGG-CCCGGATGTGAGAAG-3',反向引物为5'-GGAGC-CCTTGTCTCGATGATG-3'; β -actin正向引物为5'-CATGTACGTTGCTATCCAGGC-3',反向引物为5'-CTCCTTAATGTCACGCACGAT-3'; IL-10正向引物为5'-CATCGATTTCTTCCCTGTGAA-3',反向引物为5'-TCTTGGAGCTTATTAAAGGCATTC-3'; CTLA-4正向引物为5'-CACAAGGCTCAGCTGAACCT-3',反向引物为5'-AGGTGCCCCGTGCAGATGGAA-3'。

1.4.8 细胞转染

将磁珠分选的健康人CD4⁺T细胞以CD3/CD28 T细胞激活磁珠(5 μ L/ 1×10^6 个细胞)预活化24 h,收集细胞,预冷PBS洗涤后,重悬于预冷的Lonza电转缓冲液V(瑞士Lonza公司)中,将其分为空载体组(加入空载体)及AHR+P300共转染组(加入AHR、P300过表达质粒)。轻柔混匀细胞悬液后转移至预冷的电转杯中,采用Lonza 4D-Nucleofector电转仪进行电穿孔。电转后立即加入预温的含20% FBS的RPMI 1640培养基中,轻柔转移至6孔板中,置于37 $^{\circ}$ C、5% CO₂培养箱中继续培养,48 h后收集细胞进行后续实验。

1.4.9 Co-IP

收集转染后的CD4⁺T细胞,预冷PBS洗涤后,加入IP裂解液(含蛋白酶抑制剂cocktail),冰上裂解40 min,4 $^{\circ}$ C、12 000 r/min离心10 min后取上清。分为Anti-P300组、IgG组和Input组。Anti-P300组、IgG组各取200 μ g蛋白质样品,分别加入4 μ g抗P300抗体或抗正常兔IgG; Input组取30 μ g蛋白质样品,于4 $^{\circ}$ C下旋转孵育过夜。各组加入30 μ L Protein A/G磁珠,于4 $^{\circ}$ C下旋转孵育4 h,置于磁力架上吸附磁珠,弃上清,用裂解液洗涤磁珠4次;然后加入2 $\times$ SDS上样缓冲液,于95 $^{\circ}$ C下变性10 min,采用WB检测AHR、P300蛋白的表达水平。

1.4.10 ChIP

收集各组CD4⁺T细胞,加入1%的甲醛,室温下交联10 min,加入0.125 mol/L的甘氨酸终止交联。预冷PBS洗涤后,加入细胞裂解液和细胞核裂解液,

采用超声破碎DNA至200~1 000 bp片段。取50 μ L超声破碎产物作为Input, 其余分为3份, 分别加入目的抗体及同型对照IgG, 于4 $^{\circ}$ C下旋转孵育过夜。加入20 μ L Protein A/G磁珠, 于旋转混匀器上孵育、混匀4~6 h。经洗涤、逆交联及DNA纯化后, 针对*FOXP3*启动子区域设计引物, 扩增-78 bp至-263 bp及-431 bp至-596 bp区段。聚合酶链反应(polymerase chain reaction, PCR)产物经琼脂糖凝胶电泳鉴定, 采用ChIP-PCR验证AHR及P300在*FOXP3*启动子区域的结合位点。采用ChIP-定量PCR(quantitative PCR, qPCR)定量检测*FOXP3*启动子区域AHR、P300结合水平, 以及H3K9ac、H3K14ac乙酰化水平, 以Input样品Ct值进行标准化, 采用Input%法计算富集倍数。*FOXP3*(-78 bp至-263 bp)正向引物为5'-TGGACAGCGGAGGAAGTA-3', 反向引物为5'-CTCAGCAACCTGGAGAAGA-3'; *FOXP3*(-431 bp至-596 bp)正向引物为5'-GTGTTCCCTCTACCCAAAG-3', 反向引物为5'-CCTTAGTGCCTGTGACCT-3'。后续ChIP-qPCR检测均针对*FOXP3*启动子-78 bp至-263 bp区段进行。

1.5 统计学处理

采用GraphPad Prism 11.0统计学软件进行数据分析。计量资料经正态性检验后, 符合正态分布者以均数 $\pm$ 标准差表示。2组间比较时, 方差齐者采用独立样本*t*检验, 方差不齐者采用Welch's *t*检验; 多组间比较采用单因素方差分析, 组间两两比较采用Tukey法。两变量相关性满足正态分布者采用Pearson相关分析, 不满足正态分布或为等级资料者采用Spearman秩相关分析。以 $P<0.05$ 为差异有统计学意义。

2 结果

2.1 SLE患者CD4⁺T细胞中Tfr%及Tfr细胞中FOXP3、AHR表达水平的改变

流式细胞术结果显示, 与健康对照相比, SLE患者CD4⁺T细胞中Tfr%显著降低($P<0.001$, 图2A)。Spearman相关性分析显示, Tfr%与SLEDAI呈负相关($r=-0.803$, $P<0.001$; 图2B)。进一步检测Tfr细胞内FOXP3、AHR蛋白表达水平, 结果显示, 与健康对照相比, SLE患者Tfr细胞中FOXP3、AHR蛋白表达均显著下降(均 $P<0.01$, 图2C、2D)。Pearson相关性分析显示, Tfr细胞内AHR蛋白表达水平与FOXP3蛋白表达水平呈正相关($r=0.596$, $P<0.05$, 图2E)。

2.2 AHR激活上调FOXP3表达及Tfr细胞的分化

CCK-8结果显示, 在0~349.27 nmol/L浓度范围内ITE处理48 h对CD4⁺T细胞增殖活性无明显影响($P>0.05$, 图3A)。WB结果显示, 与Control组相比, ITE组CD4⁺T细胞核蛋白中AHR表达水平明显升高($P<0.01$, 图3B)。real-time RT-PCR结果显示, 与Control组相比, *IL-10*及*CTLA-4* mRNA表达水平显著上调($P<0.05$, 图3C)。流式细胞术结果显示, ITE组较Control组Tfr细胞FOXP3蛋白表达水平及Tfr%均显著升高(均 $P<0.01$, 图3D、3E)。

2.3 AHR招募P300结合至FOXP3启动子区域

Co-IP结果证实, AHR与P300存在蛋白质相互作用(图4A)。进一步ChIP-PCR结果显示, AHR在*FOXP3*启动子区域-78 bp至-263 bp及-431 bp至-596 bp区段存在结合位点(图4B), P300在*FOXP3*启动子区域-78 bp至-263 bp区段存在结合, 而在-431 bp至-596 bp区段未检测到明显结合(图4C), 提示AHR可招募P300结合至*FOXP3*启动子区-78 bp至-263 bp区段。

2.4 AHR激活上调FOXP3启动子区域组蛋白乙酰化并促进FOXP3表达

real-time RT-PCR及WB结果显示, 与Control组相比, ITE组*FOXP3* mRNA及蛋白质表达水平均显著升高(均 $P<0.05$, 图4D、4E)。ChIP-qPCR结果显示, ITE组*FOXP3*启动子区域H3K9ac、H3K14ac乙酰化水平明显升高($P<0.05$), 且AHR及P300在*FOXP3*启动子区域的结合水平均显著增加(均 $P<0.01$, 图4F、4G)。

2.5 AHR介导的FOXP3表达上调部分依赖P300介导的组蛋白乙酰化修饰

CCK-8检测结果显示, P300抑制剂A-485在0~93.20 nmol/L浓度范围内对CD4⁺T细胞的增殖活性无明显影响($P>0.05$, 图5A)。ChIP-qPCR结果显示, 与Control组相比, ITE组AHR及P300在*FOXP3*启动子区域的结合水平均升高(均 $P<0.05$), A-485组P300在*FOXP3*启动子区域的结合水平明显降低($P<0.05$)且AHR结合水平无明显变化($P>0.05$); 与ITE组相比, ITE+A-485组AHR结合水平差异无统计学意义($P>0.05$), 而P300结合水平降低($P<0.05$); 与A-485组相比, ITE+A-485组AHR结合水平明显升高($P<0.001$), P300结合水平无明显变化($P>0.05$, 图5B)。组蛋白乙酰化检测结果显示, 与Control组相比, ITE组H3K9ac及H3K14ac乙酰化水平均显著升高(均 $P<0.001$), A-485组H3K9ac及H3K14ac乙酰化水平均显

著降低(均 $P < 0.05$); 与 A-485 组相比, ITE+A-485 组 H3K9ac 及 H3K14ac 乙酰化水平均无明显差异(均 $P > 0.05$), 而 ITE+A-485 组 H3K9ac 及 H3K14ac 乙酰化水平较 ITE 组均明显下降(均 $P < 0.05$, 图 5C)。WB 结果显示, 与 Control 组相比, ITE 组 FOXP3 蛋白表达水平明显升高($P < 0.001$), A-485 组 FOXP3 蛋白表达水平明显降低($P < 0.001$); 而 ITE+A-485 处理组 FOXP3

蛋白表达水平较 A-485 组明显升高($P < 0.001$), 且低于 ITE 组($P < 0.05$, 图 5D)。流式细胞术结果显示, 与 Control 组相比, ITE 组、ITE+A-485 组 Tfr% 均明显升高(均 $P < 0.05$), A-485 组 Tfr% 明显降低($P < 0.001$), 而 ITE+A-485 组 Tfr% 较 A-485 组升高($P < 0.01$), 但仍低于 ITE 组($P < 0.05$, 图 5E)。

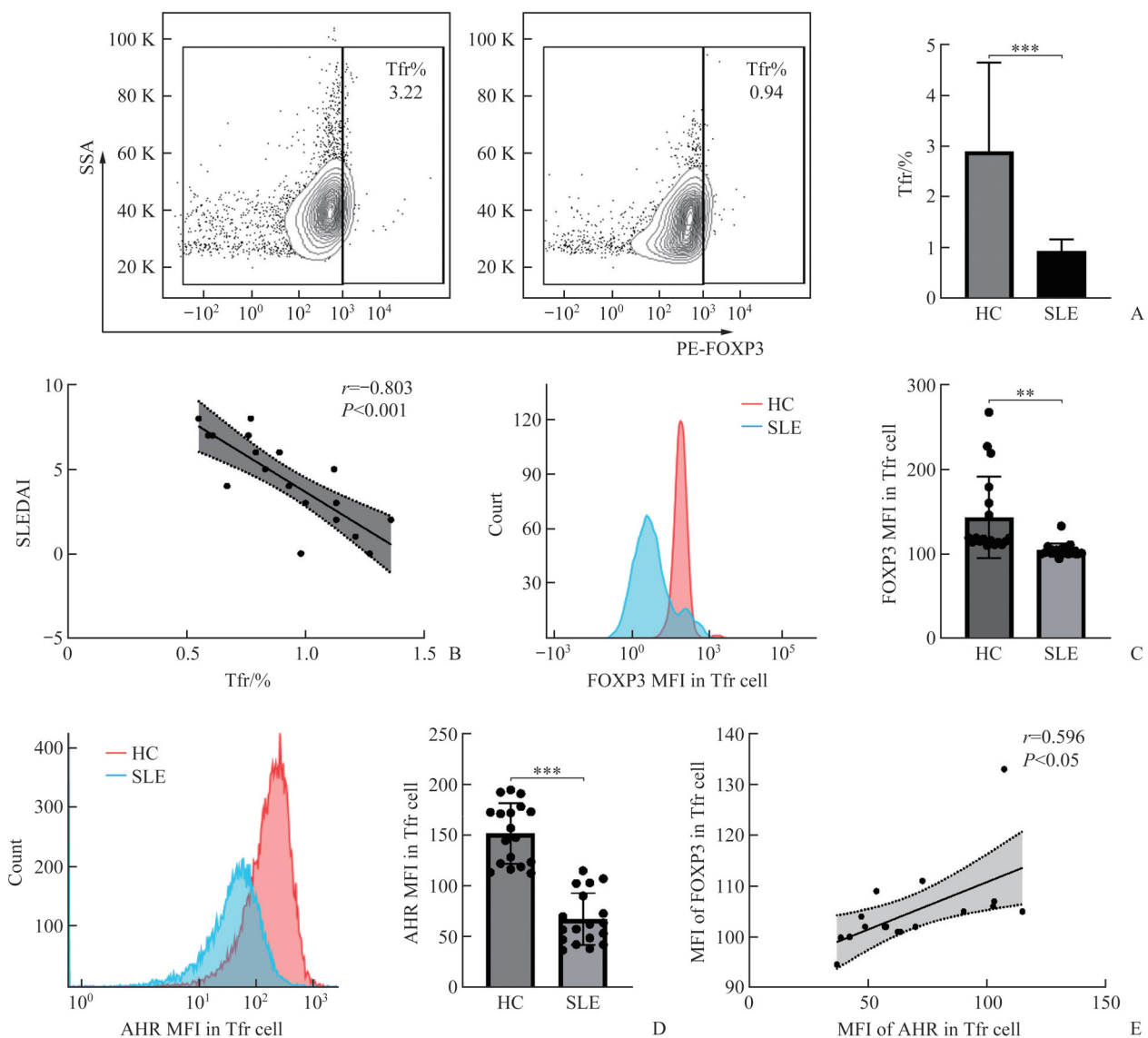


图2 SLE患者与健康对照CD4⁺T细胞中Tfr%及FOXP3、AHR表达差异

Figure 2 Differences in Tfr% and FOXP3 and AHR expression in CD4⁺ T cells between SLE patients and healthy controls

A: Flow cytometry was deployed to evaluate Tfr% in peripheral CD4⁺ T cells from SLE patients and healthy controls. B: Spearman correlation analysis between Tfr frequency and SLEDAI. C and D: Intracellular FOXP3 (C) and AHR (D) protein expression in Tfr cells was assessed by flow cytometry, respectively. E: Pearson correlation between AHR and FOXP3 expression levels within Tfr cells. ** $P < 0.01$, *** $P < 0.001$. Data are presented as mean±standard deviation. Statistical significance was determined using Student's *t*-test, Welch's *t*-test, Pearson correlation analysis or Spearman correlation analysis. $n = 18$. AHR: Aryl hydrocarbon receptor; SLEDAI: Systemic lupus erythematosus disease activity index; HC: Healthy control; Tfr%: Tfr cell proportion; MFI: Median fluorescence intensity.

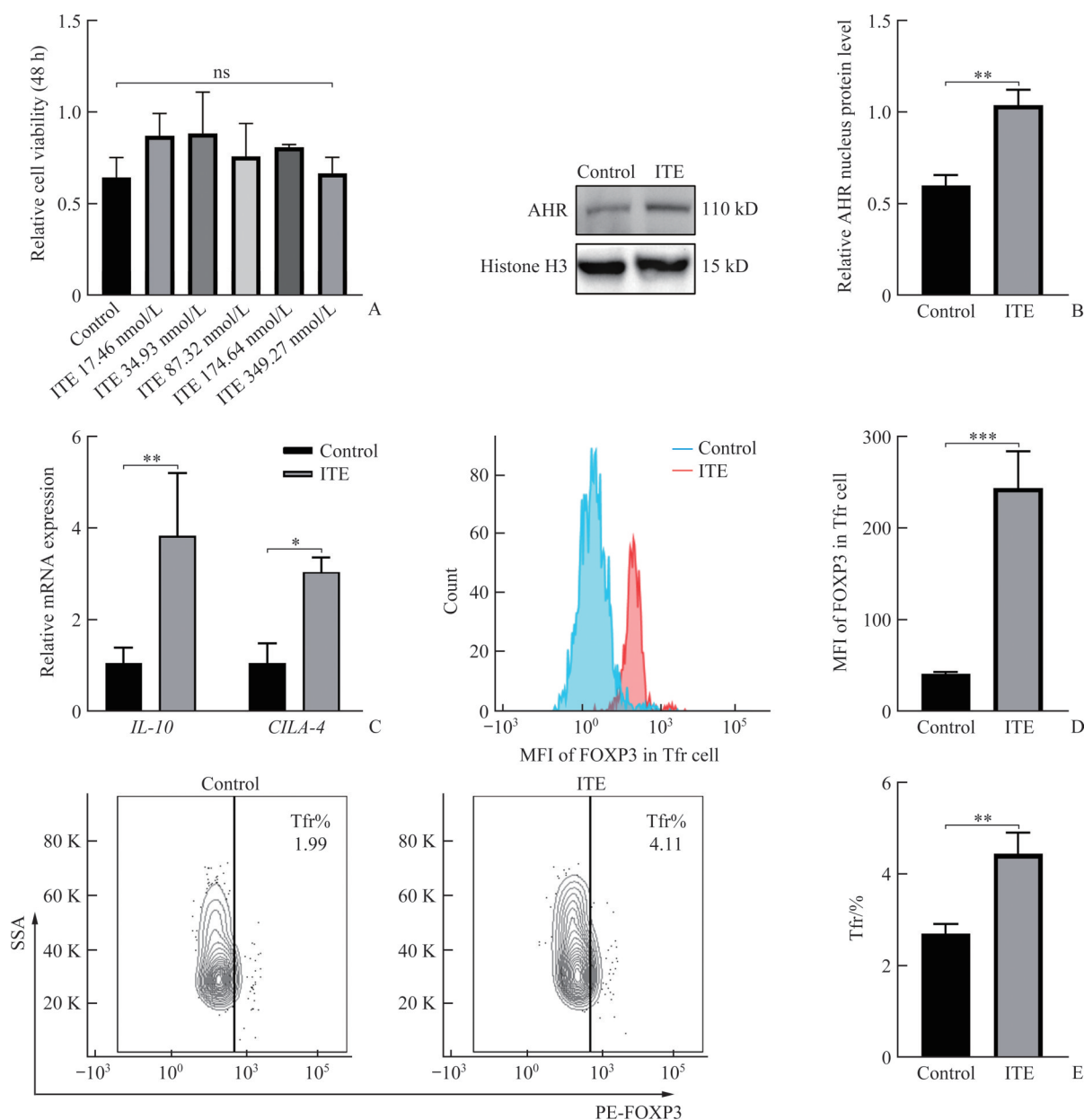


图3 AHR激活健康人CD4⁺T细胞后Tfr%及FOXP3、AHR表达变化

Figure 3 Changes in Tfr% and expression of FOXP3 and AHR following AHR-mediated activation of CD4⁺ T cells in healthy individuals

A: CCK-8 assay was used to evaluate CD4⁺T cell viability following treatment with graded concentrations of ITE. B: Nuclear AHR expression was detected by WB in CD4⁺ T cells from the Control and ITE groups. C: Real-time RT-PCR was used to detect the mRNA expression of *IL-10* and *CTLA-4* from the Control and ITE groups. D: Flow cytometry was employed to assess FOXP3 protein levels within Tfr cells between the Control and ITE groups. E: Flow cytometry was employed to assess Tfr% between the Control and ITE groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are presented as mean $\pm$ standard deviation. Statistical significance was determined using Student's *t*-test or one-way ANOVA. $n = 3$. CTLA-4: Cytotoxic T-lymphocyte associated protein 4; IL-10: Interleukin-10; WB: Western blotting; RT-PCR: Reverse transcription PCR; ITE: 2-(1'-H-Indol-3'-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester; ns: No significance.

2.6 SLE患者CD4⁺T细胞中AHR激活可上调Tfr细胞百分比、FOXP3表达水平及FOXP3启动子区AHR/P300结合水平、乙酰化水平

流式细胞术结果显示, 与SLE+DMSO组相比, SLE+ITE组Tfr%显著上调($P < 0.05$, 图6A), FOXP3

表达水平显著上调($P < 0.01$, 图6B)。ChIP-qPCR结果显示FOXP3启动子区H3K9ac、H3K14ac乙酰化水平及AHR、P300结合水平均显著上调(均 $P < 0.05$, 图6C、6D)。

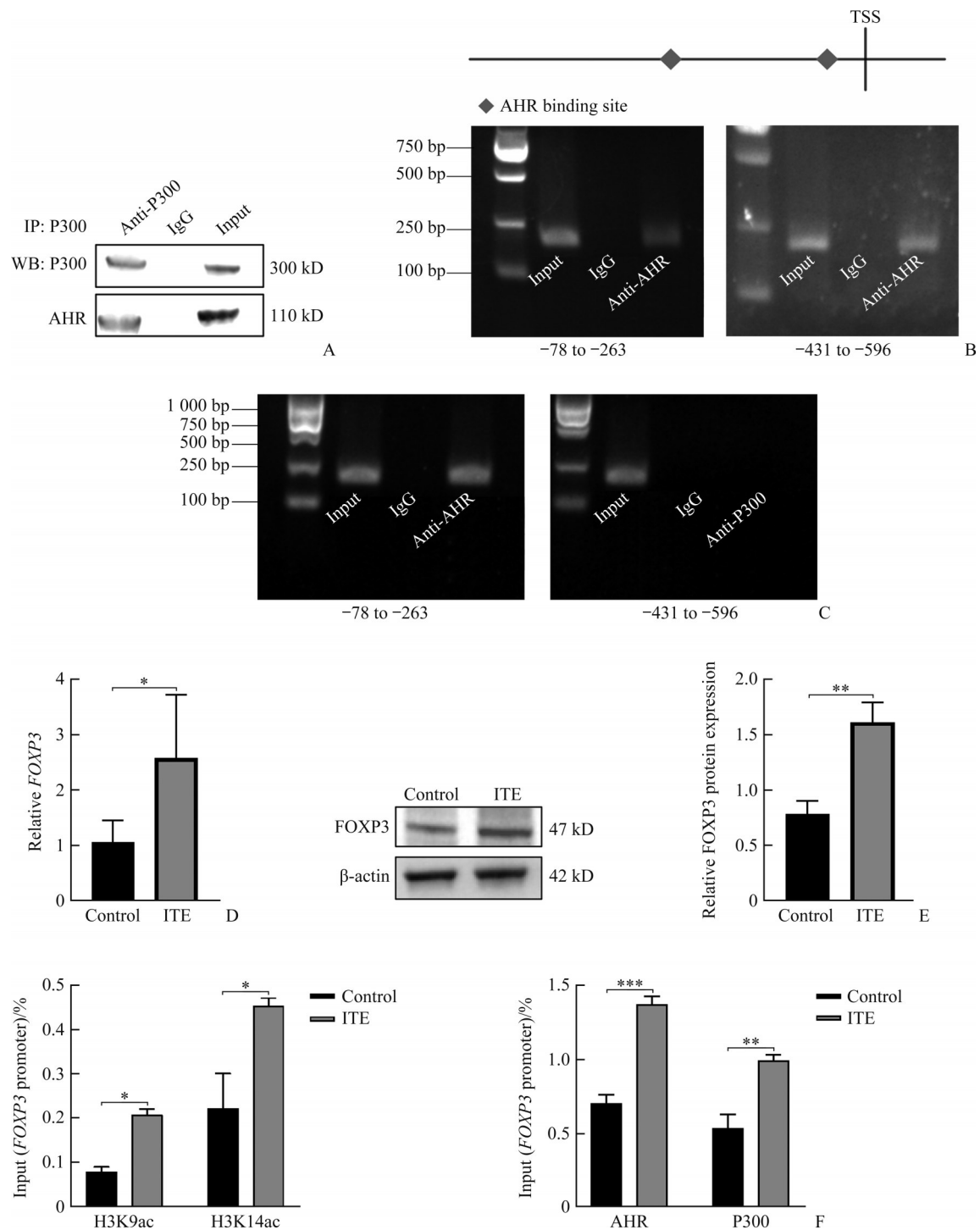


图4 AHR招募P300结合至 $FOXP3$ 启动子区域并调控其乙酰化水平

Figure 4 AHR recruits P300 to the $FOXP3$ promoter region and regulates its histone acetylation levels

A: Co-immunoprecipitation was deployed to verify the physical interaction between AHR and P300 in CD4⁺ T cells. B: Schematic illustration of AHR binding sites on the $FOXP3$ promoter and representative ChIP-PCR gel electrophoresis. C: ChIP-PCR was used to detect P300 binding sites on the $FOXP3$ promoter (-78 bp to -263 bp). D: Real-time RT-PCR was used to detect the $FOXP3$ mRNA expression levels. E: WB was used to detect the FOXP3 protein expression levels. F: ChIP-qPCR was employed to quantify H3K9ac and H3K14ac enrichment at the $FOXP3$ promoter (-78 bp to -263 bp) in CD4⁺ T cells from the DMSO and ITE groups. G: ChIP-qPCR was used to assess AHR and P300 binding levels at the $FOXP3$ promoter (-78 bp to -263 bp) in CD4⁺ T cells from the DMSO and ITE groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are presented as mean $\pm$ standard deviation. Statistical significance was determined using Student's t -test. $n = 3$. AHR: Aryl hydrocarbon receptor; ChIP: Chromatin immunoprecipitation; PCR: Polymerase chain reaction; qPCR: Quantitative PCR; TSS: Transcription start site.

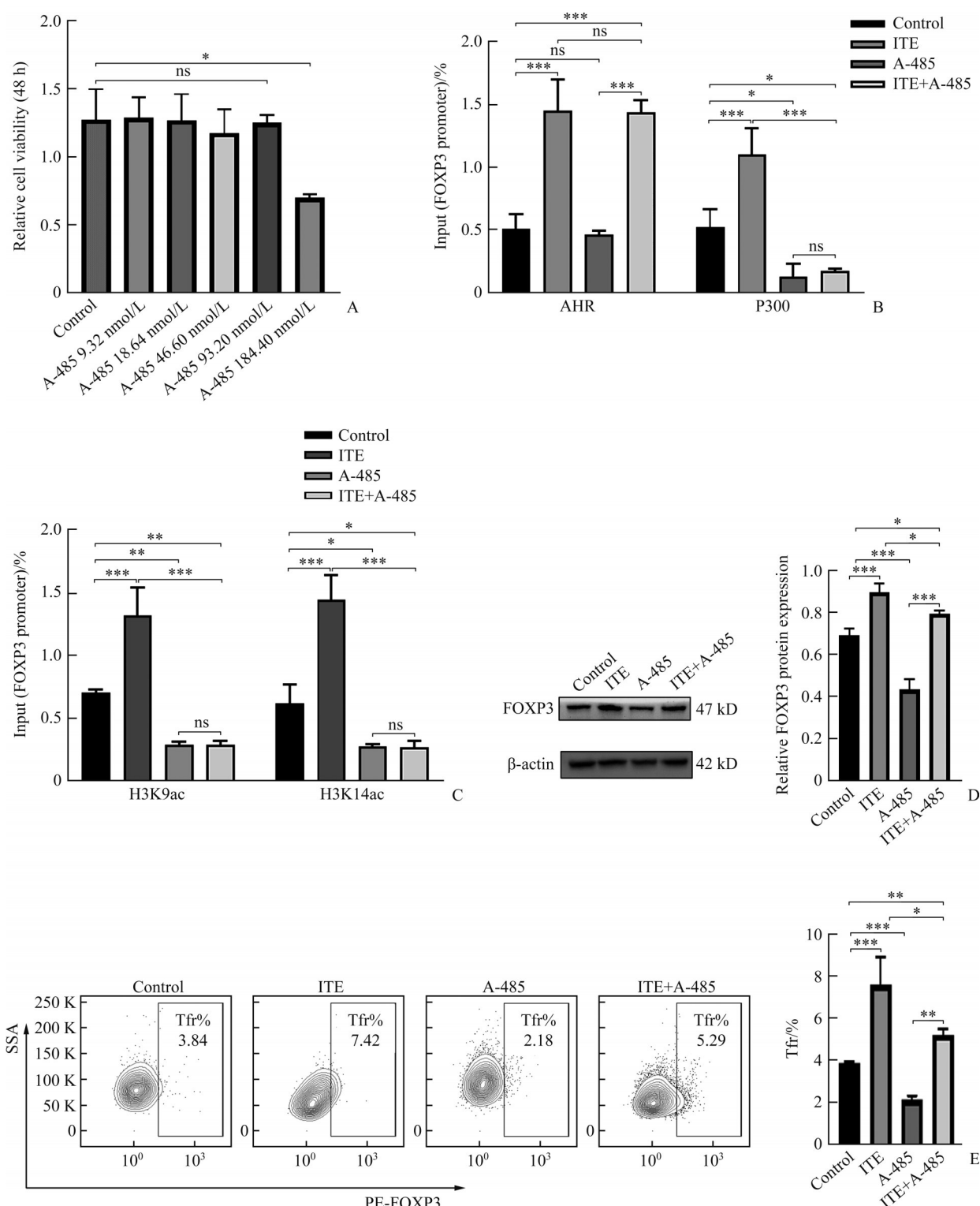


图5 AHR调控FOXP3表达上调部分依赖P300介导的组蛋白乙酰化修饰

Figure 5 AHR-mediated upregulation of FOXP3 expression is partially dependent on P300-mediated histone acetylation

A: CCK-8 assay was deployed to assess CD4⁺ T cell proliferation across A-485 concentrations. B and C: ChIP-qPCR was used to evaluate AHR/P300 binding levels and histone acetylation (H3K9ac/H3K14ac) at the *FOXP3* promoter (−78 bp to −263 bp) in Control, ITE, A-485, and ITE+A-485 groups. D: WB was used to detect FOXP3 protein expression in Control, ITE, A-485 and ITE+A-485 groups. E: Flow cytometry was employed to determine Tfr% in Control, ITE, A-485 and ITE+A-485 groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are presented as mean ± standard deviation. Statistical significance was determined using one-way ANOVA. $n = 3$.

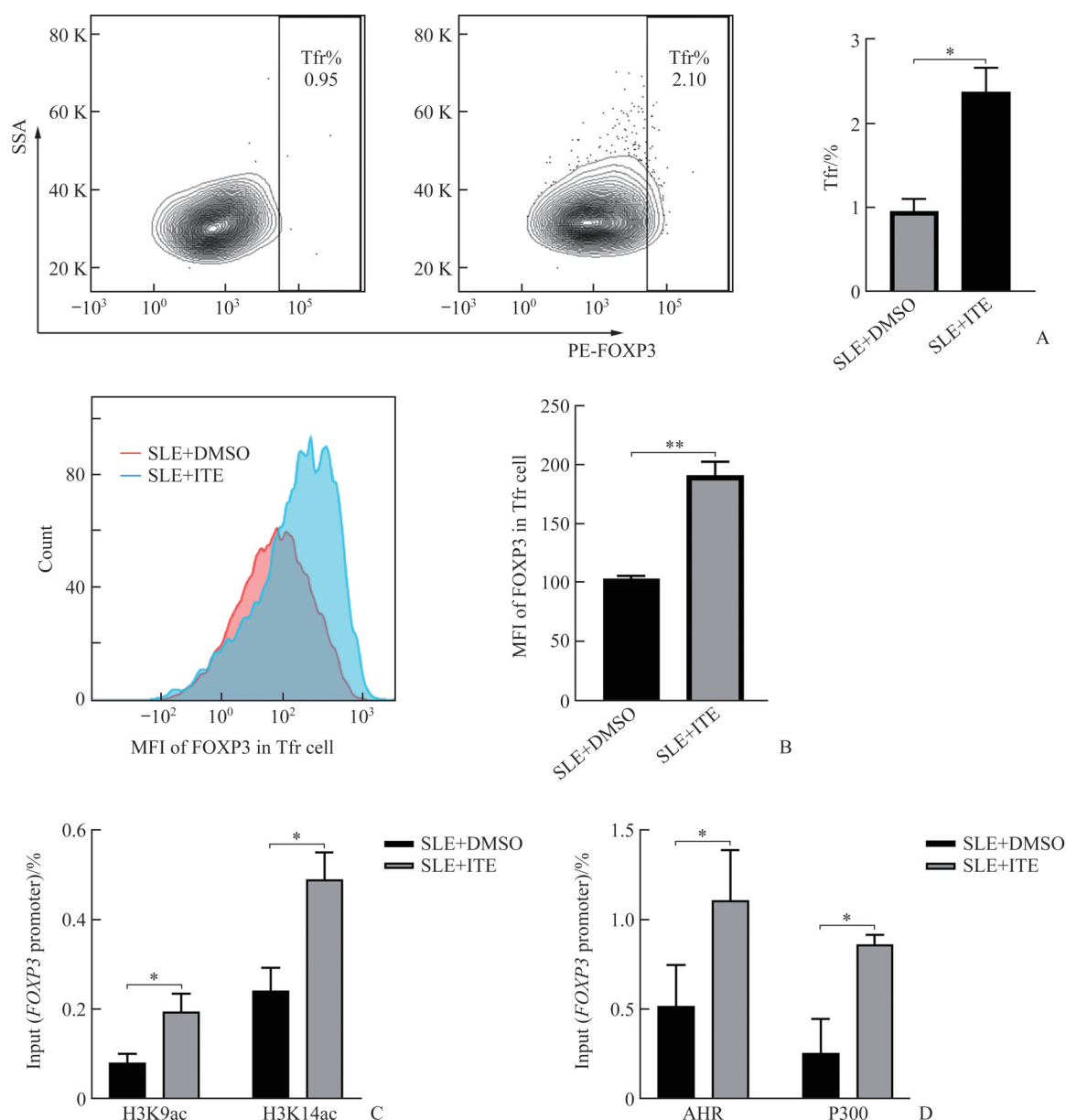


图6 在SLE患者CD4⁺T细胞中进行机制验证

Figure 6 Mechanistic validation in CD4⁺T cells from SLE patients

A: Flow cytometry was used to detect Tfr% in the SLE+DMSO and SLE+ITE groups from SLE patient CD4⁺T cells. B: Flow cytometry was used to detect intracellular FOXP3 expression in the SLE+DMSO and SLE+ITE groups from SLE patient CD4⁺T cells. C: ChIP-qPCR was deployed to quantify H3K9ac and H3K14ac levels at the *FOXP3* promoter (−78 bp to −263 bp). D: ChIP-qPCR was used to evaluate AHR and P300 binding level at the *FOXP3* promoter (−78 bp to −263 bp). **P*<0.05, ***P*<0.01. Data are presented as mean±standard deviation. Statistical significance was determined using Student's *t*-test. *n*=3.

3 讨论

作为调控生发中心反应和维持免疫稳态的关键细胞亚群, Tfr细胞的数量与功能异常在自身免疫性疾病的进展中发挥着重要作用。既往研究^[8]发现, SLE患者的Tfr%降低, 且与疾病活动度、抗双链DNA抗体水平呈负相关。本研究结果显示, 与健康

对照相比, SLE患者外周血CD4⁺T细胞中Tfr%显著降低, 且与SLEDAI呈显著负相关, 与上述报道一致。然而有研究^[19]发现SLE患者外周血Tfr%高于健康对照, 这种差异可能与Tfr细胞定义、患者人群特征(如疾病活动度)及样本量等因素有关, 该研究采用CD4⁺CXCR5⁺FOXP3⁺作为Tfr细胞表型定义, 而本研究采用CD4⁺CXCR5⁺PD-1⁺FOXP3⁺作为Tfr细胞表型

定义,可能更准确地识别具有滤泡定位能力的Tfr细胞。FOXP3作为Tfr细胞分化和功能维持的核心转录因子,其表达下调被认为是Tfr功能受损的重要原因。本研究观察到SLE患者Tfr细胞中FOXP3表达降低,同时AHR表达亦下降,且二者呈正相关,提示AHR信号通路异常可能作为上游机制参与调控FOXP3表达。

AHR为配体依赖性转录因子,参与调控多种免疫细胞的分化与功能。Ehrlich等^[20]报道,高剂量AHR配体可诱导FOXP3⁺Treg细胞分化,而低剂量则促进Th17细胞分化,提示配体剂量可能是影响AHR功能的因素之一。同样有研究^[21]发现,AHR激动剂可降低流感病毒感染小鼠的Tfh细胞比例,同时改变Tfr/Tfh,表明AHR激活对滤泡T细胞具有一定调控作用。本研究通过体外实验发现,ITE激活CD4⁺T细胞AHR后,Tfr细胞内FOXP3蛋白表达上调且Tfr%升高,提示在本实验条件下,ITE介导的AHR活化可促进免疫耐受。不同配体类别、剂量及实验模型均可影响AHR对免疫系统的调控作用,未来还需进一步探讨不同AHR调节剂对SLE患者Tfr细胞的影响。

组蛋白乙酰化等表观遗传修饰在SLE发病机制中发挥重要作用。研究^[22-23]发现活动期SLE患者外周血CD4⁺T细胞中组蛋白H3和H4整体乙酰化水平明显降低,且H3乙酰化程度与SLEDAI呈负相关,SLE患者B细胞中组蛋白H3和H4同样呈低乙酰化状态。这提示组蛋白乙酰化水平下调可能参与SLE的免疫失衡。本研究对特定基因启动子区域的分析发现,AHR激活后可特异性升高FOXP3启动子区域H3K9ac和H3K14ac的乙酰化水平,为理解SLE中FOXP3表达下调的表观遗传机制提供了新的视角。本研究通过Co-IP及ChIP-qPCR实验证实,CD4⁺T细胞中AHR与P300存在蛋白质相互作用,AHR可招募P300至FOXP3启动子区域,促进组蛋白乙酰化修饰,并在SLE患者CD4⁺T细胞中验证了上述机制。虽然因患者血液样本不足这一客观原因限制,无法在Tfr细胞层面进行乙酰化水平等验证,但仍可提示AHR-P300-FOXP3轴在SLE患者Tfr细胞调控中的重要作用。

FOXP3的转录、翻译及翻译后修饰受到多种机制调控。已有研究^[24]表明,AHR可降低FOXP3的泛素化和降解水平,稳定FOXP3表达,从而促进Treg细胞分化。本研究中,P300抑制剂A-485仅能部分逆转ITE诱导的FOXP3高表达,证实AHR介导的FOXP3表达上调部分依赖于P300介导的组蛋白乙酰化修饰,说明AHR除组蛋白乙酰化修饰途径外,还

可能通过泛素化修饰等多条通路共同维持FOXP3稳态,具体机制有待后续研究进一步阐明。

综上所述,AHR协同P300介导的FOXP3组蛋白乙酰化修饰在SLE患者Tfr细胞的分化过程中发挥了正向调控作用,且AHR调控Tfr细胞分化部分依赖于P300介导的组蛋白乙酰化。然而,本研究仍存在一定局限性,如未进行体内实验验证,未来需在SLE动物模型中进一步验证,且样本量相对有限,未来需扩大样本量以提高结果可信度。此外,不同AHR配体在免疫调控中的差异性作用尚未被系统比较。尽管如此,该发现仍为理解SLE免疫调控异常提供了新的研究思路。

作者贡献声明:任睿健 实验操作,数据分析,论文撰写;饶钰 数据分析,论文撰写与修改;胡凌雪、刘宇航、贺治杰 实验操作,数据采集;丁澍 研究设计,论文指导与修改。所有作者阅读并同意最终的文本。

利益冲突声明:作者声称无任何利益冲突。

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(责任编辑 吴旭芳)