



• 糖尿病与代谢综合征 •

高糖诱导的氧化应激通过促进Teff细胞凋亡和Treg细胞分化 加剧糖尿病免疫抑制*

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【摘要】 目的 探讨血糖控制不良的糖尿病后期CD4⁺ Foxp3⁺调节性T细胞(Treg细胞)比例升高的原因及机制,以期为预防和治疗糖尿病后期的继发感染寻找新的思路和治疗靶点。**方法** 将6~8周龄的野生型C57BL/6小鼠随机分为两组(实验组和对照组,每组5只),实验组通过注射链脲佐菌素(STZ)构建1型糖尿病小鼠,对照组注射等体积0.1 mol/L Citrate buffer;用高脂饲料饲喂6~8周龄的野生型C57BL/6小鼠2个月后将小鼠随机分为两组(实验组和对照组,每组3只),实验组通过少量多次注射STZ构建小鼠2型糖尿病模型,对照组注射等体积0.1 mol/L Citrate buffer。在小鼠糖尿病稳定发病2周后,收取小鼠的脾脏和外周淋巴结,通过流式细胞术检测其T细胞免疫反应。通过免疫磁珠分选幼稚T细胞进行原代培养,研究高糖调控T细胞分化和功能的机制。将小鼠组织单细胞悬液和T细胞培养后的样本,通过多色流式细胞术检测Treg细胞、效应T细胞(Teff细胞)的比例,以及细胞增殖标志物Ki67、细胞凋亡率、胞内活性氧(ROS)的表达水平。**结果** 在血糖控制不良的1型糖尿病和2型糖尿病小鼠发病后期,外周CD4⁺ Foxp3⁺ Treg细胞比例升高($P<0.05$);同时,这些糖尿病小鼠中Treg细胞的Ki67表达上调($P<0.01$),而非Treg细胞(Foxp3⁻ non-Treg cells)的凋亡比例升高($P<0.05$);Teff细胞在高糖处理条件下ROS的产生增加,细胞凋亡率升高($P<0.05$);高糖处理诱导了转化生长因子- β (TGF- β)的活化,并促进了Treg细胞的分化增加,阻断TGF- β 信号通路或清除ROS均能完全抑制高糖诱导的Treg细胞分化($P<0.01$)。**结论** 糖尿病中失控的高糖内环境通过诱导Teff细胞发生氧化应激产生高水平的ROS,从而引起Teff细胞的凋亡增加,并通过活化TGF- β 促进了Treg细胞的分化,最终引起糖尿病后期免疫抑制内环境的加剧。抑制晚期糖尿病患者的ROS水平,可能有利于抑制Teff细胞的凋亡和Treg细胞比例的升高,并有望为改善糖尿病后期由高血糖引起的免疫抑制和继发感染提供新的视角。

【关键词】 糖尿病 活性氧 T淋巴细胞,调节性 细胞凋亡 转化生长因子 β 高血糖症

Oxidative Stress Induced by High Glucose Aggravates Immunosuppression in Diabetes Mellitus by Promoting Effector T Cell Apoptosis and Regulatory T Cell Differentiation

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[Abstract] Objective To explore the regulatory mechanisms underlying the increased proportion of CD4⁺ Foxp3⁺ regulatory T (Treg) cells in late-stage diabetes mellitus (DM) with poorly-controlled blood glucose, and to identify new approaches and therapeutic targets for the prevention and treatment of secondary infections in the late stage of DM. **Methods** Wild-type C57BL/6 mice aged 6 to 8 weeks were randomly assigned to two groups, the experimental and the control groups ($n = 5$ per group). Mice in the experimental group were injected with streptozotocin (STZ) to induce the mouse model of type 1 diabetes mellitus (T1D), while those in the control group received injection of an equal volume of 0.1 mol/L citrate buffer. In addition, wild-type C57BL/6 mice aged 6 to 8 weeks were fed with high-fat diet for 2 months and subsequently randomly assigned to two groups, the experimental and the control groups ($n = 3$ per group). Mice in the experimental group were injected with low-dose STZ for multiple times to induce the mouse model of type 2 diabetes mellitus (T2D), while those in the control group received an equal volume of 0.1 mol/L citrate buffer. The spleen and peripheral lymph nodes of the mice were collected 2 weeks after the stable onset of diabetes, and T cell immune responses were examined by flow cytometry. Naive T cells isolated by immunomagnetic beads were cultured to investigate the mechanisms by which high glucose regulates T cell differentiation and function. The frequency of Treg cells and effector T (Teff) cells, the expression levels of Ki67, a cell proliferation marker, cell apoptosis rate, and intracellular reactive oxygen species (ROS) levels in the mouse tissue single cell suspension and T cell culture samples were assessed by multicolor flow

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cytometry. **Results** Late-stage T1D and T2D mice with poorly-managed blood glucose exhibited increased peripheral CD4⁺ Foxp3⁺ Treg frequencies ($P < 0.05$). In these diabetic mice with poorly-managed blood glucose, the expression of Ki67 in Treg cells was significantly upregulated ($P < 0.05$), while the apoptosis of non-Treg cells (Foxp3⁻ non-Treg cells) increased markedly ($P < 0.05$). Under high-glucose treatment conditions, the ROS levels in Teff cells increased significantly, and the cell apoptosis also increased significantly. High-glucose treatment induced the activation of transforming growth factor- β (TGF- β) and promoted the differentiation of Treg cells, whereas blocking the TGF- β signaling pathway or neutralizing ROS completely inhibited high glucose-induced Treg differentiation ($P < 0.01$). **Conclusion** Sustained hyperglycemic internal environment in poorly-controlled diabetic mice causes high level of ROS production in Teff cells by inducing oxidative stress, which leads to increased apoptosis of Teff cells, promotes the differentiation of Treg cells by activating TGF- β , and ultimately leads to exacerbated immunosuppressive environment in the late stages of DM. Inhibiting the high level of ROS in late-stage diabetic patients may be conducive to mitigating Teff apoptosis and increasing the frequencies of Treg cells, and may offer new perspectives for improving hyperglycemia-induced immunosuppression and secondary infections in the late stage of DM.

[Key words] Diabetes mellitus Reactive oxygen species T-Lymphocytes, regulatory Apoptosis Transforming growth factor beta Hyperglycemia

糖尿病是全球增长最快的疾病之一,预计到2045年患病人数将达到6.93亿^[1]。各种严重的并发症是晚期糖尿病患者死亡的主要原因之一^[2]。研究发现,高血糖可通过影响先天免疫反应和适应性免疫反应,增加患者对病毒、细菌和真菌感染的易感性,从而引发比非糖尿病患者更加严重的感染,如软组织感染(糖尿病足)、尿路感染、皮肤感染、呼吸道感染、肺炎等^[2-3]。因此,研究糖尿病和高糖内环境如何损害保护性免疫反应,对于预防和治疗晚期患者的继发感染具有重大意义。

CD4⁺ Foxp3⁺调节性T细胞(Treg细胞)在维持免疫稳态中起着关键作用^[4]。既往的研究认为,糖尿病后期的Treg细胞比例增加可能是导致晚期糖尿病患者免疫功能障碍的主要原因之一^[5-6]。然而,糖尿病后期Treg细胞比例增加的原因和机制仍不清楚,限制了该领域的深入研究和转化应用。因此,本研究通过探讨高糖内环境调控Treg细胞比例升高的调节机制,以期为预防和治疗糖尿病后期的继发感染提供潜在治疗靶点。

1 材料与方法

1.1 主要药物和试剂

链脲佐菌素(STZ)(纯度 $\geq 98\%$,美国Sigma公司);转化生长因子- $\beta 1$ (TGF- $\beta 1$)和未活化的TGF- $\beta 1$ 前体LAP-TGF- $\beta 1$ (美国R&D Systems公司);高脂饮食(北京小鼠友泰生物科技有限公司);葡萄糖、他莫昔芬和葵花籽油(美国Sigma公司);无葡萄糖DMEM(美国Thermo Fisher Scientific公司);0.1 mol/L Citrate buffer(pH=4.5)(中国Solarbio公司);anti-mouse CD3、anti-mouse CD28和anti-TGF- β (美国Bio X Cell公司);N-乙酰-L-半胱氨酸(NAC)(中国,Medchemexpress公司);mouse CD4⁺ CD62L⁺ T Cell Isolation Kit和mouse Regulatory T Cell Isolation Kit(美国Miltenyi Biotec公司);Zombie Yellow Fixable Viability

Kit(美国BioLegend公司);豆蔻酰佛波醇乙酯(PMA)(美国Sigma公司);Ionomycin calcium salt(上海阿拉丁生化科技有限公司);Golgi-Plug Protein Transport Inhibitor和Cytofix/Cytoperm Fixation/Permeabilization Solution Kit(美国BD Biosciences公司)。rh Annexin V Pacific Blue™、Annexin V Binding Buffer、Anti-mouse CD4 PerCP-Cy5.5 (RM4-5)、Anti-mouse CD45.2 APC-eFluor 780 (104)、Anti-mouse TCR β APC-eFluor 780 (H57-597)、Anti-mouse IL-17A PE-Cy7 (eBio17B7)、Anti-mouse IFN gamma eFluor 450 (XMG1.2)、Anti-mouse FOXP3 eFluor 450 (FJK-16 s)、Anti-mouse IL-10 APC (JES5-16E3)、Anti-mouse IL-4 PE (11B11)、Ki67 Monoclonal Antibody (SolA15) PE-Cy7、Foxp3/Transcription Factor Staining Buffer Set(美国Thermo Fisher Scientific公司)。

1.2 实验动物

6~8周龄的雌性或雄性C57BL/6小鼠购于上海南方模式生物科技股份有限公司,6~8周龄的*Tgfbri*^{ff}和ER-Cre小鼠分别购于上海南方模式生物科技股份有限公司和The Jackson Laboratory,经繁育得到*Tgfbri*^{ff} ER-Cre小鼠(用于实验时小鼠为6~8周龄)。上述小鼠均饲养于四川大学实验动物中心和四川大学华西医院实验动物中心,放置于温度22℃、12 h光照/12 h黑暗的SPF级屏障环境,允许自由饮水及摄食。本研究经四川大学华西医院动物伦理委员会批准(批准号:20220301143)。动物实验样本量依据本课题预实验研究,并参考同类研究中的常用样本量^[7-8],确定为每组3~5只;单次实验使用周龄一致、体质量差异在10%以内的雌性C57BL/6小鼠;剔除未能成功建模的小鼠(累计剔除2只小鼠);将建模组(实验组)和对照组小鼠同笼饲养,单次实验的多笼小鼠笼位相邻放置,以最小化潜在混杂因素的影响;实验未采用盲法,所有实验人员均知晓小鼠的分组情况,以正确注射药

物、监测小鼠的糖尿病发病情况并进行照护。

1.3 动物实验

1.3.1 STZ诱导的1型糖尿病(T1D)模型

利用基于计算机的随机顺序生成器将6~8周龄雌性C57BL/6小鼠随机分为两组($n=5$), 实验组于空腹8 h后单次腹腔注射220 mg/kg STZ, 对照组注射等体积的0.1 mol/L Citrate buffer(pH=4.5), 随后每周两次监测小鼠的血糖和体质量, 当小鼠连续两次随机血糖值 ≥ 16.7 mmol/L, 并出现多食、多饮、多尿和体质量减轻现象, 即鉴定为糖尿病稳定发病并纳入实验样本, 否则剔除在外^[7]。本实验模型小鼠一般在STZ注射后3 d左右发病。待糖尿病稳定发病2周后, 利用颈椎脱臼法处死小鼠, 收取小鼠脾脏、外周淋巴结制备成单细胞悬液用于流式细胞术进行免疫学分析。

1.3.2 STZ诱导的2型糖尿病(T2D)模型

将6~8周龄雌性C57BL/6小鼠饲喂高脂饲料(60 kcal% Fat)约2个月, 至其体质量达到初始体质量的2倍左右, 利用基于计算机的随机顺序生成器将其随机分为两组($n=3$)。实验组每隔3 d腹腔注射一次30 mg/kg STZ, 连续注射两次, 对照组注射等体积的0.1 mol/L Citrate buffer(pH=4.5), 并每周两次对其血糖和体质量进行监测^[9]。剔除标准同1.3.1。待糖尿病稳定发病2周后, 利用颈椎脱臼法处死小鼠, 收取小鼠脾脏、外周淋巴结制备成单细胞悬液用于流式细胞术进行免疫学分析。

1.4 原代T细胞培养

1.4.1 总脾脏细胞的培养实验

在无葡萄糖的DMEM中加入5.5、25或100 mmol/L葡萄糖, 配制完全DMEM培养基。将C57BL/6小鼠脾脏取出并制成单细胞悬液, 重悬于上述3种完全DMEM培养基中, 并置于24孔板(0.5×10^6 /孔), 加入anti-CD3($0.5 \mu\text{g/mL}$), 在37℃、体积分数为5% CO₂培养箱中培养24 h用于凋亡检测, 或培养3 d用流式细胞术分析CD4⁺ Foxp3⁺ Treg细胞和CD4⁺ IL-10⁺ T细胞(type 1 regulatory T cell, Tr1细胞)的分化情况。

1.4.2 Treg细胞和效应T细胞(Teff细胞)的培养和活性氧检测

将C57BL/6小鼠脾脏和外周淋巴结取出并制成单细胞悬液, 使用mouse CD4⁺ CD62L⁺ T cell Isolation Kit和mouse Regulatory T Cell Isolation Kit分选出Treg细胞和幼稚T细胞(naïve T细胞), 重悬于1.4.1中所述的3种完全DMEM培养基, 并置于anti-CD3($1.5 \mu\text{g/mL}$)包被的48孔板(2×10^5 /孔)中, 加入anti-CD28($1.5 \mu\text{g/mL}$), 在37℃、体积分数为5%的CO₂培养箱中激活培养24 h, 然后加入H2DCFDA($5 \mu\text{mol/L}$)孵育30 min, 并进行流式细胞术分析活性氧(ROS)产生情况。

1.4.3 小鼠脾脏T细胞和总脾脏细胞的凋亡检测

将稳定发病2周的T1D小鼠的脾脏取出并裂解红细胞, 制成单细胞悬液; 或将C57BL/6小鼠的总脾脏细胞裂解红细胞后在1.4.1中所述的3种完全DMEM培养基中培养24 h并收取培养后的细胞。然后, 加入100 μL 含有Annexin V及Zombie Yellow的试剂工作液(1:100稀释), 室温避光孵育20 min并行流式细胞术检测细胞凋亡情况。

1.4.4 Treg细胞诱导分化实验

将6~8周龄的雌性或雄性C57BL/6小鼠, 或*Tgfbri*^{-/-}(他莫昔芬处理的*Tgfbri*^{fl/fl}ER-Cre小鼠)和*Tgfbri*^{+/+}小鼠(葵花籽油处理的*Tgfbri*^{fl/fl}ER-Cre小鼠)脾脏和外周淋巴结取出并制成单细胞悬液, 使用mouse CD4⁺ CD62L⁺ T cell Isolation Kit分选出幼稚T细胞(naïve T细胞), 重悬于1.4.1中所述的3种完全DMEM中, 并置于 $1.5 \mu\text{g/mL}$ anti-CD3包被的24孔板(0.5×10^6 /孔), 加入anti-CD28($1.5 \mu\text{g/mL}$)、TGF- β 1(2 ng/mL)、LAP-TGF- β 1(10 ng/mL)、anti-TGF- β ($50 \mu\text{g/mL}$)、SB431542($5 \mu\text{mol/L}$)或NAC(10 mmol/L), 在37℃、体积分数为5%的CO₂培养箱中培养3 d后, 收集细胞进行流式细胞术分析Treg细胞的分化情况。

1.5 多色流式细胞术

用Zombie Yellow Fixable Viability Kit标记死细胞, 室温避光孵育10 min。对于细胞表面染色, 将流式抗体与细胞在4℃孵育20 min。对于细胞内细胞因子染色, 表面染色前用含有高尔基体阻断剂(1:1000稀释)、PMA(5 ng/mL)和离子霉素(250 ng/mL)的完全DMEM培养基, 在细胞培养箱中培养3~4 h, 表面染色后使用BD Cytofix/Cytoperm固定/打孔试剂盒处理细胞20 min, 最后用细胞因子抗体孵育。对于转录因子染色, 在表面染色后, 用Foxp3/转录因子固定/打孔试剂盒处理1 h, 再用转录因子抗体进行孵育。对T细胞进行总ROS检测时, 收集细胞并用含 $5 \mu\text{mol/L}$ H2DCFDA的PBS溶液在37℃避光孵育25 min。样本用BD LSRFortessa或Beckman CytoFLEX S进行检测, 数据使用FlowJo 10.6.2软件进行分析。

1.6 统计学方法

使用GraphPad Prism 9对数据进行统计学分析和作图。数值以 $\bar{x} \pm s$ 表示。除非另有说明, 两组间的比较采用非配对双尾Student's *t*检验, 两组以上的比较采用单因素方差分析(并采用Tukey's多重比较检验)。 $\alpha=0.05$ 。

2 结果

2.1 血糖控制不良的晚期T1D小鼠Treg细胞比例升高

T1D模型稳定发病2周后, 利用流式细胞术检测对照组和T1D小鼠脾脏和外周淋巴结的T细胞免疫反应。结

果显示(图1A~1C),与对照组相比,T1D小鼠外周淋巴结中 $CD4^+ Foxp3^+$ Treg细胞的比例升高,同时, $CD4^+ Foxp3^-$ 非Treg细胞(主要为效应T细胞、记忆T细胞和幼稚

T细胞)比例则降低。而同样具有免疫抑制功能的Tr1细胞在T1D小鼠脾脏中的比例也升高(图1D、1E)。不仅如此,T1D小鼠中Treg细胞与分泌炎症细胞因子IL-17A的

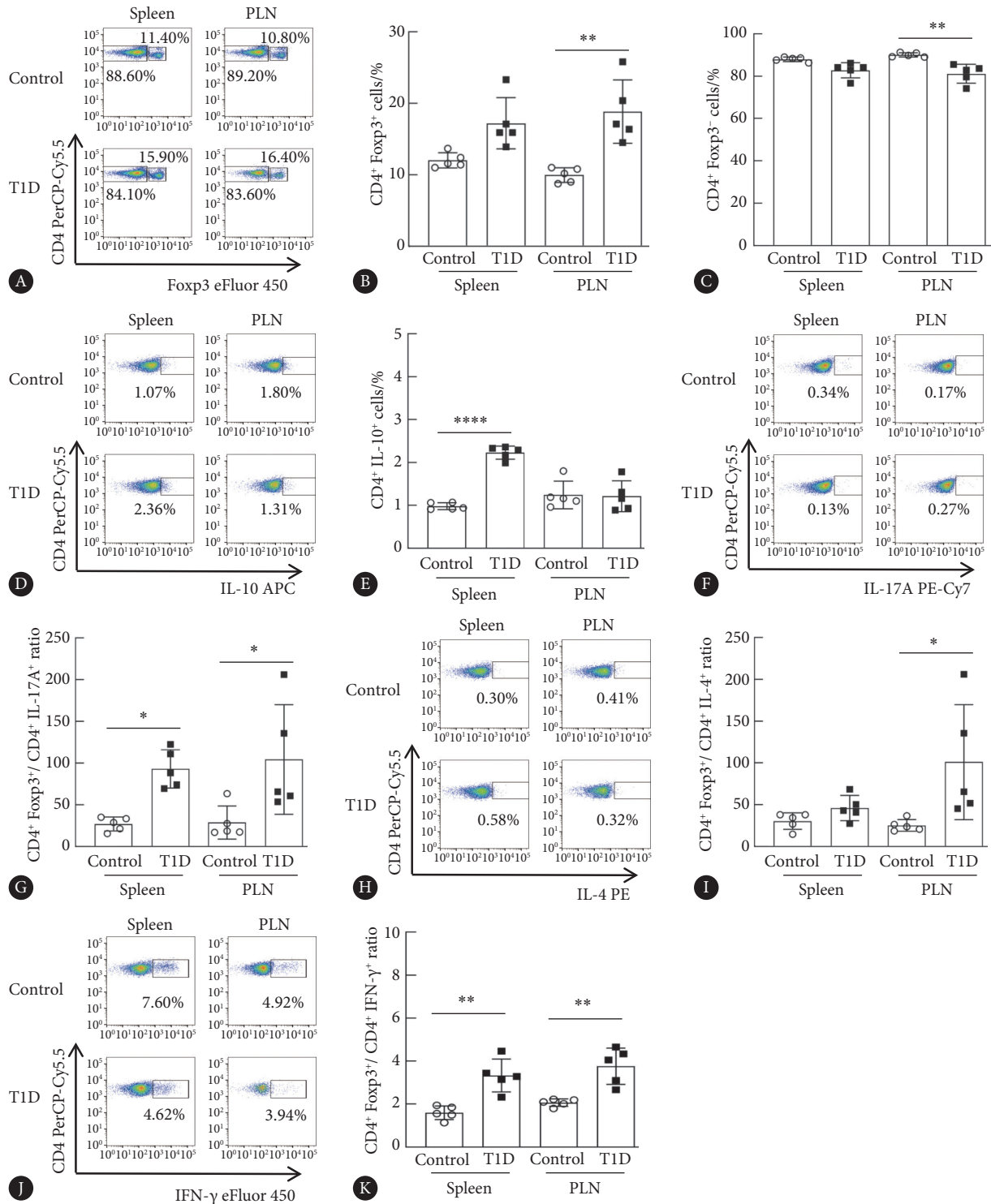


图 1 血糖控制不良的晚期T1D小鼠中Treg细胞比例升高

Fig 1 The frequency of Treg cells increased in the late-stage T1D mice with poorly-controlled blood glucose

A-E, Frequencies of $CD4^+ Foxp3^+$ regulatory T (Treg) cells (A and B), $CD4^+ Foxp3^-$ non-Treg cells (C), $CD4^+ IL-10^+$ T (Tr1) cells (D and E) in the spleen and peripheral lymph nodes (PLN). F, H, and J, Frequencies of $CD4^+ IL-17^+$ T (Th17) cells, $CD4^+ IL-4^+$ T (Th2) cells, and $CD4^+ IFN-\gamma^+$ T (Th1) cells. G, I, and K, Ratios of Treg cell frequency to indicated Teff cell frequency. The data are representative of three independent experiments ($n = 5$ mice per group). * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

Th17细胞的相对比值,与分泌IL-4的Th2细胞的相对比值,以及分泌IFN- γ 的Th1细胞的相对比值,均明显增大(图1F~1K)。证明在血糖控制不良的晚期T1D小鼠中,T细胞免疫受到了Treg细胞的明显抑制。

2.2 血糖控制不良的晚期T2D小鼠Treg细胞比例升高

T2D模型稳定发病2周后,流式细胞术检测(图2A~2C)发现,在T2D小鼠脾脏中,CD4⁺ Foxp3⁺ Treg细胞比例同样出现升高,同时,CD4⁺ Foxp3⁻非Treg细胞比例则降低,而

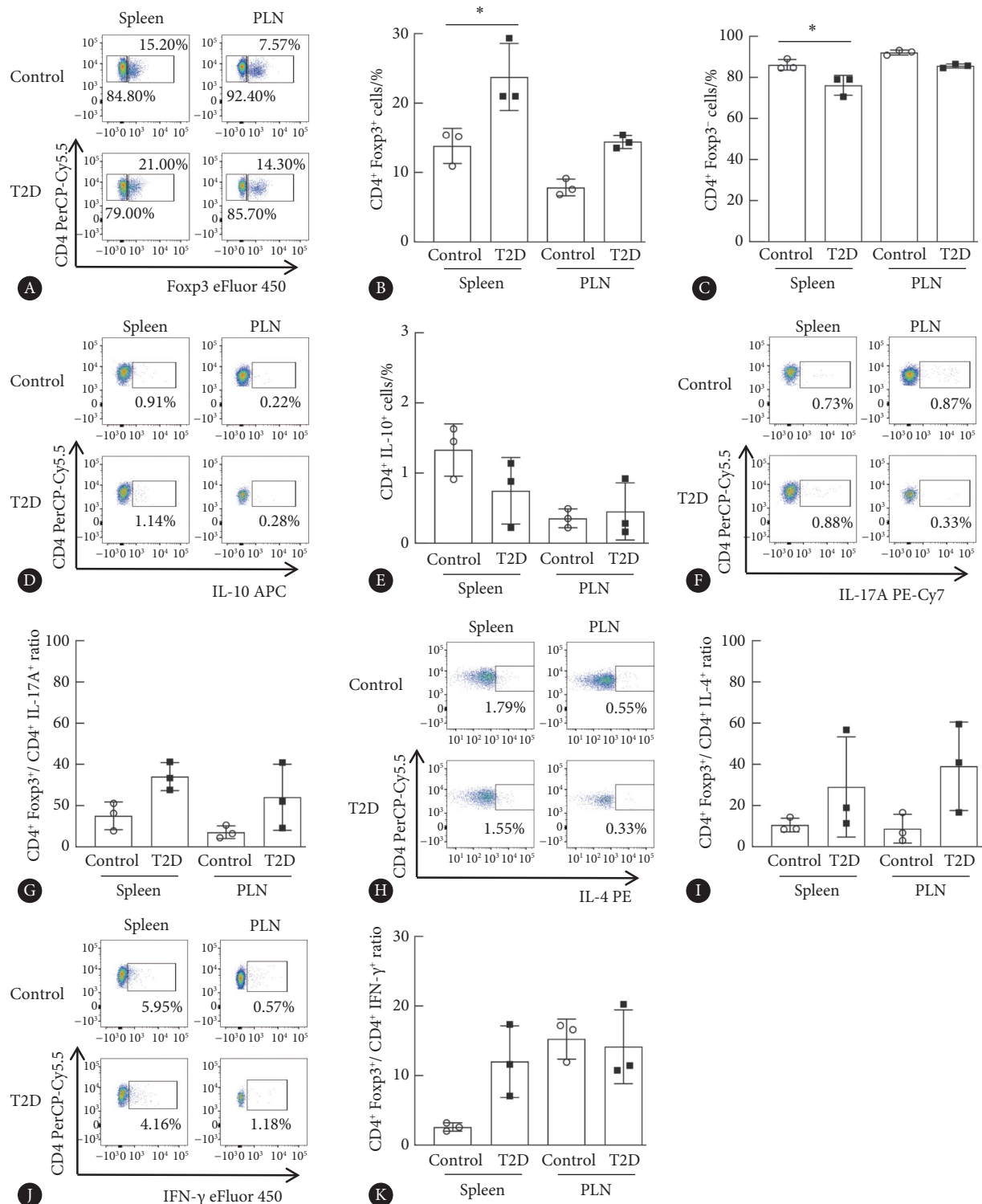


图2 血糖控制不良的晚期T2D小鼠中Treg细胞比例升高

Fig 2 The frequency of Treg cells increased in late-stage T2D mice with poorly-controlled blood glucose

A-E, Frequencies of CD4⁺ Foxp3⁺ Treg cells (A and B), CD4⁺ Foxp3⁻ non-Treg cells (C), Tr1 cells (D and E). F, H, and J, Frequencies of Th17 cells, Th2 cells, and Th1 cells. G, I, and K, Ratios of Treg cell frequency to indicated Teff cell frequency. The data are representative of two independent experiments ($n = 3$). * $P < 0.05$.

Tr1细胞的比例在两组小鼠中无明显差异($P > 0.05$) (图2D、2E)。与此同时,在T2D小鼠的脾脏和外周淋巴结中Treg细胞与Th17细胞的相对比值以及与Th2细胞的相对比值均显示出上升趋势,且在T2D小鼠的脾脏中,Treg细胞与Th1细胞的相对比值也显示出上升趋势,但差异均无统计学意义(图2F~2K)。因此,在持续高血糖的T2D小鼠中,Treg细胞的比例也出现了明显升高。

2.3 血糖控制不良的晚期糖尿病小鼠中非Treg细胞凋亡增加,而Treg细胞增殖活性增强

本研究检测了T1D小鼠脾脏和外周淋巴结T细胞的生长以及脾脏T细胞的凋亡,以分析血糖控制不良的晚期糖尿病小鼠中Treg比例升高的原因。结果显示,在脾脏和外周淋巴结中,两组小鼠的CD4⁺ Foxp3⁺非Treg细胞中Ki67的表达无明显差异($P > 0.05$),而糖尿病小鼠中Treg细胞的Ki67明显上调(图3A~3D),说明糖尿病小鼠中Treg细胞的生长和增殖活性增强。不仅如此,糖尿病组小鼠脾脏中CD4⁺ Foxp3⁺非Treg细胞的凋亡高于对照组($P < 0.05$),而两组小鼠脾脏中Treg细胞的凋亡则无明显差异($P > 0.05$) (图3E~3H)。结果显示,糖尿病中非Treg细胞的凋亡增加,而Treg细胞的生长和增殖活性增强。

2.4 高糖在原代免疫细胞培养中增加Treg细胞分化,并促进Teff细胞凋亡

为进一步验证体内实验结果,本研究使用含有不同浓度葡萄糖的完全DMEM培养基培养了小鼠总脾脏细胞,发现随着糖浓度的升高,CD4⁺ Foxp3⁺ Treg细胞的分化增加,而Tr1细胞的分化则没有发生明显变化(图4A~4D)。同时,本研究也检测了Teff细胞和Treg细胞的凋亡情况,发现在高糖条件下Teff细胞的凋亡增多,而Treg细胞在不同糖浓度条件下的凋亡没有明显改变(图4E~4H)。结果显示,高糖可以直接促进Treg细胞的分化和Teff细胞的凋亡。

2.5 高糖诱导Teff细胞发生氧化应激并增加ROS的产生

利用ROS探针H2DCFDA检测Teff细胞和Treg细胞在不同糖浓度培养基中产生ROS的情况。结果显示,随着糖浓度的升高,Teff细胞会显著增加ROS的产生,而Treg细胞中ROS的产生则未发生明显变化(图5A~5C)。不仅如此,在相同培养条件下,Teff细胞比Treg细胞产生了更高水平的ROS($P < 0.05$) (图5C)。结果显示,与Treg细胞相比,Teff细胞更容易发生高糖诱导的氧化应激,从而引发细胞凋亡,导致Teff细胞比例降低,从而升高Treg细胞比例。

2.6 高糖诱导的氧化应激通过ROS介导的TGF- β 活化诱导Treg细胞分化

为了研究高糖对Treg细胞分化的促进作用是否依赖

ROS介导的TGF- β 活化,本研究使用不同糖浓度培养T细胞,并加入活化的TGF- β 1或未活化的TGF- β 1前体(LAP-TGF- β 1)。结果见图6。在TGF- β 1条件下,3种糖浓度中Treg细胞分化无明显变化;而在LAP-TGF- β 1存在的条件下,高糖能促进CD4⁺ Foxp3⁺ Treg细胞分化。在加入TGF- β 的中和抗体anti-TGF- β 和TGF- β 受体1的抑制剂SB431542后,高糖诱导的Treg细胞分化则被完全抑制,证明高糖通过活化TGF- β 诱导了Treg细胞分化。为进一步验证此机制,本研究提取了他莫昔芬(*Tgfb1*^{-/-})和葵花籽油(*Tgfb1*^{+/+})处理的*Tgfb1*^{fl/fl} ER-Cre小鼠的幼稚T细胞,在不同糖浓度下进行培养。结果显示,在对照组(*Tgfb1*^{+/+})中,加入LAP-TGF- β 1后,高糖能促进Treg细胞分化,而在TGF- β 受体1敲除的*Tgfb1*^{-/-}组中,高糖诱导的Treg细胞分化被完全抑制。为了证明高糖诱导的TGF- β 活化是由ROS介导的,本研究通过在T细胞培养体系中同时加入LAP-TGF- β 1和ROS清除剂NAC,研究Treg细胞的分化情况。结果发现,在加入了NAC后,高糖诱导的Treg细胞分化同样被完全抑制。流式图见网络资源附件。综上所述,高糖诱导的T细胞氧化应激通过增加ROS的产生介导TGF- β 的活化,从而促进Treg细胞的分化。

3 讨论

糖尿病视网膜病变、肾病、神经病变和心血管疾病是导致糖尿病患者死亡的主要并发症^[2, 10-11]。目前已知,1型和2型糖尿病及其相关并发症均涉及炎症和免疫异常^[6, 12-13]。高血糖引起的并发症也很大程度上和促炎细胞因子水平升高有关,这些细胞因子是脂质过氧化和氧化应激的标志物^[5, 14];而高血糖患者体内的促炎细胞因子增加,免疫细胞被激活,从而参与细胞和体液免疫反应^[15-16],但这些炎症水平的升高是否由轻度感染引起的继发炎症尚不明确。Treg细胞在控制免疫平衡和维持免疫耐受中起着关键作用^[17-19]。虽然研究人员已经报道了随着糖尿病的进展和恶化,Treg细胞的比例显著升高,但高糖内环境导致Treg细胞比例升高的机制仍不清楚。ZHEN等^[5]预测,外周Treg细胞比例的增加可能与胸腺Treg发育的增多或外周Teff细胞被诱导为Treg的增多有关。

为了探讨血糖控制不良的糖尿病个体中Treg细胞比例上升的机制,本研究首先从T1D和T2D小鼠模型入手,发现在糖尿病中持续的高糖内环境的确可以增加Treg细胞的比例;同时,Treg细胞与促炎因子IL-17A、IL-4以及IFN- γ 的相对比值均呈升高趋势,即T细胞免疫呈现免疫耐受状态。这些发现提示Treg细胞比例增加可能是导致晚期糖尿病患者免疫抑制内环境的主要原因之一。接

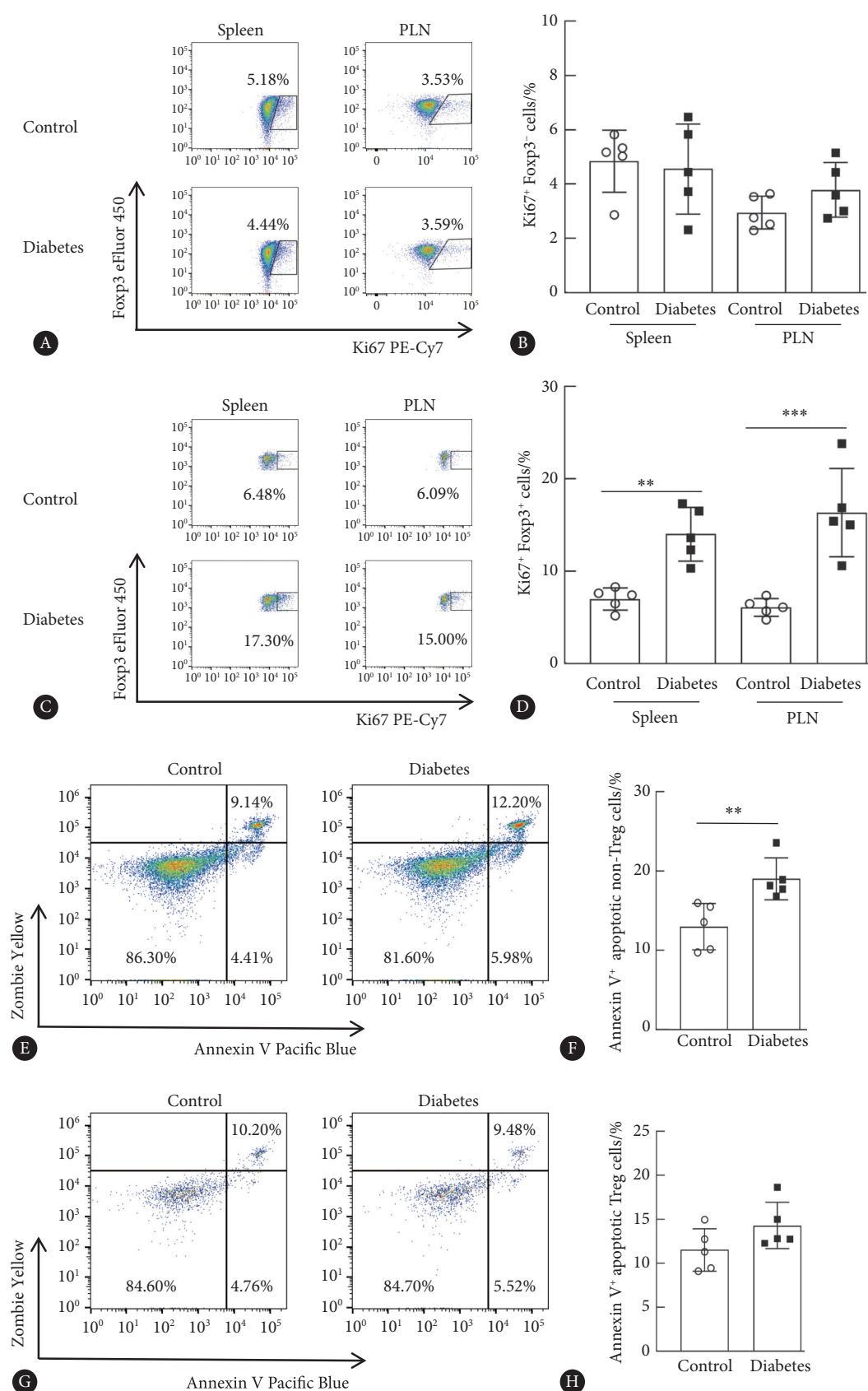


图3 稳定发病的糖尿病小鼠非Treg细胞凋亡增加, 而Treg细胞活性增强

Fig 3 The apoptosis of non-Treg cells and the growth of Treg cells increased in diabetic mice

A-D, Frequencies of Ki67⁺ Foxp3⁻ non-Treg cells and Ki67⁺ Foxp3⁺ Treg cells. E-H, Frequencies of apoptotic CD4⁺ Foxp3⁺ Treg cells and CD4⁺ Foxp3⁻ non-Treg cells in the spleen. The data are representative of two independent experiments ($n = 5$). ** $P < 0.01$, *** $P < 0.001$.

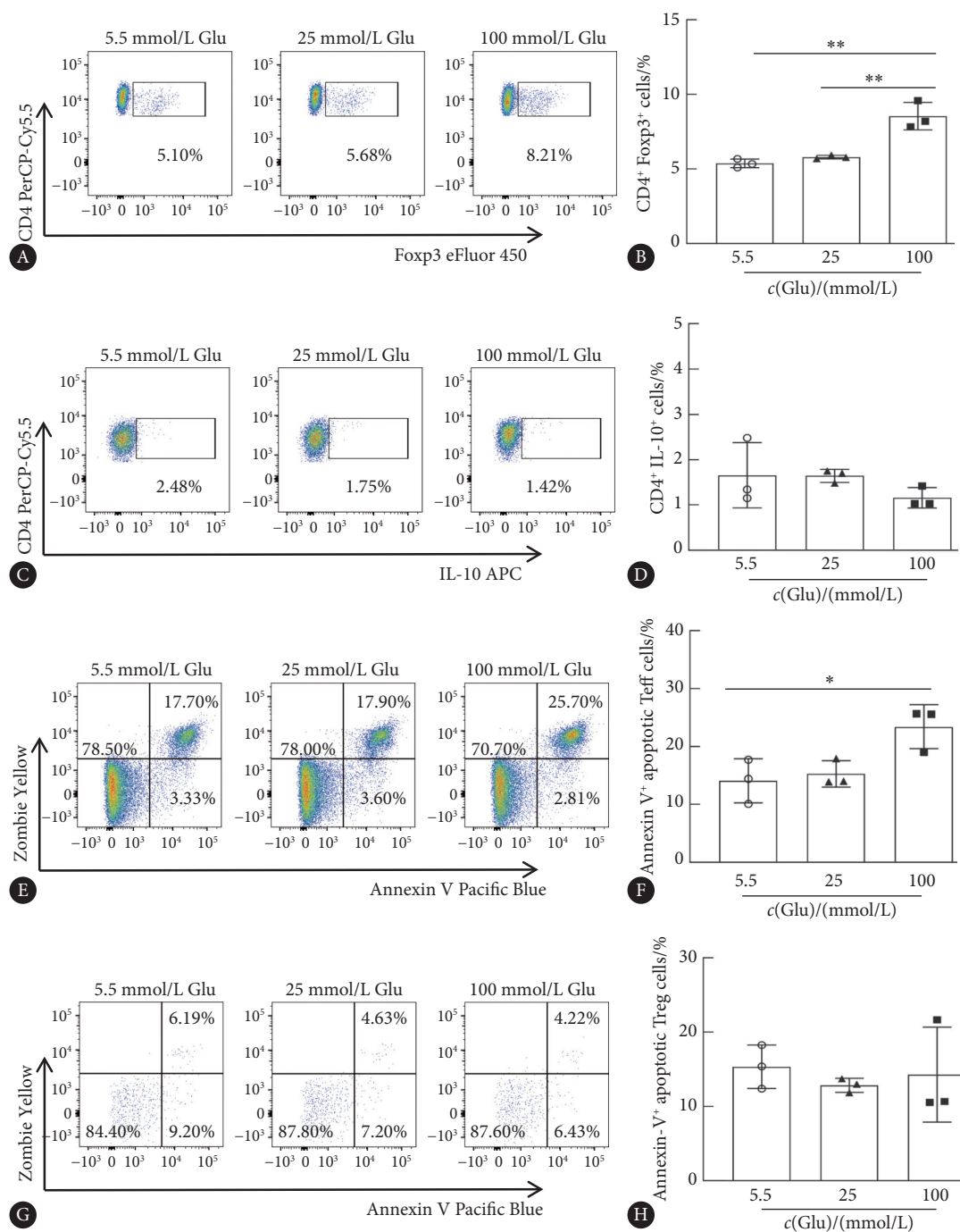


图 4 高糖在体外培养中直接促进Treg细胞分化并诱导Teff细胞凋亡

Fig 4 High glucose promotes Treg cell differentiation and Teff cell apoptosis *in vitro*

Total splenocytes were cultured in complete DMEM containing 5.5, 25, or 100 mmol/L glucose, with plate-bound anti-mouse-CD3 (0.5 μ g/mL) for 24 h ($n = 3$). A-D, Frequencies of CD4⁺ Foxp3⁺ Treg cells (A, B) and Tr1 cells (C, D). E-H, Frequencies of apoptotic CD4⁺ Foxp3⁺ Treg cells and CD4⁺ Foxp3⁻ non-Treg cells. * $P < 0.05$, ** $P < 0.01$.

着,本研究探讨了糖尿病中Treg细胞比例上升的原因。结果发现,血糖控制不良的晚期糖尿病小鼠中Treg细胞的生长更快,而包括Teff在内的非Treg细胞的凋亡增加,这使得Treg细胞的比例出现相对升高。

基于以上结果,本研究继而探究了高糖内环境诱导Treg细胞比例升高和Teff细胞凋亡增加的机制。已有研究证实,高糖内环境会诱导ROS介导的氧化应激^[20-22],而

氧化应激可以调控和影响T细胞凋亡^[23-25]。通过检测Treg细胞和Teff细胞中ROS的水平,我们发现随着糖浓度的升高,Teff细胞会发生严重的氧化应激,而Treg细胞的ROS产生则不受糖浓度的影响;并且,Teff细胞的ROS产生水平均高于Treg细胞。这些数据证明Teff细胞在高糖条件下诱导了ROS的大量产生,从而引发了Teff细胞的凋亡。既往研究发现,高糖能够通过活化TGF- β 从而调控

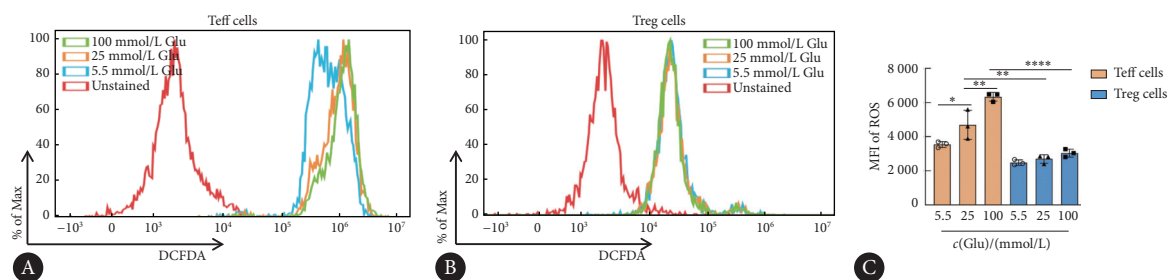


图5 高糖处理促进Teff细胞ROS的产生, 但不影响Treg细胞ROS的产生水平

Fig 5 High glucose induced more ROS production in Teff cells, but not in Treg cells

Teff cells and Treg cells were cultured in complete DMEM containing 5.5, 25, or 100 mmol/L glucose, with plate-bound anti-mouse-CD3 (1.5 μ g/mL), soluble anti-mouse-CD28 (1.5 μ g/mL) for 24 h, and then the production of ROS was determined ($n = 3$). A and B, ROS levels of Teff cells (A) and Treg cells (B) in indicated culture conditions. C, The mean fluorescence intensity (MFI) of ROS levels. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

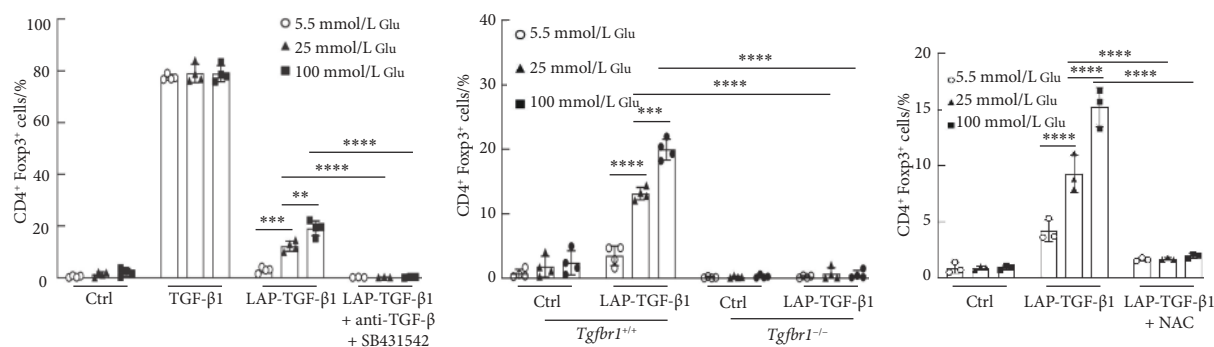


图6 高糖通过ROS诱导的TGF-β活化促进Treg细胞分化

Fig 6 High glucose promotes Treg cell differentiation through ROS-mediated TGF-β activation

Naive T cells from indicated mice were cultured in complete DMEM containing 5.5, 25, or 100 mmol/L glucose, with plate-bound anti-mouse-CD3 (1.5 μ g/mL), soluble anti-mouse-CD28 (1.5 μ g/mL), with or without LAP-TGF- β 1 (10 ng/mL), TGF- β 1 (2 ng/mL), anti-TGF- β (50 μ g/mL), SB431542 (5 μ mol/L) and NAC (10 mmol/L) for three days ($n = 3$ or 4). ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

细胞命运^[26-27]。因此, 笔者预计高糖可能通过ROS诱导的TGF- β 活化促进了Treg细胞的分化^[28]。通过在T细胞培养体系中加入LAP-TGF- β 1, 本研究发现随着糖浓度的升高, Treg细胞的分化增加, 而在阻断或敲除TGF- β 信号通路后, 高糖诱导的Treg细胞分化则被完全抑制。不仅如此, 加入ROS的清除剂NAC同样可以完全抑制高糖诱导的Treg细胞分化。这些数据充分证实, 高糖能够通过ROS诱导的TGF- β 活化促进Treg细胞的分化, 继而引起Treg细胞分化的增加。基于上述结果, 后续研究应着重关注高糖引起的免疫抑制内环境对血糖控制不良的晚期糖尿病患者继发感染的具体影响, 以及在临床工作中研究靶向清除ROS是否能够有效提高晚期糖尿病患者的抗感染能力。

综上所述, 本研究验证了糖尿病中持续失控的高血糖内环境会引起Treg细胞比例的上升, 并证明高糖诱导的氧化应激和ROS大量产生主要发生在非Treg细胞中。Teff细胞ROS的大量产生, 一方面介导了Teff细胞的凋亡增多, 另一方面介导了TGF- β 活化并诱导Treg细胞的分化, 通过这两条途径最终导致了Treg细胞比例的显著上

升和免疫抑制内环境的加剧。本研究从T细胞免疫的角度强调了糖尿病患者血糖控制的重要性, 并提示靶向氧化应激及其诱导的Teff细胞凋亡和Treg细胞分化, 有望改善糖尿病后期由高血糖引起的免疫抑制和继发感染。然而, 本研究仅使用了1型糖尿病和2型糖尿病小鼠模型开展研究, 未对血糖控制不良的糖尿病患者的T细胞进行研究和验证, 在一定程度上限制了本研究结论的临床价值。因此, 后续研究需要对血糖控制不良的糖尿病患者体内Treg细胞的变化情况进行长期监测和机制探究, 以充分揭示Treg细胞的变化在血糖控制不良的糖尿病患者免疫失衡中发挥的作用。

* * *

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Author Contribution MA Xiao is responsible for data curation, formal analysis, methodology, visualization, and writing--original draft. LI

Zhenhong is responsible for methodology and visualization. CHEN Wenjing is responsible for formal analysis and methodology. ZHANG Wei is responsible for resources, validation, and writing--review and editing. ZHANG Dunfang is responsible for conceptualization, funding acquisition, methodology, project administration, supervision, and writing--review and editing. All authors consented to the submission of the article to the Journal. All authors approved the final version to be published and agreed to take responsibility for all aspects of the work.

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