enhanced T-cell differentiation by FOXN1-overexpressing iTEPCs.

After killing the transplanted mice and analyzing splenic cells for CD3 expression (Fig 2, *A*), both $CD4^+$ and $CD8^+$ single-positive cells could be detected in the spleens of iTEPC-grafted mice (Fig 2, *B*). Additionally, endogenous CD3 expression on T cells was significantly greater ($P = .028$) in the iTEPC-coFoxn1 group (Fig 2, *C*). iTEPC-coFoxn1–transplanted animals contained more $CD4^+$ single-positive T cells in the spleen compared with the iTEPC-mTurq2–transplanted mice and contained significantly more $CD4^+$ ($P = .0023$) and $CD8^+$ ($P = .0234$) mature T cells compared with the MEF control group (Fig 2, *D*). T cells generated in mice transplanted with iTEPC-coFoxn1 organoids had a broader T-cell receptor (TCR) repertoire than MEF or iTEPC-mTurq2–transplanted mice (see the Methods section in this article's Online Repository, Fig E1), and a higher TCR diversity complexity score (see Fig E3 in this article's Online Repository at www.jacionline.org).

To demonstrate whether the generated T cells can be activated on antigen stimulation, we cultured isolated splenocytes *in vitro* with anti-CD3/CD28 antibodies and measured the production of IFN-γ (Fig 2, *E*). Splenocytes from iTEPC-coFoxn1 mice generated significantly more $CD8^+IFN\text{-}\gamma^+$ T cells than the stimulated splenocytes from MEF ($P = .019$) and iTEPC-mTurq2 ($P = .044$) mice and a similar number of $CD8^+IFN\gamma^+$ T cells as mice grafted with human fetal thymus (Fig 2, *F*). This demonstrates that iTEPC-coFoxn1 induces generation of functional T cells.

The here reported proof of principle to generate functional thymic organoids from autologous iPSCs has several potential clinical applications, such as treatment of primary and acquired immune defects (DiGeorge Syndrome and AIDS), assistance in T-cell immune reconstitution after allogeneic hematopoietic stem cell transplantation, and overcoming certain aspects of immune senescence during aging. Limitations include the small size, the immature developmental stage of the organoids, and the nonregulated nature of FOXN1 expression. The MEFs used as "cellular glue" for the organoids after reaggregation could be replaced with mesenchymal stromal cells or fibroblasts derived from the same iPSC line. These adaptations to the procedures described here, together with recent advancements in organ bioengineering, such as organ decellularization or generation of an artificial 3-dimensional matrix,[10] can result in a large enough thymic mass for sufficient T-cell output. Combined with FOXN1 gene therapy, such an iPSC strategy could provide the basis of patient-specific thymic organoid transplants in the future.

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Hedgehog signaling promotes T_H2 differentiation in naive human CD4 T cells

To the Editor:

Hedgehog (Hh) proteins are intercellular signaling molecules that control development and tissue homeostasis. They also regulate thymocyte development and peripheral T-cell activation in mice and human subjects and have recently been shown to promote T_H2 differentiation and function in mice.[1-4] Sonic Hedgehog homologue (SHH) is involved in homeostasis of many epithelial tissues, and because these tissues are the sites of allergic disease, it is important to understand how Hh signaling influences human CD4 T_H differentiation. Here we show that Hh signaling promotes human T_H2 differentiation by using materials and methods described in Furmanski et al[3] and Yanez et al[5] and in the Methods section in this article's Online Repository at www.jacionline.org.

We used quantitative RT-PCR to evaluate gene expression of components of the Hh signaling pathway in naive human CD4

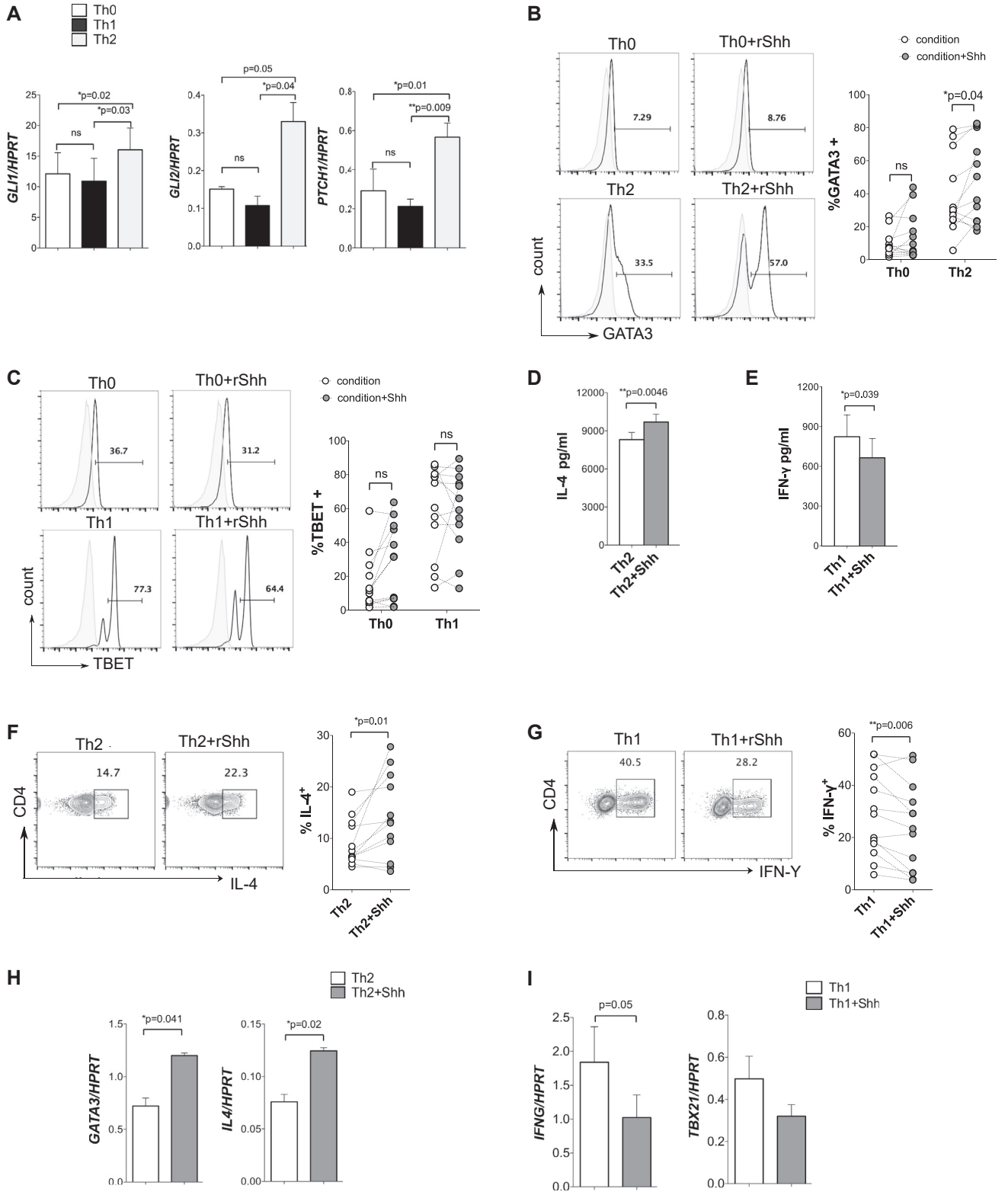

FIG 1. Shh treatment increases T_H2 differentiation *in vitro*. Naive CD4 T cells (n = 12 donors) stimulated under T_H-skewing conditions with or without rShh **(B-I)** analyzed at 48 hours **(A)**, at day 4 **(B-E)**, and at day 7 plus restimulation **(F-I)** are shown. Plots indicate means ± SEMs; each *point* represents an individual donor. Fig 1, *A*, Gene expression (quantitative RT-PCR; n = 3). FACS histograms show intracellular expression (gated on $CD4^+$ cells) of GATA-3 (Fig 1, *B*) and T-bet (Fig 1, *C*). *Gray overlays* show control stain. Scatterplots show percentages of positive cells. Fig 1, *D* and *E*, Cytokine concentration (ELISA) in supernatants. Fig 1, *F* and *G*, FACS plots show CD4 and intracellular cytokine expression. Scatterplots show cytokine-positive percentages. Fig 1, *H* and *I*, Gene expression (quantitative RT-PCR; n = 3). $*P < .05$ and $**P < .01$, paired 2-tailed *t* test. *ns*, Not significant.

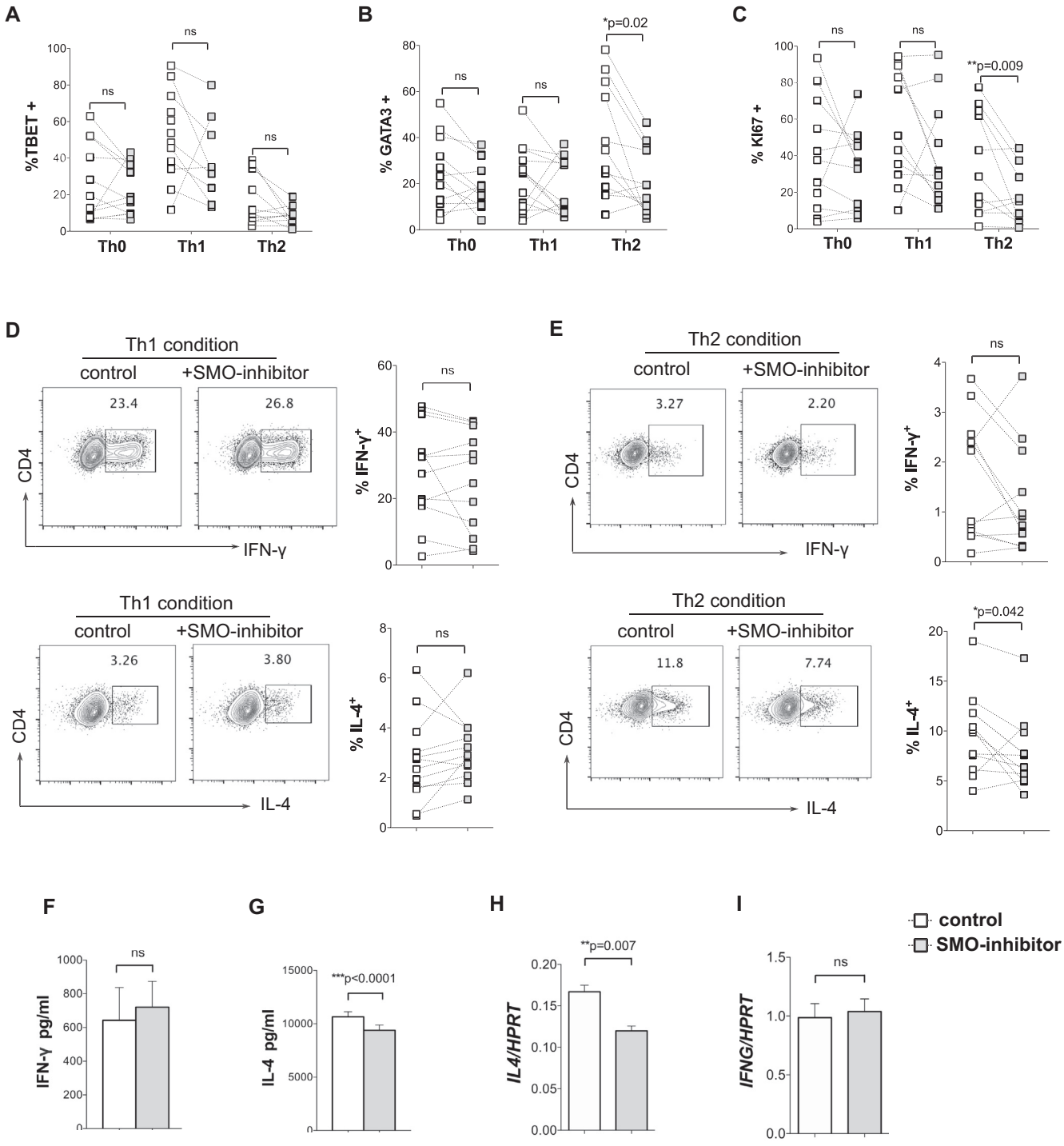

FIG 2. SMO inhibition decreases T_H2 differentiation *in vitro*. Naive CD4 T cells (n = 12 donors) stimulated under T_H-skewing conditions with SMO inhibitor *(gray squares)* or DMSO (control; *open bars/squares*) on day 4 (**A-C, F,** and **G**) and day 7 plus restimulation (**D, E, H,** and **I**). Scatterplots show means ± SEMs; each *point* represents an individual donor. Fig 2, *A-C*, Percentage of $CD4^+$ cells that were positive for intracellular staining against T-bet (Fig 2, *A*), GATA-3 (Fig 2, *B*), and Ki-67 (Fig 2, *C*). Fig 2, *D* and *E*, FACS plots show expression of CD4 and intracellular IFN-γ *(upper plots)* or intracellular IL-4 *(lower plots)* in cells cultured under T_H1 (Fig 2, *D*) or T_H2 (Fig 2, *E*) conditions. Scatterplots show percentages of $CD4^+$ cells that stained positive with the stated cytokine. Fig 2, *F* and *G*, Cytokine concentration (ELISA) in supernatants from T_H1 (Fig 2, *F*) and T_H2 (Fig 2, *G*) cultures. Fig 2, *H* and *I*, Gene expression (quantitative RT-PCR) in cells from T_H2 (Fig 2, *H*) and T_H1 (Fig 2, *I*) cultures (3 random donors). $*P < .05$, $**P < .01$, and $***P < .001$, paired 2-tailed *t* test. *ns*, Not significant.

T cells stimulated for 48 hours in T_H0-, T_H1-, or T_H2-polarizing conditions. Expression levels of the Hh-responsive transcription factors glioma-associated oncogene 1 *(GLI1)* and *GLI2* and the Hh cell-surface receptor patched 1 *(PTCH1)* were greater in CD4 T cells cultured under T_H2-skewing conditions compared with those cultured under T_H0 or T_H1 conditions (Fig 1, *A*), suggesting that Hh signaling is involved in human T_H differentiation or function. Because *GLI1* and *PTCH1* are Hh target genes, their greater expression in T_H2-differentiated cells indicates that this population has overall greater Hh-mediated transcription.

To test the influence of SHH signaling on T_H differentiation, we stimulated purified naive human CD4 T cells from 12 independent, randomly selected anonymous donors for 4 days under skewing conditions with or without a single dose of recombinant Shh (rShh). Treatment with rShh significantly enhanced expression of the T_H2 transcription factor GATA-3 in cells stimulated under T_H2 conditions, whereas GATA-3 expression under T_H0 conditions and T-bet expression under T_H0 or T_H1 conditions were not affected (Fig 1, *B* and *C*). Treatment of T_H2-skewing cultures with rShh also increased the concentration of IL-4 in supernatants after 4 days of culture compared with control T_H2-skewing cultures (Fig 1, *D*). Interestingly, the concentration of IFN-γ was lower when rShh was added compared with control T_H1 cultures (Fig 1, *E*). After 7 days of culture and anti-CD3/CD28 restimulation, the proportion of CD4 T cells that expressed IL-4 was significantly increased in the presence of rShh under T_H2 conditions (Fig 1, *F*). In contrast, the percentage of cells that expressed IFN-γ was reduced in T_H1 plus rShh cultures compared with T_H1 cells (Fig 1, *G*). Shh treatment increased *GATA3* and *IL4* expression in T_H2 cultures (Fig 1, *H*), whereas rShh treatment decreased *IFNG* and *TBX21* (T-bet) expression in T_H1 cultures (Fig 1, *I*). These data indicate that Hh signaling promotes T_H2 differentiation in human CD4 T cells, with simultaneous repression of IFN-γ and T-bet.

We then investigated whether pharmacologic inhibition of the Hh signaling pathway by treatment with an inhibitor of the nonredundant Hh signal transduction molecule smoothened (SMO; PF-04449913) would impair T_H2 differentiation.[6] The proportion of cells that expressed the T_H1 lineage–specific transcription factor T-bet was not affected by SMO inhibitor treatment under skewing conditions (Fig 2, *A*). Likewise, no differences were found in expression of GATA-3 under neutral or T_H1 conditions (Fig 2, *B*). However, SMO inhibitor treatment significantly reduced the proportion of CD4 T cells that expressed GATA-3 and Ki-67 (a marker of proliferation) when cultured under T_H2-skewing conditions (Fig 2, *B* and *C*). SMO inhibition did not affect the percentage of cells that expressed IFN-γ under T_H1 conditions, and as expected, IL-4 expression was low under T_H1 conditions in both control and SMO inhibitor–treated cultures (Fig 2, *D*). However, when cultured under T_H2 conditions, the percentage of cells that expressed IL-4 was significantly reduced by SMO inhibitor treatment (Fig 2, *E*). Analysis of cytokine concentrations in culture supernatants by means of ELISA showed that IFN-γ levels were similar in both groups under T_H1 conditions (Fig 2, *F*), but under T_H2 conditions, significantly lower concentrations of IL-4 were found in the SMO inhibitor group compared with the control group (Fig 2, *G*). Finally, we investigated transcript levels of *IL4* and *IFNG* by using quantitative RT-PCR. In T_H2-skewed cells *IL4* expression was significantly lower in SMO inhibitor–treated cultures than control cultures (Fig 2, *H*), whereas *IFNG* transcript levels were not different between groups under T_H1 conditions (Fig 2, *I*). Taken together, these analyses indicate that attenuation of Hh signal transduction by treatment with the SMO inhibitor reduced T_H2 differentiation but did not affect T_H1 fate.

Here we show that Hh signaling promotes T_H2 differentiation in human CD4 T cells. We found that treatment of naive CD4 T cells with rShh under T_H2-skewing conditions increased expression of the transcription factor GATA-3, a reliable indicator of T_H2 transcriptional identity. In support of this, *IL4* expression was enhanced and IL-4 cytokine production was increased in T_H2 cultures on treatment with rShh. In contrast, rShh treatment antagonized T_H1 differentiation in T_H1 cultures, leading to lower *IFNG* and *TBX21* expression and a lower proportion of cells expressing intracellular IFN-γ. Attenuation of Hh signal transduction by pharmacologic SMO inhibition reduced T_H2 differentiation: both *GATA3* expression and *IL4* expression were significantly decreased.

In murine T_H differentiation Hh signaling promotes T_H2 differentiation, skewing the overall pattern of transcription to a T_H2-like profile, and *Il4* is a GLI2 target gene in murine T cells.[3] Importantly, Hh pathway activation in T cells has physiologic relevance in a murine model of allergic asthma because by favoring T_H2 polarization and cytokine production, it contributes to disease severity.[3,7]

In human subjects a genome-wide association study linked components of the Hh signaling pathway to allergic asthma,[8] and a recent study found that children with asthma presented with greater levels of SHH in airway epithelia than healthy control subjects.[9]

Here we provide *in vitro* evidence that Hh signaling enhances T_H2 differentiation in human CD4 T cells. One strength of our study is that our experiments were performed with cells isolated from 12 different unknown leukocyte cone donors, and we obtained consistent experimental results from all donors independent of their age or sex (of which we had no knowledge). A weakness of our study is that it was limited to *in vitro* experimentation. In the future, it will be interesting to assess the T_H differentiation status of T-cell populations isolated from samples from patients with asthma to obtain further *ex vivo* evidence that Hh signaling is involved in human T_H2 responses. This will be important to our understanding of human atopic diseases, such as asthma, in which T_H2 T-cell responses drive disease.

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Early-onset airway damage in early-career elite athletes: A risk factor for exercise-induced bronchoconstriction

To the Editor:

Recent data indicate that elite athletes are at risk of developing exercise-induced bronchoconstriction (EIB).[1-3] EIB is defined as the acute narrowing of the airways during or immediately after exercise.[2] The prevalence of EIB in athletes ranges between 3.7% and 54.8% depending on the sport discipline and the environment in which the athletes exercise.[1] The pathophysiological mechanisms driving EIB are not fully understood. One of the hypotheses states that high ventilatory rates in combination with environmental factors such as cold dry air, air pollution, and/or chlorine by-products can lead to an increased mechanical load to the airways, resulting in epithelial shedding and damage.[4] In line with this hypothesis, we showed that in adolescent and young adult elite swimmers, epithelial damage with increased sputum uric acid and serum club cell protein 16 (CC16) levels, is present.[5] As damage-associated molecular patterns result from epithelial damage, airway innate immune cells, such as macrophages, can get activated, resulting in the release of proinflammatory cytokines such as TNF-α, IL-6, IL-8, IL-1β, and IL-1α.[5] Only few studies have investigated the prevalence of EIB in early-career elite athletes. Pedersen et al[6] did not find increased EIB or signs of airway inflammation in 13- to 16-year-old elite swimmers leading to the conclusion that elite swimmers develop EIB throughout their career. This urges for screening tools to detect EIB in young elite athletes early in and during their sports career. We hypothesize that markers of epithelial damage, which might be the first pathological event upon airway triggering, could be suitable biomarkers for the detection of EIB. Therefore, we tested 117 12- to 13-year-old early-career elite athletes performing more than 12 hours of sports per week from different Flemish prevalent sport disciplines (swimming, basketball, and football) and compared them with control subjects (see this article's Online Repository at www.jacionline.org). We investigated the prevalence of EIB with the eucapnic voluntary hyperventilation (EVH) test adapted for young elite athletes[7] as well as different markers of airway inflammation and epithelial damage (see this article's Online Repository at www.jacionline.org). Subjects' characteristics are presented in Table I. Interestingly, swimmers showed significantly higher FEV_1 and forced vital capacity (FVC) compared with control subjects (Table I). Symptoms during exercise were investigated in the sport disciplines (see Fig E1 in this article's Online Repository at www.jacionline.org). In this study, we found that 24.5% of the tested early-career elite athletes (12-13 years old) suffer from EIB. This is slightly higher than the mean prevalence (15%) of EIB in young elite athletes (5-18 years old) as reported in a meta-analysis by de Aguiar et al.[8] Besides the differences in age group, this higher prevalence can be due to the use of the EVH test instead of an exercise challenge test, such as a 6-minute running test at 85% to 90% of the maximal heart rate, which is used in most of the previous studies in athletes between 12 and 18 years old. We opted to use the EVH test because it is performed in a standardized setting similar for all sport disciplines and it is accepted by the International Olympic Committee to diagnose EIB. In our study, early-career elite swimmers (33.3%) have the highest prevalence of EIB followed by basketball (27.3%) and football players (12.8%) (Fig 1). Several studies in adult elite athletes have already shown that EIB is more common in swimmers as well as in athletes performing sports in a cold environment.[3] We now show that already at the start of their sports career, swimmers have a higher EIB prevalence compared with others, probably because of the long-term exposure to chlorine by-products during intensive exercise. In addition to the presence of EIB in early-career elite athletes, signs of epithelial damage were detected in 12- to 13-year-old athletes. Indeed, in all athletes (ie, in swimmers and in basketball and football players), baseline serum CC16 level was increased compared with the level in age-matched controls (Fig 1). No significant differences were found in the percentages of sputum neutrophils and eosinophils between athletes and control subjects (see Fig E3 in this article's Online Repository at www.jacionline.org), leading to believe that at this young age epithelial damage is already present, but cellular inflammation is still missing in almost all the athletes (see Table E3 in this article's Online Repository at www.jacionline.org). Interestingly, higher serum CC16 levels correlated significantly with higher maximal fall amplitude in FEV_1 after the EVH test ($r = -0.1937$; $P = .0262$; Fig 1). Sputum uric acid was increased in all athletes compared with the level in age-matched controls but was most striking in basketball players (Fig 1). In contrast, no significant differences in serum uric acid levels were found among the different athletes and control subjects (data not shown). However, EIB+ athletes have higher serum uric acid levels, but not higher CC16 levels compared with those in EIB− athletes ($P = .044$; Fig 1). No significant differences were observed in sputum proinflammatory cytokine mRNA levels (IL-1α, IL-1β, IL-6, IL-8, IL-17A, and TNF-α) between the 4 groups (see Fig E2 in this article's Online Repository at www.jacionline.org). However, sputum IL-8 mRNA levels (but

METHODS

Human naive CD4 purification and culture

Human PBMCs were freshly isolated from randomly selected, unknown leukocyte cone donors (UK National Health Service [NHS] Blood and Transplant Centre) by means of gradient centrifugation with Lymphoprep (Axis Shield, Oslo, Norway). Donors to the UK NHS Blood and Transplant Centre are aged between 17 and 65 years, and we had no knowledge of their age, sex, or identity. Ethical approval was authorized by the local NHS Research Ethics Committee.

Naive CD4 T cells ($CD3^+CD4^+CD45RA^+CD45RO^-$) were magnetic bead purified from PBMCs by using the EasySep Isolation Kit (STEMCELL Technologies, Vancouver, British Columbia, Canada). The purity of naive CD4 T cells was analyzed by using flow cytometry and exceeded 95%. After magnetic bead isolation, naive CD4 T cells were rested for 3 to 5 hours and then plated in 96-well round plates at 1×10^6 cells/mL. Cells were stimulated in complete RPMI (supplemented with 10% FBS, 1% penicillin-streptomycin, and 10^{-5} mol/L 2-mercaptoethanol) with 5 μg/mL plate-bound anti-CD3 antibody (clone UCHT1) and anti-CD28 antibody (eBioscience, San Diego, Calif). For T_H0 conditions, no cytokines were added. For T_H1 conditions, anti–IL-4 (5 μg/mL), rIL-12 (20 ng/mL), and rIFN-γ (10 ng/mL) were added. For T_H2 conditions, anti–IFN-γ (2.5 μg/mL) and rIL-4 (20 ng/mL) were added.

After 4 days, cells were expanded in human rIL-2 (100 U/mL) for 3 days in fresh medium containing the same skewing cytokines and neutralizing antibodies but in the absence of anti-CD3 and CD28 stimulation. Cells were then restimulated for 16 hours by addition of soluble anti-CD3 and anti-CD28 (1 μg/mL) before gene expression and cytokine analysis. Where stated, rShh (R&D Systems, Minneapolis, Minn) was added at a final concentration of 0.5 μg/mL at the initiation of culture and again on day 4, when the medium was changed and the cells were expanded by addition of rIL-2.

Where stated, SMO inhibitor (PF-04449913 [Pfizer, New York, NY] dissolved in dimethyl sulfoxide [DMSO]) was added to cultures for a final concentration of 0.374 μg/mL, and an equivalent concentration of DMSO alone was added to the control wells (DMSO at 1:10,000 final dilution). This treatment or control was added to the corresponding wells every day until the end of the experiment.

For intracellular cytokine staining, CD4 T cells were stimulated for 4 hours with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, St Louis, Mo), 500 ng/mL ionomycin (Sigma-Aldrich), and 3 μg/mL Brefeldin A (eBioscience).

Flow cytometry

Cells were stained with combinations of directly conjugated antibodies from Thermo Fisher (Waltham, Mass) or BioLegend (San Diego, Calif) in fluorescence-activated cell sorting (FACS) buffer (5% FBS and 0.01% sodium azide in 1× PBS) acquired on a C6 Accuri flow cytometer (BD Biosciences, San Jose, Calif) and analyzed with FlowJo software (version 10.6; TreeStar, Ashland, Ore). For intracellular staining, CD4 T cells were stained with anti-CD4 for cell-surface staining and then incubated with Fixation/Permeabilization solution (eBioscience) for 20 minutes in the dark. After this, cells were washed twice with permeabilization buffer and then stained with specific antibodies in permeabilization buffer for 40 minutes. After incubation, cells were washed with permeabilization buffer and resuspended in FACS buffer for FACS analysis.

ELISA

IFN-γ and IL-4 cytokines were measured with Ready-Set-Go! Kits (eBioscience), according to the manufacturer's instructions.

Quantitative RT-PCR

RNA was extracted with the PicoPure Kit (Applied Biosystems, Foster City, Calif). cDNA was synthesized by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and were analyzed on an iCycler (Bio-Rad Laboratories, Hercules, Calif) with SYBR Green Supermix (Bio-Rad Laboratories), according to the manufacturer's guidelines. RNA levels obtained from each sample were measured relative to the housekeeping gene hypoxanthine phosphoribosyltransferase *(HPRT)*. All primers were purchased from Qiagen (Hilden, Germany).

Statistical analysis

The paired 2-tailed Student *t* test was used for statistical analysis for comparison of *in vitro* treatment of cells from a given subject.