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Rapid Communication

The TBX21 transcription factor T-1993C polymorphism is associated with decreased IFN-γ and IL-4 production by primary human lymphocytes

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ABSTRACT

T-bet is a transcription factor that drives the Th1 immune response primarily through promoting expression of the IFN-γ gene. Polymorphisms in the T-bet gene, *TBX21*, have been associated with immune-mediated diseases such as asthma and systemic sclerosis. We found that the *TBX21* promoter polymorphism T-1993C is associated with a significant decrease in IL-4 and IFN-γ production by stimulated primary human lymphocytes from healthy participants.

1. Introduction

The transcription factor T-bet, "T-box expressed in T cells," is a member of the T-box family and is a master regulator of T-cell differentiation. As a potent transactivator of *IFNG*, T-bet stimulates production of the hallmark Th1 cytokine, IFN-γ, and drives Th1 polarization. T-bet simultaneously opposes commitment to the Th2 and Th17 lineages [1,2]. T-bet function has been investigated extensively in murine knockout models. In addition to directing the CD4+ cell lineage, these models have demonstrated important roles for T-bet in dendritic cells, CD8+ T cells, B cells, natural killer (NK) cells, and NKT cells. T-bet functioning is required in these cell types for defense against intracellular organisms and for antineoplastic responses [3,4]. T-bet has also been shown to play an influential role in inflammatory processes such as inflammatory bowel disease, autoimmune diseases such as rheumatoid arthritis, and immune-mediated conditions like asthma [2].

In humans, several single nucleotide polymorphisms (SNPs) in *TBX21* are known to affect T-bet expression levels and are associated with disease susceptibility. The *TBX21* T-1993C promoter polymorphism (rs4794067) is associated with decreased T-bet expression levels [5,6]. *TBX21* T-1993C is associated with an increased risk of developing asthma [6] and aspirin-induced asthma [7] and with a decreased risk of developing systemic lupus erythematosus [8] and autoimmune hepatitis type 1 [9]. Other *TBX21* SNPs have been associated with genital HSV infection (rs17244587) [10] and systemic sclerosis (rs11650354) [11]. Additionally, T-bet status is of interest with regards to cancer development and prognosis, because Th2-dominant cytokine profiles are known to be tumor-supportive [12]. Although there have been many associations with disease susceptibility in humans, to our knowledge, there have been no studies which directly measure cytokine production by primary human lymphocytes harboring *TBX21* polymorphisms. In this study, we investigated the effect of the *TBX21* T-1993C promoter polymorphism on production of IFN-γ and IL-4 by stimulated primary human lymphocytes from healthy donors.

2. Materials and methods

2.1. Participant blood sample and questionnaire collection

Participants were recruited at the University of North Carolina at Chapel Hill (UNC-CH) Health Care Blood Donation Center in the platelet donation clinic from August 2010 to December 2010. In addition to medication and disease exposure restrictions, platelet donors are required to be adults who are in good health. Blood samples were collected from 220 donors and complete questionnaires were obtained from 210 of those people (men 90; women 120) with a mean age of 31.8 years (SD 15.5; min 17 max 73).

Abbreviations: SNP, single nucleotide polymorphism; IFN-γ, interferon-γ; IL-4, interleukin-4; Th cells, T helper cells; T-bet, T-box expressed in T cells; NK, natural killer; HSV, herpes simplex virus; UNC-CH, University of North Carolina at Chapel Hill; IRB, institutional review board; EDTA, ethylenediaminetetraacetic acid; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cell; FBS, fetal bovine serum; IC, intracellular; PMA, phorbol 12-myristate 13-acetate; RT, room temperature; PFA, paraformaldehyde.

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Informed written consent for involvement in the study and for blood-product biobanking was obtained from all participants. The protocol was approved by the UNC-CH institutional review board (IRB). The blood samples were collected in two 10 ml EDTA vacutainers and one pro-coagulation vacutainer. Questionnaires were completed during the platelet donation process. All blood samples were transported to the BioSpecimen Processing facility on the UNC-CH campus for processing and product storage within 3 h of the blood draw.

2.2. TBX21 T-1993C polymorphism genotyping

DNA was isolated from coagulated whole blood and then stored by the UNC-CH BioSpecimen Processing facility on the UNC campus. The Puregene [Qiagen, Valencia, CA] modified salting-out precipitation method was used for DNA extraction in a high-throughput automated method on the Autopure DNA extraction robot. *TBX21* (rs4794067) genotyping was performed by the UNC-CH Mammalian Genotyping Core using the TaqMan PCR technique and manufacturer-supplied protocols, reagents and software [Applied Biosystems, Foster City, CA; Catalog No. C_11626504_10]. Briefly, 5–10 ng of genomic DNA was amplified in 5 μL reactions containing 1× Universal TaqMan Master Mix and 1× probe mix according to the manufacturer's instructions. PCR amplification was performed and allelic discrimination was conducted by comparing the post-amplification intensities of allele-specific reporter dyes to pre-amplification intensities for each sample relative to negative control reactions. End-point reading of the genotypes was performed with a 7900HT Real-Time PCR System [Applied Biosystems, Foster City, CA].

2.3. PBMC isolation and cryopreservation

Peripheral blood mononuclear cells (PBMCs) were isolated from anti-coagulated whole blood by centrifugal separation using Histopaque [Sigma, St. Louis, Mo]. The cells were washed and frozen in 20% fetal bovine serum (FBS) and 10% DMSO freezing solution. The cells were stored overnight in −80 °C and then transferred to liquid nitrogen for long term storage.

2.4. Assessment of intracellular cytokine production

The cryopreserved PBMCs were defrosted by rapid re-warming in a 37 °C water bath and immediately washed with RPMI supplemented with FBS. The intracellular cytokine assay was performed twice. The cells for batch 1 were allowed to recover for 10 days, and for batch 2 they recovered for 17 days. The samples were monocyte-depleted by exclusion of adherent cells. Cell viability was confirmed with Trypan Blue staining.

Intracellular (IC) staining was performed according to a modified BD Pharmingen alternative IC staining protocol. Briefly, the monocyte-depleted PBMCs were assessed for viability, counted, and activated with 1 μg/ml phorbol 12-myristate 13-acetate (PMA) [Sigma, St. Louis, Mo] plus 0.5 μl/mL Ionomycin [Sigma, St. Louis, Mo] for 4 h in 5% CO2 at 37 °C in the presence of 1 μl/ml GolgiPlug protein transport inhibitor (Brefeldin A, 1.0 μg/ml) [BD, Cat. No. 554715]. The cells were then washed and stained for cell-surface antigens using fluorochrome-conjugated antibodies against CD3 and CD16+CD56, to identify T cells and natural killer cells respectively, or the appropriate Ig negative control. Only the second batch was stained for CD3. After a 15 min incubation at room temperature (RT) in the dark, the cells were washed then fixed and permeabilized by addition of Fixation/Permeabilization solution [BD, Cat. No. 554722]. The cells were then incubated in this solution for 20 min at RT in the dark.

After washing, the cell pellets were resuspended in Perm/Wash buffer [BD, Cat. No. 554723] which contained the intracellular fluorochrome-conjugated anti-cytokine antibodies at optimal concentrations and incubated for 30 min at RT in the dark. The antibody cocktail consisted of anti-IFN-γ PerCP-Cy5.5/anti-IL6 V450/anti-IL-4 APC. Isotype matched antibodies to the cytokines were utilized to control for non-specific binding and a set of CD19 controls on separate cells was used for gating. The cells were then fixed in RPMI/2% paraformaldehyde (PFA) and analyzed immediately using a Dako Cyan flow cytometer and Summit software. Forward and side scatter properties were utilized to determine the lymphocyte population.

2.5. Statistical analysis

Statistical analysis was performed using SAS software version 9.2 (SAS Institute, Inc., Cary, NC). The nonparametric method of the Wilcoxon rank-sum test was used to assess the differences in the quantity of cytokine production between the *TBX21*-1993CC and -1993TT genotypes. Analyses were conducted for the two batch datasets separately, as well as for the combined batch dataset. The linear mixed effect model for repeated measurements, using the PROC MIXED procedure, was utilized to handle standard data analysis for the combined datasets. The parameter of interest was estimated using the unstructured covariance matrix. The statistical testing of the allele effect was conducted using the CONTRAST statement in the MIXED procedure, and the test statistic and the *P*-value were reported. The measure of the intracellular staining was quantified by mean fluorescence intensity as determined by flow cytometry. A two-sided *P*-value of less than 0.05 was considered significant.

3. Results

3.1. TBX21 genotype analysis

SNP genotyping identified 12 participants homozygous for *TBX21*-1993C (rs4794067) (5.5%), 74 participants who were -1993TC heterozygotes (34.1%), and 131 participants who were homozygous for the wild-type -1993T allele (60.4%). The genotype frequencies of rs4794067 in our group were very similar to those reported in the human dpSNP for the European group HapMap-CEU: CC 0.044, CT 0.389, TT 0.566 from 226 sample counts (http://www.ncbi.nlm.nih.gov/SNP).

3.2. T-bet-1993CC homozygote status is associated with decreased IFN-γ and IL-4 production

The homozygous -1993TT and -1993CC cells were analyzed twice in separate batches. Batch 1 had 10 controls with the -1993TT genotype and 8 cases with the -1993CC genotype, and batch 2 had 7 controls and 5 cases. Four samples were excluded from analysis due to poor recovery as indicated by having a < 50% viability count by Trypan Blue staining. An additional two samples were not analyzed in batch 2, due to a limited number of total cryopreserved cells.

Analysis of batch 1 revealed that both IL-4 and IFN-γ production by stimulated lymphocytes was significantly decreased in cells with the *TBX21*-1993CC compared to the -1993TT genotype ($P = 0.004$ and $P = 0.03$ respectively) (Fig. 1). In batch 2, the levels of IL-4 and IFN-γ cytokine production were also significantly lower in the -1993CC compared to -1993TT group ($P = 0.01$ and $P = 0.02$ respectively) (Fig. 2).

Combining measurements from the two batches also demonstrated significant differences in cytokine production by each