TH17 cells contribute to uveitis and scleritis and are inhibited by IL-27/STAT1 in the retina (1) Detection of TH17 cells in human blood

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Method Article

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Abstract

Introduction

This study implicates interleukin-17 \(IL-17)-producing T-cells \(TH17 cells) in etiology of human uveitis and scleritis. We show that blood of patients with uveitis or scleritis contained more TH17 cells than blood of healthy individuals. The numbers of TH17 cells increased during active uveitis/scleritis and decreased following treatment and in a mouse model, treatment with an IL-17-specific antibody reduced severity of ocular inflammation. These results suggest that TH17 cells may mediate eye diseases by inducing production of TNFa, as large amounts of this cytokine are found in retinal cells of mice with ocular inflammation. IL-2 was found to promote TH17 expansion while IFNg, produced by another T-cell type, inhibits TH17 proliferation by upregulating IL-27 expression. This study provides explanations for efficacy of IL-2R antibody therapy in uveitis and suggests that antagonism of TH17 by IFNg and IL-27 could be used for treatment of chronic inflammation. In this protocol we described the detection of TH17 cells in human blood. IL-17-expressing T helper cells are implicated as etiologic agents of several chronic inflammatory diseases of rodents. Peripheral blood mononuclear cells \(PBMC) of normal human subjects and patients with uveitis \(intraocular inflammatory diseases) or scleritis \(sight-threatening idiopathic inflammatory ocular disease) were analyzed to examine whether TH17 cells are also etiologic agents of human chronic inflammatory diseases. The steps of this protocol are: Isolation of human peripheral blood mononuclear cells \(PBMC) and CD4+ T cells Expansion of Th17 cells in human PBMC by IL-2 Intracellular cytokine staining assay RT-PCR and Quantitative RT-PCR \(qRT-PCR) DNAse Digestion of RNA Samples First Strand cDNA Synthesis Other protocols related to our Nature Medicine paper can be found here: "Analysis of the expression of IL-17 in mouse PBMC, lymph node and retina":http://www.natureprotocols.com/2007/08/30/th17_cells_contribute_to_uveit_1.php "Western Blot Analysis":http://www.natureprotocols.com/2007/08/30/th17_cells_contribute_to_uveit_2.php "Analysis by confocal microscopy":http://www.natureprotocols.com/2007/08/31/th17_cells_contribute_to_uveit_3.php "Chromatin immunoprecipitation":http://www.natureprotocols.com/2007/08/31/th17_cells_contribute_to_uveit_4.php "Retinal cell isolation":http://www.natureprotocols.com/2007/08/31/th17_cells_contribute_to_uveit_5.php

Reagents

• 1 X PBS \(without Ca^{2+} and Mg^{2+}). • 24 well and 96 well sterile plates. Screw cap tube conical 15 ml \(Sarstedt) and Blue Max 50 ml Polypropylene \(Becton Dickinson) sterile tubes. • Isolymph \(Gallard-Schlesinger Industries, Inc. Norway). • Hemacytometer • Human cytokines IL-2, IL-6, IL-23, TGF-β1 \(R&D, MN). • Antibodies for FACS were conjugated to various chromogenic molecules: Human PerCP- CD3, PE-CD4, FITC-CD8, APC-CD56, and -CD20, PEcy5-conjugated antibody to human CD16 IL-17 \(e-Bioscience), CD25, CD45RO, CD62L or IFN-γ and isotype controls \(BD Bioscience, CA). • Fetal calf serum \(FCS), \(Hyclone, Logan, UT) • 10 ml BD Vacutainer \(sodium heparin) Becton Dickson, Frankin Lakes, NJ. • 5 ml

Polystyrene round-bottom tube \(12 X 75 mm style; BD Bioscience) • Rosette Sep kit \(Stem Cell Tech, VA) • Complete T lymphocyte culture medium: RPMI 1640 \(Invitrogen, CA), 10% fetal bovine serum \(HyClone, UT), Penicillin \(100 U/ml) and Streptomycin \(100 µg/ml), HEPES \(10 mm, GiBcoBRL NY), L-Glutamine \(2 mm, cellgro Wash. DC. DC). • FACS buffer: 1 X PBS, 1% FBS, 10 mm HEPES, 0.05% NaN3 \(see below). • Human recombinant IL-2 \(biological activity is ≥ 1 X 107 units/mg \(PerproTech Inc.) • Complete T lymphocyte culture medium \(see above). • TRIzol Reagent \(Invitrogen) • NanoDrop \(NP-1000) Spectrophotometer \(NanoDrop Technologies, Inc, Wilmington, DE) • SuperScript III cDNA synthesis system reagents \(Invitrogen) • Generate Human IL-17 full-length cDNA fragments. • RT-PCR primers: \(A) Human β-actin, 5'-TACGACTGGCATCGTGATGGACTCCGGTGACG-3' & 5'-CG TCATACTCCTGCTTGCTGATCCACATCTGC-3'. \(B) Human IL-17, 5'-AAGACCTCATTGGTGTCACTGCTAC -3' & 5'-ATCTCTCAGG GTCCTCATTGCG-3'. \(C) Mouse β-actin, 5'-GTGGGCCGCTCTAGGCACCAA-3' and 5'-TCTTTGCCAATAG TGATGACTTGGC-3' \(D) Mouse IL-17, 5'- ATGCTGTTGCTGCTGCTGAGCC -3' & 5'-GGTCTTCATTGCG GTGGAGAG -3'. • Quantitative RT-PCR \(qRT-PCR) primers and probes: \(A) Human β-actin, 5'-CCTGGCACCCAGCACAAT-3', 5'-GCCGATCCACACGGAG TACT-3' & 5'-FAM-ATCAAGATCATTGCTCCTCCTGAGCGC-BHQ1-3'; \(B) Human IL-17, 5'-ACTGCTACTGCTGCTGAGCCT-3', 5'-TGGGCATCCTGGATT TCG-3' & 5'FAM-AGGCCATAGTGAAGGCAGGAATC-BHQ-1-3'. \(C) Mouse β-actin 5'-CAAGTCATCACTATTGGCAACGA-3', 5'-CCCAAGAAGGAA GGCTGGA-3' &5'FAM-CGGTTCCGATGCCCTGAGGCTC- BHQ-1-3'. \(D) Mouse IL-17, 5'-CTCCAGAAGGCCCTCAGACTAC-3', 5'-AGCTTTCCCTCCG CATTGACACAG-3' & 5'FAM-TCTGGGAAGCTCAGTGCCGCCACCAGC-BHQ-1-3' • PMA \(phorbol 12-myristate 13-acetate; Ionomycin; Calcium \(Sigma). • BD cytofix/Cytoperm kit \(BD Bioscience). • FACS buffer: 500 ml 50 ml 10X HBSS \(GIBCO BRL; Cat#14185-052) 5.0 ml FCS 5.0 ml NaN3 \(5%) 5.0 ml 1M HEPES 435 ml dH_20 \(cell culture grade)

Equipment

• Centrifuges: Sorvall Legend RT \(Germany). • Micro-centrifuges: Eppendorf 5417R and 5415D • Mupid-2 Mini-gel electrophoresis system \(Cosmo Bio Co., LTD, Japan). • BD FACScalibur \(BD Bioscience). • 7500 real-time PCR System \(ABI)

Procedure

Isolation of human peripheral blood mononuclear cells \(PBMC) and CD4+ T cells 1| Transfer blood in heparin vacutainer tubes into 50 ml BLUE MAXTM BD Falcon tube 2| Carefully layer 30 ml diluted blood over 12ml of Isolymph \(do not allow layers to mix) 3| Centrifuge for 30 min \(1,800 rpm) at room temperature. 4| Harvest buffy coat layer \(whitish inter-phase layer containing PBMC) \(~2-5 ml) 5| Transferred to fresh 50 ml Falcon tube and bring to 50 ml with PBS 6| Wash twice in PBS by centrifugation for 10 min in 1,200 rpm at 4 °C 7| Suspend pellet in 5 ml cold complete medium and count cells in hemacytometer. 8| 15-20 ml of buffy coat yields >50×10^6 buffy coat cells 9| Isolate CD4+ T cells from buffy coat with Rosette Sep kit as suggested by manufacturer. **Expansion of Th17 cells in human PBMC by IL-2** **Cell cultures and ELISA** 1| Prepare fresh culture medium containing requisite

antibiotics and serum 2| Re-suspend PBMC or purified CD4+ T cells \(1 X 106 per ml) in presence or absence of IL-2 \(100 u/ml), TGF-β1, IL-6 or IL-23 at 10 ng/ml or IFN-γ at 100 U/ ml. 3| Culture cells in 24 well plate \(2 ml per well) at 37 °C for 4 days in 5% CO_2 4| Harvest culture supernatants \(0.5 ml/well) for ELISA. 5| Multiplex ELISA was performed using Pierce Search Light Technology \(Pierce Boston Technology, Woburn, MA). 6| Harvest cells by pipetting gently up and down with 2 ml Serological pipette 7| Transfer to 15 ml tubes and centrifuge at 1200 RPM \(Sorvall Legend RT) for 10 min. **Intracellular cytokine staining assay** 1| Activate PBMC or CD4+ T cells in the presence or absence of IL-2 for 4 days 2| Stimulate cultures with PMA \(20 ng/ml) and Ionomycin \(1 mm) for 4 h 3| Terminate the reaction by addition of Golgistop to cultures for 1 additional hour 4| Transfer cells into 15 ml of SARSTEDT tubes and wash cells twice in FACS buffer 5| Suspend cells in FACS buffer \(1-2×10^6/ml) 6| Transfer 1 ml aliquots \(1-2×10^6) into separate 5 ml Falcon tubes 7| Centrifuge for 1,200 rpm for 10 min. 8| Perform intracellular staining using BD Cytofix/Cytoperm kit as suggested by manufacturer 9| Subject stained cells to FACS analysis immediately or within 48 h \(keep cells at 4 °C). In our lab, data collection was with FACSCalibur and analyzed using FLOWJO analysis software. **RT-PCR and Quantitative RT-PCR \(qRT-PCR)** **RNA Isolation using TRIZOL Reagent** 1| Homogenize samples in 1 ml of TRIZoL reagent per 50-100mg of tissue \(<15 ml) 2| Transfer to Nalgene tubes and incubate for 5 minutes at room temperature. 3| Add 0.25 ml of chloroform per 1 ml of TRIZoL reagent. 4| Shake vigorously by hand or vortex for 15 seconds. 5| Incubate at room temperature for 2-3 minutes. 6| Centrifuge at 12,000 g \(about 9,000 rpm on SV600) for 15 min at 4 °C. 7| Transfer aqueous phase to fresh Nalgene tubes. 8| Add equal volume of isopropyl alcohol. 9| Mix well and incubate samples at room temperature for 10 minutes. 10| Centrifuge at 12,000 g for 10 minutes at 2-8 °C. 11| Discard supernatant and add 1 ml of 75% ETOH to pellet. 12| Using a 2 ml pipette, scrape the RNA pellet away from tube into the ETOH. 13| Transfer ETOH/RNA mixture to fresh 1.5 ml eppendorf tube. 14| Rinse nalgene tube with more 75% ETOH and transfer to the eppendorf tube. 15| Bring the final volume of RNA/ETOH sample to 1.5 ml with 75% ETOH 16| Centrifuge at max speed in refrigerated microfuge for 10 minute 17| Discard supernatant and add 500 µl 75% ETOH 18| Centrifuge at 10,000 rpm in refrigerated microfuge for 5 minute 19| Pulse the sample and using P100 pipette carefully remove residual ETOH. 20| Air-dry RNA pellet for 15 minutes. 21| Redissolve RNA in DEPC treated water and store in aliquots at -70 °C. 22| Determine RNA concentration using a spectrophotometer. **DNAse Digestion of RNA Samples** 1| To 1.5 ml microcentrifuge tube add the following \(all RNA grade): RNA =X µl 10X Buffer =50 µl DNAse I =1 µl DEPC Water ==>500µl **Note**: Can scale down to 50 µl 2| Digest for 20 minutes at 37 °C. 3| Add 30 µl 20 mM EDTA \(RNA grade) and heat at 95 °C for 5 mins. 4| Phenol/chloroform extraction \(1x): add 500 µl \(Phenol/chloroform 5| Vortex \(1 minute) and centrifuge for 5 minutes at 14,000 rpm \(RT). 6| Transfer aqueous phase \(top) to another RNAse-free 1.5 ml tube. 7| Chloroform extraction: Add 400 µl of chloroform \(with isoamyl alcohol) 8| Vortex \(1 minute) and centrifuge for 5 minutes at 14,000 rpm \(RT). 9| Transfer aqueous phase \(top) to another RNAse-free 1.5 ml tube. 10| Determine the volume of sample 11| Precipitate by adding 1/9 volume of 3 M NaOAc, pH 5.2. 12| Add 2 volumes cold absolute \(95-100%) alcohol 13| Incubate on dry ice \(or -70 °C) for 15 minutes. 14| Centrifuge at 4 °C, 14,000 rpm for 15 minutes. 15| Remove supernatant and add 500 µl of ice-cold 75% EtOH. 16| Incubate for 5 minutes at RT. 17| Centrifuge at 4 °C, 14,000 rpm for 5 minutes. 18| Remove supernatant and add 500 µl of ice-

cold 75% EtOH. 19| Incubate for 5 minutes at RT. 20| Centrifuge at 4 °C, 14,000 rpm for 5 minutes. 21| Remove supernatant 22| Spin for 30 seconds 23| Remove supernatant with 100 µl pipetteman 24| Air dry for 15 minutes. 25| Suspend in DEPC water. **First Strand cDNA Synthesis** _Use only DEPC-treated H_2O, RNase-free tubes, and ART tips_ 1| Wipe down lab bench and pipetteman with 10% H_2O_2 and change gloves frequently 2| Mix all components briefly and spin down. 3| Preset heat blocks or thermocycler to 37 °C, 50 °C and 70 °C, respectively 4| Into a 0.5 ml microcentrifuge tube add 27.5 µl RNA \(1-10 µg) 5| Add 2.5 µl Oligo\(dT) to each tube 6| Incubate mixture at 70 °C for 10 minutes 7| Quench on ice for 1 minute and leave on ice 8| Add the following reagents in the following order: 5 X First Strand buffer 10 µl a. M DTT 5.0 µl 10 mM dNTP 2.5 µl RNAse out 1.0 µl 9| Incubate for 2 minutes at 50 °C 10| Add 2.5 µl Superscript III \(Invitrogen; Catalog # 18080-093) 11| Mix gently by tapping tubes, pulse 12| Incubate at 50 °C for 60 minutes **Note**: Up to this point, be extremely careful to prevent RNase contamination. For preparation of more than 5 RNA samples, Superscript can be added together with the other reagents 13| Incubate at 70 °C for 15 minutes to terminate reaction 14| Incubate tubes at 37 °C for 2 minutes 15| Add 1.0 µl \(3.5 units) of RNase H, mix gently by tapping tubes 16| Incubate at 37 °C for 30 minutes 17| Then at 95 °C for 5 minutes 18| Pulse samples by brief centrifugation 19| Add 6 µl of 4 M LiCl to each tube, vortex and pulse 20| Add 150 µl of cold \(-20°C) absolute EtOH and vortex 21| Freeze in dry ice for 30 minutes 22| Spin at 14,000 rpm for 15 minutes and remove supernatant 23| Add 150 µl of cold 75% EtOH 24| Spin for 15 minutes as above and remove supernatant 25| Air-Dry for 15 minutes 26| Suspend pellet in 50 µl of DEPC-treated H2O 27| Incubate at 70 °C, 10 minutes: to completely dissolve the pellet 28| Save 0.5-1.0 µl for cDNA quantification 29| Store at -20 °C **Note**: Verify that all RNA preparations are of high quality by spectrophotometry \(optical density at 260 Angstrom: >1.9) and 30| Verify integrity of the RNA by Formamide electrophoresis using 1.5-2.0 µg RNA \(use mini-gel apparatus). Examine intensity of 18S and 28S ribosomal RNA. 31| For RT-PCR, incubate samples at 95 °C for 10 min to activate the AmpliTaq Gold and carry out amplification for 30-35 cycles of 30 s each at 95 °C, 60 °C, and 72 °C. Follow this by a final 10-min extension at 72 °C. PCR primers used span at least an intron. 32| Perform real-time 5'-nuclease fluorogenic RT-PCR analysis on an ABI 7500 real-time PCR System using Taqman primer/probe sets \(ABI) and PCR parameters as recommended for TaqMan Universal PCR master mix kit \(PE Biosystems). Calculate relative levels of gene expression by extrapolation from standard curves generated using purified human IL-17 cDNA fragments. Standard curves from our lab showed excellent linearity, indicating precise quantitative relationship between cDNA copy number and fluorescence intensity within the dynamic range of the assay. 33| Normalise the mRNA expression levels to the levels of ACTB \(encoding β-actin) and/or GAPDH housekeeping genes

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