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T cell activation Rho GTPase activating protein (*TAGAP*) is upregulated in clinical and experimental arthritis

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ABSTRACT

Genome-wide association studies have identified various susceptibility variants and loci associated with incidence of rheumatoid arthritis (RA) in different populations. One of these is T cell activation Rho GTPase activating protein (*TAGAP*). The present study sought to measure the expression of *TAGAP* in RA patients, $CD4^+$ T cells subsets from healthy humans and in mice with collagen-induced arthritis. Peripheral blood mononuclear cells (PBMC) from RA patients and tissues of arthritic mice at different stages of the disease were used for the evaluation of *TAGAP* mRNA expression. Increased *TAGAP* expression was observed in RA patients compared to healthy controls, and there were differences in the expression level of *TAGAP* in the tissues of mice with experimental arthritis. Gene expression in $CD4^+$ T cells from healthy humans was greatest 4 h after activation and protein expression was greatest after 24 h. The expression of *TAGAP* was not correlated with $CD4^+$ lymphocyte subsets which were enriched for functionally defined subsets (Th17, Treg, Th1), further indicating its utility as an indicator of lymphocyte activation. These findings indicate that increased TAGAP expression is a distinguishing feature of inflammatory disease and further highlight the role of TAGAP in RA susceptibility.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation in the synovial membranes of joints that leads to bone damage and loss of joint function [1]. RA affects around 0.5–1% of the adult population in developed regions [2–4] although regional variations have been observed [5,6]. The disease is two to three times more common in women than in men, which may be related to hormonal factors [7]. Although the etiology of RA is still unknown, up to 60% incidence of the disease is attributed to genetics [5], and the rest being attributed to environmental factors [9]; these environmental factors may include infections, trauma and life-style that may play role in initiating RA in genetically susceptible individuals.

The strongest evidence for a genetic association with RA comes from twin studies which indicate that monozygotic twins have higher concordance rate than di-zygotic twins. Similarly, the concordance rate is higher in non-twin siblings than in the general population, with estimated heritability ranging from 50% to 60% [7–10]. To date, more than 100 single nucleotide polymorphisms (SNPs) [11–14] and more than 50 genes have been shown to be associated with incidence of RA, including *PTPN22, STAT4, CTLA4, CD28,* and *CCR6* [15–19]. The gene encoding T cell activation Rho GTPase Activating Protein (*TAGAP*) is another such gene, located on chromosome 6q25 in humans, within a 200 kb block of linkage disequilibrium, and encodes a member of Rho-GTPase protein family that releases GTP from GTP-bound Rho [20].

TAGAP acts as a molecular switch and is also thought to be important in modulating cytoskeletal changes [21] in the activation of T cells [22] and is therefore of particular interest in the context of T cell-driven autoimmune disease processes. Furthermore, risk loci at the *TAGAP* locus have also been identified for various autoimmune diseases, including type 1 diabetes, coeliac disease, Crohn's disease and RA [23–26].

In the present study we sought to investigate *TAGAP* expression in healthy individuals, and its role in RA. We compared *TAGAP* in RA patients and controls; in addition to human cells, we extended our study to an in vivo model of RA, collagen-induced arthritis (CIA), to measure the expression of *TAGAP* at different stages of the disease. We then demonstrated that *TAGAP* was associated with immune activation by

Abbreviations: CFA, complete Freud's adjuvant; CIA, collagen-induced arthritis; PBMC, peripheral blood mononuclear cell; RA, rheumatoid arthritis; TAGAP, T cell activation Rho GTPase activating protein

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Table 1
Patient characteristics for gene expression analysis of blood samples.

Number of individuals	16
Age (years; median with range)	60; 34–84
Disease duration (years; median with range)	10; 0.75–58
Male:female	2:14
Treatments (1–4/patient)	
Methotrexate (%)	62.5
Folic acid (%)	31.25
TNF inhibitor (%)	37.5
Prednisone (%)	12.5
Gold injection (%)	6.25
Celecoxib (%)	6.25
Sulfasalazine (%)	6.25

measuring the kinetics of *TAGAP* expression in PBMC, $CD4^+$ T cells and $CD4^+$ subsets from healthy individuals.

2. Materials & methods

2.1. Study subjects

The study subjects for the analysis of *TAGAP* protein and gene expression in PBMC included blood samples from healthy individuals. All the samples were obtained from aphaeresis cones and provided by North London Blood Transfusion Service. The study group for comparative mRNA expression analysis of *TAGAP* and *IFNG* in PBMC comprised 16 RA patients; all met American College of Rheumatology (ACR) 1987 criteria for classification of RA [27]. The demographics of the RA patients are shown in Table 1. The blood samples were obtained with informed consent from patients attending the Rheumatology Clinic at Charing Cross Hospital, London.

2.2. Mice

Arthritis was induced in DBA/1 mice by immunization with type II collagen in complete Freund's adjuvant (CFA) [29]; all procedures were performed pursuant to ethical and regulatory approval. At different stages of disease, mice were culled and then paws removed and snap frozen in liquid nitrogen. The paws were pulverized with the BioPulverizer™ (BioSpec). The resultant powder was homogenized in 500 µL of Trizol reagent [Invitrogen] using a Sample Grinding Kit [GE Healthcare]. The aqueous phase of the Trizol extraction was added to an RNA extraction column [RNeasy Mini Kit, Qiagen] and then the mRNA purification was completed according to the manufacturer's instructions. The mRNA was quantified by Nanodrop and reverse transcribed to make cDNA. Cells from lymph nodes and spleens were processed in a similar fashion but without the use of the BioPulverizer™.

2.3. Antibodies

Anti-human CD3, anti-human CD28, anti-human CD4 PerCP-Cyanine5.5, anti-human CD8a, anti-human CD45RO and anti-human CD45RA antibodies were purchased from eBioscience. Anti-*TAGAP* and goat anti-rabbit IgG Fc antibodies were purchased from Abcam. Anti-human CCR6 and anti-human CD161 antibodies were purchased from Biolegend Ltd.

2.4. Reagents

Lympholyte®-H (Human) Cell Separation Media [Cedarlane], Phosphate Buffered Saline (PBS) [BDH Prolabo], RPMI 1640 complete medium [Gibco Life Technologies], Fetal Bovine Serum (FBS) [Gibco life technologies], Penicillin-Streptomycin (Pen-Strep) [Lonza Biowhittaker], Fixation/Permeabilization Concentrate [eBioscience], Fixation/Permeabilization Diluent [eBioscience], CD4 MicroBeads (Human) [Miltenyi Biotec], RNeasy Mini Kit [Qiagen], High Capacity cDNA Reverse Transcription Kit [Applied Biosciences], Mastermix: EfficienSee FAST qPCR™ Mastermix Plus dTTP [Eurogentec].

2.5. Cell preparation

In brief, human PBMC were purified from apheresis cones using Lympholyte®-H (Human) Cell Separation Media following manufacturer's protocol. Cells from the cone were diluted with phosphate buffered saline (PBS), underlayered with Lympholyte solution and separated by centrifugation at 20 °C at 2000 rpm for 20 min. The PBMC were isolated by aspiration and washed twice with PBS by centrifuging at 1500 rpm at 4 °C for 5 min. PBMC were cultured in RPMI 1640 complete medium containing 10% FBS and 1% Pen-Strep.

2.6. Purification of $CD4^+$ T cell subsets

For the purification of $CD4^+$ T cells from PBMC, a magnetic bead/column-based purification kit from Miltenyi Biotec was utilized, according to the manufacturer's instructions. The purity of the isolated $CD4^+$ T cells was tested by flow cytometric analysis and determined to be greater than 95%. For the enrichment of Th17 precursor cells, PBMC were stained with antibodies and then flow sorted into subsets defined by expression of CD4, CCR6 and CD161. A portion of cells of each subset (5×10^5) was stimulated with TPA (20 ng/mL), ionomycin (1 µM) in the presence of brefeldinA (6.25 µg/mL) for two hours then stained intracellularly with antibodies for IL-17, TNFα and FoxP3; remaining cells were snap frozen for gene expression analysis.

2.7. T cell stimulation

For measurement of the kinetics of *TAGAP* expression, T cells were stimulated for up to 24 h with plate-bound anti-CD3 mAb (1.0 µg/ml) and soluble anti-CD28 mAb (0.5 µg/ml) in RPMI 1640 complete medium.

2.8. Flow cytometry

After each time point, the cells were centrifuged for 5 min at 1500 rpm at 4 °C. After removing the supernatant, the cell pellet was washed with FACS buffer. The cells were stained by surface antibodies for 30 min at 4 °C; antibodies were diluted 1:100 in FACS buffer. After 30 min, the cells were washed with FACS buffer by centrifuging for 5 min at 1500 rpm at 4 °C. Supernatant was removed and the cells were fixed and permeabilised according to the manufacturer's instructions.

After permeabilisation, the cells were ready for intracellular staining. The cells were stained with anti-*TAGAP* antibody and incubated for 30 min at 4 °C. The cells were washed with permeabilisation wash buffer and then stained by secondary antibody and incubated for 15 min at 4 °C. The cells were washed with permeabilisation wash buffer by centrifuging for 5 min at 1700 rpm at 4 °C and the pellet was resuspended in FACS buffer for FACS analysis.

2.9. Quantitative real time PCR analysis

Total RNA was isolated from PBMC and $CD4^+$ T cells using RNeasy Mini Kit as recommended by the manufacturer. Quality of total isolated RNA was then assessed using the Nano drop Spectrophotometer [Labtech International, UK, ND-1000]. Using 500 ng of total RNA, complementary DNA (cDNA) was created using a High Capacity cDNA Reverse Transcription Kit following the manufacturer's protocol. cDNA (100 ng) was then used for gene expression analysis using Taqman gene expression assays for human *TAGAP* (Hs00299284_m1), human HPRT1 (Hs99999909_m1), human GAPDH (Hs99999905_m1), human IFNG (Hs00989291_m1), human IL17A (Hs00174383_m1), human TNF (Hs00174128_m1), mouse *TAGAP* (Mm01304651_m1) and mouse