T cell activation Rho GTPase Activating Protein (TAGAP) is upregulated in Clinical and Experimental Arthritis

Short title: Characterization of *TAGAP* expression

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Keywords

TAGAP, rheumatoid arthritis, T cells, experimental arthritis

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

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 4hrs after activation and protein expression was greatest after 24hrs. 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.

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 200kb 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 measuring the kinetics of *TAGAP* expression in PBMC, CD4⁺ T cells and CD4⁺ subsets from healthy individuals.

MATERIALS & METHODS

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.

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 500µL 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™.

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.

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].

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.

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 x 10⁵) was stimulated with TPA (20ng/mL), ionomycin (1µM) in the presence of brefeldinA (6.25µg/mL) for two hours then stained intracellularly with antibodies for IL-17, TNFα and FoxP3; remaining cells were snap frozen for gene expression analysis.

T cell stimulation

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

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 30min 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 15min 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.

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 500ng of total RNA, complementary DNA (cDNA) was created using a High Capacity cDNA Reverse Transcription Kit following the manufacturer's protocol. cDNA (100ng) 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 *HPRT* (Mm00446968_m1) [Applied Biosystems, Life Technologies]. The cDNA was loaded on a MicroAmp Optical 384-well reaction plate [Applied Biosystems, Life Technologies] along with the gene expression assay and Mastermix: EfficienSee FAST qPCR™ Mastermix Plus dTTP. The plate was then loaded on to the Applied Biosystems ViiA7™ Real-time PCR system using Applied Biosystems QuantStudio Real-Time PCR Software.

Statistics

To determine statistical differences we used Student's t-test or ANOVA, as detailed in the Figure legends.

RESULTS

***TAGAP* gene expression is altered in RA patients and murine experimental arthritis**

TAGAP (Fig. 1A) and *IFNG* (Fig. 1B) mRNA expression was investigated in PBMC from RA patients (see Table 1) and healthy controls. *TAGAP* level was significantly higher in RA patients compared to healthy controls (Fig. 1A). There was no correlation between *TAGAP* and *IFNG* expression level in patient and control samples and no effect of broadly-defined treatment groups (Fig. 1C). We examined the *TAGAP* mRNA expression levels in the lymph nodes, spleens and paws of DBA/1 mice that had been immunized with bovine type II collagen and CFA [29]. In spleens and lymph nodes there was a decrease in the expression of *TAGAP* as the disease progressed (Fig. 1D, E); the expression of *TAGAP* was moderately increased in affected paws, compared to unaffected paws in the same mice (Fig. 1F).

Kinetics of *TAGAP* protein expression following stimulation of CD4⁺ T cells

By flow cytometry analysis, it was shown that *TAGAP* protein expression was present on CD4⁺ T cells, as well as CD8⁺ T cells in the unstimulated state (Fig. 2A). The expression was increased by anti-CD3/28 stimulation in CD4⁺ cells over a 24 hour period, but the increase in expression in CD8⁺ cells was negligible (Fig. 2B). The gene expression of *TAGAP*, *IFNG*, *IL17A* and *TNF* was measured in purified CD4⁺ T cells that were stimulated with anti-CD3/28 (Fig. 2C). *TAGAP* gene expression peaked 2 hours after stimulation and declined to a level below unstimulated cells. In contrast, *IFNG*, *IL17A* and *TNF* were induced more strongly, reaching a peak at 4 and 6 hours after stimulation then declining slightly.

***TAGAP* expression within freshly isolated Th17-enriched lymphocytes**

Circulating CD4⁺ T cells expressing high levels of CCR6 are an enriched source of Th17 precursors and CD4⁺CCR6⁺ cells expressing the activation-associated surface marker CD161 are further enriched for this lymphocyte subset, the prevalence of which is increased in RA [30]. A difficulty in measuring *TAGAP* in functionally defined T cell subsets is that the stimulation that is necessary to induce cytokine expression to identify subsets (e.g. Th1 and Th17) may induce expression of *TAGAP*. To determine whether the expression of *TAGAP* was enriched in lymphocyte subsets biased towards the Th17 lineage, we sorted CD4⁺, CD4⁺CCR6⁺CD161⁻ and CD4⁺CCR6⁺CD161⁺ cells (Fig. 3A) and confirmed the relative enrichment of IL-17 producing cells from each subset (Fig. 3B, C). After extracting mRNA from each freshly isolated, unstimulated subset, the relative expression of *TAGAP* was determined by qPCR and found to be similar between freshly isolated CD4⁺CCR6⁺CD161⁻ and CD4⁺CCR6⁺CD161⁺ cells (Fig. 3D). Genes associated with functionally-defined lymphocyte subsets (Th17, Treg and Th1), were measured in each sorted subset (Figure 3E) but was not correlated with the gene expression profile of *TAGAP*.

DISCUSSION

To date, studies conducted on the *TAGAP* gene focus on the association of polymorphisms with particular diseases. For example, variations in the *TAGAP* gene are linked with various immune mediated pathologies, such as celiac disease, Crohn's disease, type 1 diabetes and RA [23-26]. Polymorphisms in the *TAGAP* gene are a shared risk factor between Crohn's disease and celiac disease and between RA, type-1 diabetes and celiac disease. Some variants of *TAGAP* suggest a potentially protective role for anal sepsis in ileocolic Crohn's disease [28]. No study so far has investigated *TAGAP* expression in RA.

In the present study, we found that the gene expression of *TAGAP* and *IFNG* was greater in PBMC from RA patients compared to healthy controls however, there was not a significant correlation between these genes that would suggest co-expression within IFN γ -expressing Th1 lymphocytes, which are known to infiltrate the synovial membrane in RA[31]. An investigation into *Tagap* gene expression in the tissues of collagen-induced arthritis mice at different stages of the disease – pre-onset, day of onset and day 10 post-onset – revealed a downward trend in lymph node and spleen gene expression and increased expression in affected paws; the change in expression mirrors the migration of leukocytes to sites of antigen presentation to sites of disease as the immune response progresses.

We observed the expression of TAGAP protein, via FACS, in CD4⁺ T cells and CD8⁺ T cells from healthy humans and our observations were broadly in agreement with Berge T et al [32]. Within sorted subsets of unstimulated CD4⁺ T cells with differing Th17 potential, there was no difference in the gene expression of *TAGAP*. As there is no correlation between *IFNG* and *TAGAP* gene expression in RA patients, and no differential expression of *TAGAP* within lymphocyte subsets with dissimilar Th17-enriched precursor prevalence, the expression of TAGAP in CD4⁺ lymphocytes may therefore be associated with activated phenotype rather than a functional phenotype such as Th1 or Th17 lymphocytes, which are increased in the circulation of RA patients.

In CD4⁺ T cells that had been stimulated in vitro, the maximal TAGAP protein expression was observed at 24 hours, whereas expression on CD8⁺ T cells was unchanged. *TAGAP* gene expression in healthy CD4⁺ T cells was measured for 24h after stimulation, and was highest between 2 and 6 hours after stimulation and declined thereafter. These findings are similar to the findings by Mao *et al.*, 2004 where *TAGAP* transcript was transiently expressed after 4 hrs of activation [22]. We also measured *IFNG*, *IL17A* and *TNF* mRNA expression level in the same stimulated CD4⁺ T cells and found that these genes are upregulated after 2h of activation and peaked between 4 and 6 hours after stimulation; thus, at the gene level *TAGAP* appears to be more transiently induced then down-regulated compared to *IFNG*, *IL17A* and *TNF*.

1 This is the first study of its kind to extend the known association of the *TAGAP* gene polymorphism with
2 RA to a potential association with inflammatory disease processes. Recently a study by Tamehiro N
3 et.al[33] demonstrated a key role for TAGAP in murine Th17 lymphocytes. The loss of TAGAP reduced
4 in vitro differentiation of Th17 differentiation and ameliorated the clinical features of experimental
5 autoimmune encephalomyelitis; a key mechanistic finding was the interaction of TAGAP with RhoH, and
6 in doing so competing with ZAP70, resulting in reduced TCR signalling[33]. The previous finding that
7 RhoH overexpression can bypass β -selection in the thymus[34], indicates TAGAP may have a significant
8 role in steady state T cell development however, further research will be required to elucidate the precise
9 role played by TAGAP in immune cell activity. As TAGAP is expressed at significantly higher levels in
10 RA patients than in controls, its expression level may be useful as a marker of T cell activity and may
11 therefore help in the stratification of patients or as a potential therapeutic target.

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8

9 **CONFLICT OF INTEREST**

10 The authors have no competing interests.

REFERENCES

1. Firestein GS. Evolving concepts of rheumatoid arthritis. *Nature*. 2003; 423: 356-61.
2. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *N Engl J Med*. 2011; 365: 2205-19.
3. Turesson C, Jacobsson L, Bergström U. Extra-articular rheumatoid arthritis: prevalence and mortality. *Rheumatology (Oxford)*. 1999; 38: 668-74.
4. Moreland L. Unmet needs in rheumatoid arthritis. *Arthritis Res Ther*. 2005; 7: S2-8.
5. Chang M, Rowland CM, Gracia VE, et al.. A Large-Scale Rheumatoid Arthritis Genetic Study Identifies Association at Chromosome 9q33.2, *PLoS Genetics*. 2008; 4: e1000107.
6. Gibofsky A, Winchester RJ, Patarroyo M, Fotino M and Kunkel HG. 1978. Disease associations of the Ia-like human alloantigens: contrasting patterns in rheumatoid arthritis and systemic lupus erythematosus. *J Exp Med*. 148:1728-1732.
7. Silman AJ, Macgregor AJ, Thomson W, et al. Twin concordance rates for rheumatoid arthritis: results from a nationwide study. *Br J Rheumatol*. 1993;32:903-7.
8. Bax M, Van heemst J, Huizinga TW, Toes RE. Genetics of rheumatoid arthritis: what have we learned?. *Immunogenetics*. 2011;63:459-66.
9. Macgregor AJ, Snieder H, Rigby AS, et al. Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. *Arthritis Rheum*. 2000;43:30-7.
10. Seldin MF, Amos CI, Ward R, Gregersen PK. The genetics revolution and the assault on rheumatoid arthritis. *Arthritis & Rheumatism*. 1999; 42:1071–79.
11. Okada Y, Wu D, Trynka G, et al. Genetics of rheumatoid arthritis contributes to biology and drug discovery. *Nature*. 2014;506:376-81.
12. Stahl EA, Raychaudhuri S, Remmers EF, et al. Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. *Nat Genet*. 2010;42:508-14.
13. Okada Y, Terao C, Ikari K, et al. Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population. *Nat Genet*. 2012;44:511-6.
14. Eyre S, Bowes J, Diogo D, et al. High-density genetic mapping identifies new susceptibility loci for rheumatoid arthritis. *Nat Genet*. 2012;44:1336-40.
15. Prasad P, Kumar A, Gupta R, Juyal RC, Thelma BK. Caucasian and Asian specific rheumatoid arthritis risk loci reveal limited replication and apparent allelic heterogeneity in north Indians. *PLoS ONE*. 2012;7:e31584.

16. Hinks A, Barton A, John S, et al. Association between the PTPN22 gene and rheumatoid arthritis and juvenile idiopathic arthritis in a UK population: further support that PTPN22 is an autoimmunity gene. *Arthritis Rheum.* 2005;52:1694-9.
17. Kochi Y, Okada Y, Suzuki A, et al. A regulatory variant in CCR6 is associated with rheumatoid arthritis susceptibility. *Nat Genet.* 2010;42:515-9.
18. Lei C, Dongqing Z, Yeqing S, Oaks MK, Lishan C, Jianzhong J, et al. Association of the CTLA-4 gene with rheumatoid arthritis in Chinese Han population. *Eur J Hum Genet.* 2005;13:823-8.
19. Kobayashi S, Ikari K, Kaneko H, Kochi Y, Yamamoto K, Shimane K, et al. Association of STAT4 with susceptibility to rheumatoid arthritis and systemic lupus erythematosus in the Japanese population. *Arthritis Rheum.* 2008;58:1940-6.
20. Smyth DJ, Plagnol V, Walker NM, Cooper JD, Downes K, Yang JHM, et al. Shared and Distinct Genetic Variants in Type 1 Diabetes and Celiac Disease. *N Engl J Med.* 2008; 359: 2767–2777.
21. Connelly TM, Berg AS, Harris LR, et al. T cell activation Rho GTPase-activating protein expression varies with inflammation location and severity in Crohn's disease. *J Surg Res.* 2014;190:457-64.
22. Mao M, Biery MC, Kobayashi SV, et al. T lymphocyte activation gene identification by coregulated expression on DNA microarrays. *Genomics.* 2004;83:989-99.
23. Eyre S, Hinks A, Bowes J, et al. Overlapping genetic susceptibility variants between three autoimmune disorders: rheumatoid arthritis, type 1 diabetes and coeliac disease. *Arthritis Res Ther.* 2010;12:R175.
24. Franke A, McGovern DP, Barrett JC, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet.* 2010;42:1118-25.
25. Festen EA, Goyette P, Green T, et al. A meta-analysis of genome-wide association scans identifies IL18RAP, PTPN2, TAGAP, and PUS10 as shared risk loci for Crohn's disease and celiac disease. *PLoS Genet.* 2011;7:e1001283.
26. Chen R, Stahl EA, Kurreeman FA, et al. Fine mapping the *TAGAP* risk locus in rheumatoid arthritis. *Genes Immun.* 2011;12: 314-8.
27. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* 1988;31:315-24.
28. Connelly TM, Sehgal R, Berg AS, et al. Mutation in *TAGAP* is protective of anal sepsis in ileocolic Crohn's disease. *Dis Colon Rectum.* 2012;55:1145-52.

29. McCann FE, Palfreeman AC, Andrews M, Perocheau DP, Inglis JJ, Schafer P, Feldmann M, Williams RO and Brennan FM. Apremilast, a novel PDE4 inhibitor, inhibits spontaneous production of tumour necrosis factor- α from human rheumatoid synovial cells and ameliorates experimental arthritis. *Arthritis Res Ther*. 2010;12:R107.
30. Cosmi L, Palma RD, Santarlasci V, Maggi L, Capone M, Frosali F, et al. Human interleukin 17-producing cells originate from a CD161⁺CD4⁺ T cell precursor. *J Exp Med*. 2008; 205: 1903-16.
31. Choy E. Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis. *Rheumatology (Oxford)*. 2012; 51: v3-11.
32. Berge T, Leikfoss IS, Brorson IS, Bos SD, Page CM, Gustavsen MW, et al. The multiple sclerosis susceptibility genes TAGAP and IL2RA are regulated by vitamin D in CD4⁺ T cells. *Genes Immun*. 2016;17:118-27.
33. Tamehiro N, Nishida K, Yanobu-Takanashi R, Goto M, Okamura T, Suzuki H. T-cell activation RhoGTPase-activating protein plays an important role in T(H)17-cell differentiation. *Immunol Cell Biol*. 2017, May 2.
34. Tamehiro N, Oda H, Shirai M, Suzuki H. Overexpression of RhoH Permits to Bypass the Pre-TCR Checkpoint. *PLoS One*. 2015 Jun 26;10(6):e0131047. doi:10.1371/journal.pone.0131047. eCollection 2015.

1 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

2

FIGURE LEGENDS

Figure 1. *TAGAP* gene expression level in RA patients and murine collagen-induced arthritis (A) *TAGAP* and (B) *IFNG* gene expression in the freshly isolated PBMC from RA patients and healthy controls; * $p < 0.05$, t-test. (C) Correlation between *TAGAP* and *IFNG* gene expression; legend – ● methotrexate in combination with another therapy, ○ methotrexate alone, ♦ treatment without methotrexate. The gene expression of *Tagap* was measured in (D) the spleens, (E) the lymph nodes and (F) the paws of DBA/1 mice at different stages of collagen-induced arthritis – unimmunized mice (Naïve), pre-onset (Pre), the 1st day of disease incidence (d1), the 10th day of disease (d10), unaffected paws (Un) and affected paws (Af); * $p < 0.05$, ** $p < 0.01$, ANOVA.

Figure 2. *TAGAP* gene and protein expression is induced by antigen stimulation. Flow cytometric measurement of *TAGAP* protein expression level in (A) $CD4^+$ and $CD8^+$ lymphocytes from healthy individuals; representative FACS plots of *TAGAP* express in cells stimulated with anti- $CD3/28$ for 0h, 2h, 8h and 24h. (B) Kinetics of *TAGAP* protein expression at different time points in $CD4^+$ and $CD8^+$ T cells during anti- $CD3/CD28$ stimulation; $n=3$ donors, * $p < 0.05$, ANOVA. (C) *IL17A*, *IFNG*, *TNA* and *TAGAP* mRNA expression up to 24hr post stimulation in human $CD4^+$ T cells from normal donors; values are mean $\pm$ SEM, $n=8$ donors, * $p < 0.05$, ANOVA.

Figure 3. *TAGAP* gene expression in human $CD4^+$ T cell subsets. (A) Cells were sorted into populations consisting of (i) $CD4^+$, (ii) $CD4^+CCR6^+CD161^-$ and (iii) $CD4^+CCR6^+CD161^+$ lymphocytes; post-sort purity check from a representative donor. (B, C) The increasing proportion of Th17 from each subset was enumerated and confirmed by intracellular cytokine staining after stimulation; labels (i-iii) correspond to subsets from (A). (C) For 8 donors the increased prevalence of Th17 was confirmed in $CD4^+CCR6^+CD161^+$ lymphocytes; ** $p < 0.01$, t-test. (D) For 4 donors, the gene expression of *TAGAP* as measured in freshly sorted, unstimulated lymphocyte subsets. (E) Expression of canonical genes associated with functionally-defined lymphocyte subsets in sorted subsets as indicated; ** $p < 0.01$, t-test.