IL-6 Production Through Repression of *UBASH3A* Gene via Epigenetic Dysregulation of Super-Enhancer in CD4⁺T Cells in Rheumatoid Arthritis

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Abstract

Background.

UBASH3A as a negative regulator of T-cell receptors (TCRs) signaling is a susceptible factor for rheumatoid arthritis (RA) patients who exhibit immune dysfunction. We studied UBASH3A expression in CD4⁺ T cells from healthy donors and RA patients, the involvement of super-enhancer (SE) in the control of UBASH3A expression, and the contribution of UBASH3A to proinflammatory cytokine production.

Methods.

The UBASH3A mRNA and protein levels were examined by quantitative PCR and Western blotting, respectively. Locked nucleic acid was administered to cells to inhibit enhancer RNA (eRNA) expression. Factors recruited to *UBASH3A* loci displaying SE architecture were studied by chromatin immunoprecipitation. CD4⁺ T cells were transfected with *UBASH3A* plasmids, and cytokine levels were measured by cytometric bead array.

Results.

UBASH3A mRNA and protein expression was lower in CD4⁺ T cells from RA patients than in those from healthy donors. *UBASH3A* mRNA levels were reduced by eRNA_1 and eRNA_3 knockdown. In RA patients, BTB and CNC homology 2 (BACH2), the silencing transcription factor, accumulated at the *UBASH3A* loci in CD4⁺ T cells, whereas the SE-defining factor, mediator complex subunit 1 (MED1)/bromodomain 4 (BRD4), did not. However, opposite phenomena were observed in healthy donors. Although stimulation of TCRs expressed on CD4⁺ T cells from RA patients led to interleukin (IL)-6 production, UBASH3A over-expression significantly inhibited its production.

Conclusions.

Transcription of *UBASH3A* is suppressed via epigenetic regulation of SE in CD4⁺ T cells from RA patients. Decreased UBASH3A levels lead to excessive activation of TCR signaling, resulting in enhanced production of IL-6.

Background

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by synovitis and progressive joint destruction [1–3]. The expression of proinflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-1 β , and matrix metalloproteinase 3, increases in the synovial fluid of patients with RA, and lymphocytes infiltrate the synovial membrane, resulting in RA pathogenesis [4, 5]. Among

the drivers of RA progression, IL-6 is a critical factor that induces a number of RA-related pathogenic events, such as imbalances between Th17 and Tregs, overproduction of autoimmune antibodies, systemic inflammation, and joint destruction when ectopically overexpressed [6]. Reflecting the role of these factors, their inhibitors have been successfully applied in clinical practice [7]. Although some patients experience RA remission, others fail to achieve remission or experience relapse, suggesting that identification of RA drivers remains limited in terms of the pathogenesis and progression of RA.

A set of such unidentified RA drivers appears to be associated with the patient's genetic background because 101 single nucleotide polymorphisms (SNPs) for susceptibility gene loci have been identified by genome-wide association studies (GWASs) in large cohorts [8]. Most SNPs related to disease are generally located in non-coding regions instead of protein-coding mRNA gene loci. Importantly, most of these RA-susceptibility SNPs were mapped to enhancer regions. Compared with typical enhancers, super-enhancers (SEs), large enhancer clusters consisting of individual enhancers, harbor 3.2 times more RA-susceptibility SNPs, suggesting that RA-susceptibility SNPs are strongly associated with transcriptional regulation via SEs [9, 10]. Strikingly, in CD4⁺ T cells, 26% of RA-susceptibility SNPs (27/101) were found to be located within the activated SEs [11]. SEs serve as pivotal enhancers to define cell identity through directing global gene regulation. The function of SEs has recently been delineated to induce dynamic chromatin looping between the SE regions and the promoter regions of the target genes with the aid of enhancer RNAs (eRNAs) transcribed from the SE regions.

Considering the previous findings of RA-associated SNP mapping in SEs, we reasoned that the dysfunction of a SE is causal for RA progression by dysregulating the expression of RA-related gene(s) directed by the SE. Therefore, we examined CD4⁺ T cells consisting of several subsets of helper T (Th) cells because CD4⁺ T cells serve as drivers in RA pathogenesis by producing inflammatory cytokines. Using an *in-silico* approach with multiple databases, we elucidated a RA-susceptibility gene using our gene mining criteria. This gene encodes ubiquitin-associated and SH3 domain-containing protein A (UBASH3A), which has been reported to attenuate T-cell receptor (TCR) signaling by targeting the TGF- β -activated kinase 1 and NF- κ B signaling axis. Because the RA-associated SNP (rs189352) has been reported in a SE of the *UBASH3A* locus, we assessed UBASH3A expression and its regulatory mechanism in CD4⁺ T cells, and found that UBASH3A expression was indeed downregulated in CD4⁺ T cells from RA patients, presumably due to dysfunction of the *UBASH3A* SE.

Methods

In-silico study (bioinformatic tools)

dbSUPER (<https://asntech.org/dbsuper/>) is a database containing 82,234 SEs in 102 human and 25 mouse tissue/cell types. RA-susceptibility genes were detected by GWAS, as reported previously [12]. BioGPS (<http://biogps.org/#goto=welcome>) is a database that summarizes the patterns of mRNA expression specific to human and mouse tissues and cells that have been identified by oligonucleotide array analysis.

Preparation of CD4⁺ T cells

CD4⁺ T cells were prepared as described previously [2]. First, CD4⁺ T cells were collected from peripheral blood mononuclear cells of healthy donors (n=11; male:female=3:8; age, 42.5±7.3 years; data are expressed as the mean ± standard deviation) and of RA patients (n=24; male:female=8:16; age, 49.3±5.1 years; CDAI: 136.5±48.6; SDAI: 140.3±50.9; DAS28: 6.1±1.2) using CD4⁺ microbeads from the CD4⁺ T Cell Isolation Kit (130-096-533; Miltenyi Biotec, Auburn, CA, USA) (Additional file 1: Table S1). CD4⁺ T cells were sorted into subsets by fluorescence-activated cell sorting (FACS) gating. Cells were then seeded into six-well plates at a density of 1 × 10⁶ cells/well and incubated in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum and 1× antibiotics at 37°C under 5% CO₂ atmosphere. Jurkat cells were treated with anti-CD28 (PV1) or anti-CD3 (2C11) for 6 h to activate TCR signaling [13].

Immunoblotting (IB)

CD4⁺ T cells were prepared from PBMC of HD (n=3; male/female 1/2) and patients with RA (n=3; male/female 1/2). CD4⁺ T cell line Jurkat as a positive control was also prepared. IB was performed as described previously [14]. Briefly, cells were lysed in TNE buffer (50 mM Tris-hydrochloride [pH 7.4], 0.5% [v/v] Nonidet P-40, 150 mM sodium chloride, 5 mM ethylenediaminetetraacetic acid, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, and 10 mg/mL aprotinin), and whole-cell extracts were prepared. Proteins (7 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Protran BA83; GE Healthcare, Chalfont St. Giles, UK) for blocking. The membranes were incubated with anti-phospho-NF-κB antibody (Ser 536, # 3033; Cell Signaling Technologies, Danvers, MA, USA), anti-NF-κB antibody (# 8242; CST), anti-UBASH3A antibody (GTX116432; GeneTex; Irvine, CA, USA), and anti-β actin antibody (A1978; Sigma-Aldrich, St. Louis, MO, USA). The bound antibodies were visualized with anti-secondary antibodies against mouse and rabbit immunoglobulin G (IgG, GE Healthcare) conjugated with horseradish peroxidase and chemiluminescence reagent (ECL[™] Prime western blotting Detection Reagent; GE Healthcare).

Cytometric bead array (CBA)

CBA was performed as described previously [14]. Briefly, the supernatant from cultured CD4⁺ T cells was collected 24 h after transfection of the cells with plasmid cloning DNA (pcDNA) 3.1-UBASH3A (courtesy of Professor Patrick Concannon) and empty vector. Next, 20 µL of the supernatant was reacted with 0.2 µL of capture beads of the Human Flex Set (558279 for IL-1 beta, 558276 for IL-6, 560383 for IL-17A, and 558273 for TNF; BD Biosciences, San Diego, CA, USA) and 19.8 µL of RPMI medium for 1 h at room temperature. Then, 0.2 µL of phycoerythrin detection reagent and 19.8 µL of RPMI medium were added, and the mixture was allowed to react for 1 h. The supernatant was rinsed two times with FACS buffer, centrifuged, and suspended in 150 µL of FACS buffer. Finally, each cytokine was detected by FACS, and its concentration was analyzed by FCAP Array.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed as described previously [15]. Briefly, total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed using SuperScript®VILO™ Master Mix (Life Technologies). On a Step One Plus system (Applied Biosystems), qPCR was performed with TaqMan®Fast Universal PCR Master Mix (Applied Biosystems) and the TaqMan® Gene Expression Assay (Applied Biosystems) primer/probe pairs to measure the expression of the following genes (Additional file 2: Table S2): *UBASH3A*, interleukin-1 beta (*IL-1β*), *IL-6*, *IL-17A*, tumor necrosis factor-alpha (*TNF-α*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Histological examination

A patient with RA (n=1) and a patient with dermatomyositis (DM, n=1) who met the diagnostic criteria consented to provide lymph node samples. This study was conducted with the approval of the ethics committee of the University of Occupational and Environmental Health. Lymph node tissue samples were collected, fixed in 10% formaldehyde, and embedded in paraffin. Immunofluorescence staining was performed as described previously [14]. Briefly, the biopsy samples were immersed in phosphate-buffered saline with Tween (PBST; 0.05% [v/v], pH 6.0) containing sodium citrate (5 mM) to inactivate antigens. The samples were blocked with a serum-free protein block (Dako, 2016-08) and reacted with polyclonal antibodies that recognize human CD4 (1:200; ab67001; Abcam, Cambridge, MA) and *UBASH3A* (1:200; ab197168, Abcam). The slides were rinsed with PBST and then incubated for 1 h with an anti-rabbit IgG secondary antibody labeled with fluorescein isothiocyanate and an anti-mouse IgG secondary antibody labeled with rhodamine (1:500; DakoCytomation, Glostrup, Denmark). Nuclei were stained with 4',6-diamidino-2-phenylindole (1:200; MERCK). The stained samples were observed using an all-in-one fluorescence microscope.

Chromatin immunoprecipitation-PCR (ChIP-PCR)

ChIP-PCR was performed as described previously [15]. CD4⁺ T cells collected from healthy donors and RA patients were cultured in RPMI 1640 medium. Chromatin was cross-linked with 1% formaldehyde for 10 min and fragmented to 200–500 bp by sonication. DNA was extracted using the EZ ChIP Kit (Merck Millipore, Taufkirchen, Germany) in accordance with the manufacturer's instructions. DNA was immunoprecipitated using a series of antibodies. PCR was performed using specific primers (Additional file 2: Table S2).

Plasmid transfection

According to the manufacturer's instructions, pcDNA3.1-*UBASH3A* and empty vector were transfected into CD4⁺ T cells (1 × 10⁵ cells) collected from healthy donors and RA patients using Lipofectamine 3000 and P3000 reagents (Thermo Fisher Scientific). In contrast, locked nucleic acid (LNA) was designed to knock-down the expression of eRNA (Additional file 2: Table S2). According to the manufacturer's instructions,

LNA targeting eRNA and control LNA were transfected into CD4⁺ T cells (1 x 10⁵ cells) with RNAi MAX (Thermo Fisher Scientific) reagents for 72 h.

Genotyping

SNP-PCR was performed according to the manufacturer's instructions to determine the genotypes of rs1893592-A/C as described previously [16]. On a Step One Plus system (Applied Biosystems), PCR was performed in 10 µL of reaction volume containing 1 µL of genomic DNA (20 ng/µL), 5 µL of TaqMan Universal PCR Master Mix, 0.25 µL of VIC/FAM-labeled probe (200 nM), and 3.75 µL of double-distilled water. The probes used are listed in Supplementary Table 2. The series of reactions was analyzed using the Allelic Discrimination Sequence Detection Software (Applied Biosystems).

Statistical analysis

All quantified data are expressed as the mean ± standard deviation. Differences between two groups were tested for statistical significance by Student's unpaired two-tailed t-test or Dunnett's multiple comparison test. Statistical significance was set at P<0.05. Statistical analyses were performed using SPSS statistical software (v. 21.0; IBM Corp., Armonk, NY, USA).

Results

Identification of UBASH3A as an RA-susceptibility gene by in-silico mining

On the basis of previous findings that RA pathogenesis is associated with SNPs located in SEs, we presumed that dysregulated expression of RA-susceptibility genes drives RA progression. Under the concept that expression of such RA-susceptibility genes is directed by SEs harboring SNPs, we performed *in-silico* screening of RA-susceptibility genes that are associated with SNPs in the SEs and that are highly expressed in CD4⁺ T cells (Fig. 1A). Under this criterion, *UBASH3A* was extracted. The gene locus of *UBASH3A* is encompassed in a SE (designated as *UBASH3A*-SE hereafter) registered in dbSUPER, and a SNP (rs1893592) with RA-susceptibility identified by the GWAS report [8] is positioned in this *UBASH3A* locus (Fig. 1B).

Downregulated expression of UBASH3A in CD4⁺ T cells from RA patients

We then investigated if the expression level of *UBASH3A* was indeed dysregulated during RA progression, reflecting the BioGPS database. Peripheral blood mononuclear cells collected from RA patients and the control group of healthy donors were separated into CD4⁺ T and CD4⁻ cells by FACS gating, and *UBASH3A* mRNA levels were quantified by RT-qPCR. As shown in Fig. 1C, *UBASH3A* expression in CD4⁺ T cells from the RA patients was significantly reduced compared with that of healthy donors. Although a significant correlation of the SNP genotype of the rs1893592-A/C allele with RA susceptibility was previously reported [8], an association of *UBASH3A* expression with this SNP genotype in the examined RA patients could not be identified in the limited number of subjects (Fig. 1D). We then examined the

expression levels of *UBASH3A* among subsets of CD4⁺ T cells that were separated into Th1, Th2, Th17, and regulatory T cells by cell sorting [3]. However, no significant difference in *UBASH3A* expression was observed among the subsets (Additional file 3: Figure S1). To verify the downregulated expression of *UBASH3A* in RA patients, *UBASH3A* protein levels were then examined by Western blotting. Reduced expression of *UBASH3A* protein in RA patients was confirmed (Fig. 1E). When compared with dermatomyositis patients by double immunofluorescence staining of CD4 and *UBASH3A* in CD4⁺ T cells from lymph nodes, less staining of *UBASH3A* was noted in RA patients (Fig. 1F).

Enhancer RNAs (eRNAs) transcribed from *UBASH3A*-SE facilitate *UBASH3A* expression in CD4⁺ T cells.

To explore the molecular basis for the observed decrease in *UBASH3A* expression in RA patients, we assessed the activity of *UBASH3A*-SE, because SEs are prime regions governing the transcription of adjacent genes. Because SE activity is dependent on the expression of eRNAs transcribed from the SE, we measured the expression of a set of eRNAs (*UBASH3A* eRNAs). The eRNA candidates were examined by *in-silico* screening based on H3K27ac marks documented in lymphoblastoma in the ENCODE database, as well as the registered transcript signals obtained by RNA-seq analysis of lymphoblastoma (GM12878) and leukemia cells (K562) in dbSUPER (Fig. 2A). According to the peaks of H3K27ac, we constructed PCR primers (Fig. 2A and B) that could detect the expression of three transcripts (designated as *UBASH3A* eRNA_1–eRNA_3 hereafter) by qRT-PCR in CD4⁺ T cells (Fig. 2C). To assess if these eRNA candidates were functional as eRNAs that facilitate *UBASH3A* expression, the effect of their knockdown on *UBASH3A* expression was investigated by an Locked nucleic acid (LNA)-mediated RNAi approach (Additional file 2: Table S2). CD4⁺ T cells were transfected with LNAs targeting both strands of the eRNAs_1–3 and the expression levels of *UBASH3* and eRNAs_1–3 were tested. The LNAs targeting *UBASH3A* eRNA_1 and eRNA_3 attenuated their expression (Fig. 2C), but such an effect was unlikely for the LNAs targeting *UBASH3A* eRNA_2. Reflecting the attenuated expression of eRNA_1 and eRNA_3, *UBASH3A* mRNA levels were reduced (Fig. 2D). Thus, these findings suggest that *UBASH3A* expression is under control of *UBASH3A*-SE, at least, the regions encoding eRNA_1 and eRNA_3.

Recruitment of silencing transcription factor to the *UBASH3A*-SE of CD4⁺ T cells from RA patients

UBASH3A expression in CD4⁺ T cells was lowered by RA progression, and therefore we reasoned that the activity of *UBASH3A*-SE is compromised during RA progression, and the identified regulatory regions encoding the *UBASH3A* eRNAs are dysfunctional. To investigate this idea, we explored several possible mechanisms, and identified the presence of a putative binding site (TGCTGAGATGCA) for BTB and CNC homology 2 (BACH2), a silencing transcription factor, by mining the binding sites for MafK, a heterodimer partner for BACH2 (Fig. 3A) [17]. High expression of BACH2 was previously reported in lymphocytes, including B and T cells [18], while MED1 (mediator complex subunit 1) and BRD4 (bromodomain-containing protein 4) are core factors underlying SE function and are detectable on the eRNA-encoding SE regions [18]. Consistent with the observed expression of *UBASH3A* eRNA_1 and _2, clear recruitment of MED1 and BRD4 was observed on two coding regions in healthy CD4⁺ T cells by ChIP assay. However, in

CD4⁺ T cells from RA patients, this recruitment was reduced, but BACH2 recruitment was evident (Fig. 3B), suggesting reduced activity of these two regulatory SE regions. Although another transcriptional repressor (Blimp1) is expressed in T cells, its recruitment to the examined coding regions was not observed (Fig. 3B). Because no clear difference in H3K27ac levels was detected in healthy and RA CD4⁺ T cells, and UBASH3A was expressed at certain levels in both groups (Fig. 1C and F), we presumed that SEs were partially active at this *UBASH3A* locus.

Ubash3a Attenuated Tcr Signaling In Cd4 T Cells

UBASH3A has been reported as an inhibitory factor for TCR signaling in CD4⁺ Jurkat cells [19]; therefore, we assessed the inhibitory function of UBASH3A in CD4⁺ T cells. TCR signaling activation by antibodies against CD3 and CD28 [13] was monitored by the phosphorylation of NF-κB, a TCR signaling downstream mediator, under the expression of UBASH3A (Fig. 4A and B). Transient expression of *UBASH3A* transcript induced by its expression vector was attenuated to some extent in the cells from both groups when treated with CD3 and CD28 antibodies (Fig. 4A). However, under this treatment, the induced phosphorylation of NF-κB was clearly inhibited by UBASH3A expression in CD4⁺ T cells (Fig. 4B and C), consistent with previous reports [19].

UBASH3A acted as a suppressor for IL-6 induction in CD4⁺ T cells

Cytokines produced in T cells with activated TCR signaling are clinically significant in RA progression. Therefore, we investigated if the observed function of UBASH3A attenuating TCR signaling suppresses cytokine production in TCR signaling-activated CD4⁺ T cells, by measuring cytokines associated with RA progression in CD4⁺ T cells from healthy donors under UBASH3A-overexpressing conditions, with or without treatment with CD3 and CD28 antibodies (Fig. 5A).

Activation of CD4⁺ T cells from healthy donors led to robust production of *IL17*, *IL6*, and *TNF* mRNA (Fig. 5B) as well as IL-6 and TNF-α protein in the culture media (Fig. 5C). In CD4⁺ T cells from RA patients, augmented expression of all the tested cytokines (IL-17, IL-1β, IL-6, and TNF-α) was observed as expected even without treatment (Fig. 5D). The treatment could be further effective in potentiating the production of cytokines, particularly IL-6 and TNF-α (Fig. 5E). Most notably, overexpression of UBASH3A in CD4⁺ T cells from healthy donors (Fig. 5B and C) and RA patients (Fig. 5D and E) suppressed the treatment-augmented cytokine production but not TNF-α protein in CD4⁺ T cells from healthy donors, confirming the suppressive function of UBASH3A for activated TCR signaling in CD4⁺ T cells.

NF-κB was recruited to the IL6 promoter in CD4⁺ T cells after TCR stimulation

We then examined how activated TCR signaling regulates IL-6 expression in CD4⁺ T cells of healthy donors. Because activated NF-κB was observed in CD4⁺ T cells with activated TCR signaling, and NF-κB binding sites (GGGATTTTCC) are present in the *IL6* promoter, we performed a ChIP assay to determine if activated NF-κB is recruited to this promoter (Fig. 6A). As shown in Fig. 6B, following antibody treatment,

clear recruitment of NF- κ B was noted at the NF- κ B binding site in the region proximal to the transcription initiation site but was not observed at a distal region.

Discussion

Using *in-silico* mining, we identified *UBASH3A* as an RA-susceptibility gene, and further found that *UBASH3A* expression was downregulated in CD4⁺ T cells from RA patients when compared with those from healthy donors (Fig. 1). Consistent with previous reports, *UBASH3A* expression was effective in attenuating activated TCR signaling in CD4⁺ T cells treated with antibodies against CD3 and CD28. Because this inhibitory function of *UBASH3A* in terms of proinflammatory cytokine production was observed in CD4⁺ T cells from both healthy donors and RA patients (Fig. 5), it is unlikely that *UBASH3A* is associated with the pathogenic state of RA. As expected, activation of NF- κ B induced by CD3/CD28 antibody stimulation was inhibited by *UBASH3A* expression. Reflecting the induced production of proinflammatory cytokines by CD3/CD28 antibody stimulation in CD4⁺ T cells, NF- κ B recruitment was more visible at the NF- κ B binding site in the distal *IL6* promoter in CD4⁺ T cells following antibody treatment (Fig. 6). Because NF- κ B serves as a major inducer for IL-6 as well as other proinflammatory cytokines, regulated production of these cytokines in CD4⁺ T cells appears to be, at least in part, directed by NF- κ B (Fig. 5).

The presence of the RA-susceptibility SNP (rs1893592) in *UBASH3A*-SE led us to assess the SNP allelic differences in this SE function among RA patients. The C allele at this SNP has been shown to be an RA-susceptibility allele in peripheral blood mononuclear cells and, in type 1 diabetes patients, this allele was reported to abnormally splice *UBASH3A* transcript, thereby reducing *UBASH3A* protein production [19]. However, no clear association of this SNP allelic difference (C/A) with the expression level of *UBASH3A* among RA patients was detected (Fig. 1). Additional subjects are needed to address this issue.

SEs govern gene regulatory networks in a cell type-specific manner through the function of eRNAs transcribed from SEs, and hence the expression of eRNAs from active SEs is considered as an indicator of their enhancer activity. The role of eRNAs in the function of SEs has been illustrated to facilitate and stabilize dynamic chromatin looping between factors such as Yin Yang 1 (YY1) and CTCF, and the cohesion supporting formation of TAD structure is shared in chromatin looping events induced by eRNAs, while eRNAs have been shown to directly associate with MED1 and BRD4, both of which are indispensable for the transcriptional initiation process. More recently, chromatin looping between the SE regions and related gene promoter regions was demonstrated to form via the liquid–liquid phase separation state, and during the initial stages eRNAs are suggested to collect a number of proteins required for transcription in a liquid droplet. By *in-silico* assessment of the intergenic regions on *UBASH3A*-SE based on H3K27ac and transcript peaks registered in the databases, we designated *UBASH3A* eRNA_1–3, and could detect their expression in CD4⁺ T cells from healthy donors by RT-qPCR (Fig. 2). *UBASH3A*-SE was found to transcribe eRNA_1–3 (Fig. 2), suggesting that the *UBASH3A* locus is functional as a SE. This point could be further confirmed by reduced expression of *UBASH3A* by LNA-

mediated knockdown of each of the tested UBASH3A eRNAs. Because LNAs for each strand of the *UBASH3A* eRNA-coding regions were effective in knocking down the expression of eRNA_1–3 as well as *UBASH3A* transcripts, the eRNA-encoding regions on *UBASH3A*-SE seem to bidirectionally transcribe sense- and anti-sense eRNAs (Fig. 2), similar to the previously characterized eRNAs on the other SEs [10].

We identified reduced expression of UBASH3A in CD4⁺ T cells, and therefore *UBASH3A*-SE was speculated to be dysfunctional in the RA pathogenic state. To address this issue, we investigated whether the coding regions 1–3 on *UBASH3A*-SE were functional by examining the recruitment of the factors associated with eRNAs by ChIP-PCR assay. As shown in Fig. 3B, MED1, a critical subunit in the mediator complex, and BRD4 were clearly detectable with p300 (a histone acetyltransferase acting as a transcriptional co-activator) on *UBASH3A* coding regions 1 and 2 in CD4⁺ T cells from healthy donors. However, this was not observed on coding region 3. Most notably, the recruitment of MED1 and BRD4 was not obvious in CD4⁺ T cells from RA patients (Fig. 3B). To explore the molecular basis of the reduced MED1/BRD4 recruitment, we undertook *in-silico* mining for the *UBASH3A* eRNA-coding regions and identified a MafK binding site. MafK heterodimerizes with BACH2, and this heterodimer is a silencing transcriptional factor like Blimp1. Because BACH2 is highly expressed in lymphocytes including B and T cells, BACH2 and Blimp1 were examined, and BACH2 was found to be inversely recruited to *UBASH3A* regions 1 and 2 in RA CD4⁺ T cells (Fig. 3B), suggesting lowered SE activity of these eRNA-coding SE regions. From these findings, we speculated that dysfunction (at least in part) of *UBASH3A*-SE during RA progression is causal for reduced UBASH3A expression in CD4⁺ T cells in RA patients. However, the molecular mechanism of the inverse recruitment of transcriptional regulators on the *UBASH3A*-coding SE regions remains unclear, and this issue requires further investigation.

The limitations of this study include the small number of UBASH3A-stained lymph node samples collected from patients with dermatomyositis as a control for RA patients. CD4⁺ T cells were collected from peripheral blood, and thus the number of subjects was limited and appeared insufficient in which to judge the genetic association of the tested SNP (rs189352) with RA pathogenesis. Additional subjects are clearly required, not only for this aspect, but also for the other assays. In this study, the *in vivo* role of UBASH3A in RA pathogenesis remains to be studied. However, high IL-6 levels in RA patients are widely known in clinical practice to cause the progression of the pathology, such as inflammation, immune abnormalities, and joint destruction. Moreover, the IL-6 antibody has demonstrated clinical success in inhibiting RA pathogenesis [20]. Furthermore, when collagen-induced arthritis was induced in wild-type and *Ubash3a*-deficient mice, arthritis scores were significantly deteriorated in the null mutants [21]. Given that the *UBASH3A* gene locus displays a SE architecture, the present findings suggest that decreased UBASH3A expression levels resulting from SE dysfunction, at least in part, promote IL-6-associated RA pathogenesis. Thus, together with previous reports, IL-6 is presumed to be increased in RA patients with decreased expression of UBASH3A, thereby resulting in a persistent inflammatory pathological state (Additional file 4: Figure S2).

Conclusions

UBASH3A mRNA was downregulated in CD4⁺ T cells from patients with RA. Further, recruitment of BACH2 and dismissal of co-activators in CD4⁺ T-cells reduced *UBASH3A* SE activity. Given *UBASH3A* significantly attenuated IL-6 cytokine production in TCR signaling-activated CD4⁺ T-cells, low levels of *UBASH3A* resulted in high levels of IL-6. Thus, *UBASH3A* can be treated as a therapeutic event for RA.

List Of Abbreviations

dbSUPER, database of super enhancer; GWAS, genome wide association study; BioGPS, Bio gene portal system; HD, healthy donor; RA, rheumatoid arthritis; SE, Super-enhancer; DM, dermatomyositis; SNP, single nucleotide polymorphism; gapdh, glyceraldehyde-3-phosphate dehydrogenase; eRNA, enhancer RNA; Locked Nucleic Acid (LNA); ChIP, chromatin immunoprecipitation; YY-1, Yin-Yang 1; BACH2, BTB and CNC homology 2; Blimp1, B lymphocyte-induced maturation protein-1; H3K27ac, histone H3 containing the acetylated lysine 27; MED1, Mediator of RNA polymerase II transcription subunit 1; BRD4, Bromodomain-containing protein 4; NF-κB, nuclear factor-kappa B; TCR, T cell receptor; IL, Interleukin; TNF, tumor necrosis factor; PBMCs, peripheral blood mononuclear cells; RQ, Relative quantification.

Declarations

Ethics approval and consent to participate

The Ethics Committee of Medical Research, University of Occupational and Environmental Health, Japan, reviewed and approved this study (approval number: H28-067). A signed informed consent was obtained from all subjects in accordance with the Declaration of Helsinki and its subsequent modifications.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

Y. Tanaka, has received consulting fees, speaking fees, and/or honoraria from Daiichi-Sankyo, Astellas, Pfizer, Mitsubishi-Tanabe, Bristol-Myers, Chugai, YL Biologics, Eli Lilly, Sanofi, Janssen, UCB and has received research grants from Mitsubishi-Tanabe, Takeda, Bristol-Myers, Chugai, Astellas, Abbvie, MSD, Daiichi-Sankyo, Pfizer, Kyowa-Kirin, Eisai, Ono. S. Nakayamada has received consulting fees, speaking fees, and/or honoraria from Bristol-Myers, Pfizer, GlaxoSmithKline, Sanofi, Astellas, Asahi-kasei, Boehringer Ingelheim and has received research grants from Mitsubishi-Tanabe, Novartis and Novartis. Other authors declares no conflict of interest.

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Authors' contributions

KY, SK, and YT contributed to the study design, overall review, and writing of the manuscript. All other authors were involved in the performance of the study and review of the manuscript. SN, TZ, PAN, NO, and SI participated in the study design and coordination. All authors read and approved the final manuscript.

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Figures

Figure 1

Figure 1

UBASH3A in CD4⁺ T-cells is downregulated in rheumatoid arthritis (RA) patients than healthy donors (HD). (A) *In silico* screening for RA-susceptibility factors in the super enhancer database (dbSUPER), GWAS, and bio gene portal system (BioGPS). (B) Super enhancer (SE_17452) domain mapped to the *UBASH3A* locus in CD4⁺ T cells. The locus is mapped to chromosome 21 at approximately 4382000-4388000. Histone H3 containing the acetylated lysine 27 (H3K27ac) marks are provided in GM12878

which shows lymphoblastoma cell line. Rs1893592-A/C is located in the intron between exon 10 and exon 11, and shown as (*). **(C)** The mRNA expression levels of *UBASH3A* gene in CD4⁻ and CD4⁺ T cells derived from peripheral blood mononuclear cells (PBMCs) that were collected from HD (n=11) and RA patients (n=24) were evaluated by RT-qPCR. **(D)** Expression of *UBASH3A* mRNA among patients with RA (rs1893592-AA, AC and CC). **(E)** Expression levels of UBASH3A protein in CD4⁻ and CD4⁺ T cells derived from PBMCs that were collected from HD (n=3) and RA patients (n=3) were evaluated by Western blotting. β -actin was used as a loading control. **(C and D)** The amount of *UBASH3A* transcript was expressed relative to that of *GAPDH* transcript. Data are expressed as the mean $\pm$ standard deviation. Dunnett's multiple comparison test. **(F)** Protein expression of UBASH3A in CD4⁺ T cells localized in the lymph nodes of dermatomyositis (DM) and RA patients was evaluated by immunofluorescence staining. RQ, Relative quantification. *P<0.05, by Student's t-test. Scale bar = 100 μ m.

Figure 2

Figure 2

eRNA promotes *UBASH3A* transcription in CD4⁺ T-cells. (A) Genomic structure of *UBASH3A*, histone H3 containing the acetylated lysine27 (H3K27ac), and transcripts peaks. Putative eRNA_1-3 are expressed within the super-enhancer (SE_17452) in CD4⁺ T-cells. H3K27ac are marked in GM12878 lymphoblastoid cells. RNA-sequencing data are shown in GM12878 and K562 leukemia cells. **(B-D)** LNA transfection targeting eRNA was performed as described under “Methods” **(C and D)**. The amount of each transcript

was quantified by RT-qPCR, and then expressed relative to that of *GAPDH* transcript. Data are expressed as the mean±standard deviation from three independent experiments. RQ, Relative quantification.
*P<0.05, vs control, by Dunnett's multiple comparison test.

Figure 3

Figure 3

BACH2 was recruited to the *UBASH3A* locus in CD4⁺ T-cells from rheumatoid arthritis patients. (A) The *UBASH3A* locus displays super-enhancer architecture. Super-enhancer (SE_17452) domains mapped in CD4⁺ T-cells is located at the *UBASH3A* locus. The locus is mapped to chromosome 21 at approximately 4382000-4388000. Histone H3 containing the acetylated lysine27 (H3K27ac) marks are provided in GM12878 lymphoblastoid cell. BACH2-binding site is mapped to the indicated *UBASH3A* locus. ChIP

assay was performed with the indicated antibodies across the *UBASH3A* loci in CD4⁺ T-cells from PBMCs from healthy donors (HD) and rheumatoid arthritis (RA) patients. DNA regions (1-3) were amplified by ChIP-PCR. (B) ChIP-PCR results. ChIP-PCR were performed as described under “Methods” Three independent experiments were performed.

Figure 4

Figure 4

UBASH3A inhibits phosphorylation of NF- κ B dependent on TCR signaling. (A and B) CD4⁺ T-cells were transfected with pcDNA3.1-UBASH3A and empty vector. (A) At 24 h after transfection, *UBASH3A* mRNA levels were quantified by RT-qPCR. The amount of *UBASH3A* transcript was expressed relative to that of *GAPDH* transcript. **(B)** At 30 min after TCR stimulation, Western blotting was performed with a series of antibodies. Data are representative of three independent Western blotting experiments. **(C)** Phosphorylated (p)-NF- κ B/total (t)-NF- κ B was quantified, and the data were plotted on a histogram. Values are expressed as mean $\pm$ standard deviation. RQ, Relative quantification. *P<0.05, vs lane 1 or 4, by Dunnett's multiple comparison test.

Figure 5

Figure 5

UBASH3A inhibits production of IL-6 induced by TCR signaling in CD4⁺ T-cells. (A–E) CD4⁺ T-cells collected from healthy donors (HD) (B–C) and rheumatoid arthritis (RA) patients (D–E) were transfected with pcDNA3.1-UBASH3A and empty vector. (A–E) In the cells treated with or without TCR stimulation for 24h, proinflammatory cytokine mRNA levels (B and D) were measured by RT-qPCR, and cytokine levels (C and E) were measured by cytometric bead array. The amount of cytokines transcript was expressed

relative to that of *GAPDH* transcript. Data are expressed as the mean±standard deviation from three independent experiments. RQ, Relative quantification. *P<0.05, vs mock or mock+CD3/CD28, by Dunnett's multiple comparison test.

Figure 6

Figure 6

TCR signaling induces recruitment of NF-κB to the *IL-6* gene promoter in CD4⁺ T-cells. (A) NF-κB binding site in the *IL-6* gene promoter. (B) ChIP assay was performed with a series of antibodies in the CD4⁺ T-cells collected from healthy donors (HD) with or without TCR stimulation (30 mins). Results are representative of 3 independent experiments with similar findings.

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