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ORIGINAL RESEARCH

Toll-like receptor-7 activation in CD8+ T cells modulates inflammatory mediators in patients with rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is an autoimmune disorder of unknown etiology with aberrant immunological responses leading to inflammation, swelling and pain of the joints. $CD8^+$ T cells have been known to be one of the major immune modulators in the progression of RA and the presence of toll-like receptors (TLRs) on these cells further accentuate their role in RA. Herein, we report an increased expression of TLR7 in the endosomes of $CD8^+$ T cells of RA patients correlating with disease severity. The stimulation of TLR7 with Imiquimod (IMQ) in these $CD8^+$ T cells drives the signalling cascade via NFkB and pERK activation and hence an increase in the mRNA transcripts of signature cytokines and cytolytic enzymes. However, a parallel synthesis of Tristetraprolin (TTP), an mRNA destabilizing protein prevents the translation of the mRNA transcripts, leading to a rapid degeneration of the target mRNA. We thus report that a direct TLR7 ligation by its agonist increases cytokine transcript signature but not an equivalent protein surge.

Keywords Rheumatoid arthritis · $CD8^+$ T cells · TLR7 · Tristetraprolin · IMQ

Introduction

Rheumatoid Arthritis (RA) is an autoimmune disorder of unknown etiology, wherein the immune system executes abnormal responses towards body's own cells [1, 2]. The disease is characterized by the presence of auto-antibodies and symptoms like inflamed small joints, degradation of bones, z thumb deformity, and morning stiffness [3, 4]. RA affects almost 1% of the total world population being three times more prevalent in women as compared to men [5]. The increased secretion and activation of inflammatory mediators such as TNFα, IFNγ, Granzyme B, Perforin, IL-1, IL-6, IL-17 and GM-CSF leads to the swelling of the synovial membrane, synovial fluid accumulation and joint deformities [1, 6, 7]. Different genetic, environmental and lifestyle factors are reported to be connected with the incidence of RA. The alleles present in the HLA-DRB1 gene region are known to contribute the most to the onset of RA [8]. Changes in DNA sequences of genes including Protein tyrosine phosphatase non-receptor type 22 (PTPN22), TNFα, IL6R, TRAF1, STAT4, FCGR, CCL1/2 and CCR6 have been reported as genetic reasons of increased cellular hyper reactivity [9–11]. Environmental and lifestyle factors such as air pollution, smoking, obesity and low vitamin D intake have also been related to the patho-etiology of RA [12–15].

Research undertaken in the recent years has brought into light the role of $CD8^+$ T cells in mediating inflammation and disease progression in patients with RA [1, 16]. $CD8^+$ T cells are known to recognize non-self peptides presented on MHC class I complex of different cells and hence activate cytotoxic responses by production of cytotoxic mediators

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including Granzyme B, Perforin, IFNγ and TNFα [16, 17]. This cytotoxic behavior drives their primary function of clearing out infected cells by lysis or by the induction of apoptosis [18, 19]. Hyper-activated state, abundant presence in the synovium and a central memory biased phenotype of the $CD8^+$ T cells in case of RA suggests that the $CD8^+$ T cells are of grave importance in the initiation and progression of this disease [20–22].

Toll-like receptors (TLR) are a group of pathogen recognition receptor proteins that have the potential to recognize a wide array of pathogenic peptides and initiate an innate immune response [23–25]. A number of studies have shown their unconventional presence in T cells [26–28]. We recently reported the presence of TLR4 on the $CD8^+$ T cells of RA patients and that the TLR4 dependent activation of $CD8^+$ T cells significantly affected the severity of RA [1]. RA patients are known to be a reservoir of antigens that may continuously stimulate immune cells with several reports suggesting role of an infectious agent in disease development.

TLR7 is an endosomal receptor that recognizes ssRNA and initiates host defense mechanisms leading to an increased expression of different pro-inflammatory cytokines [29, 30]. ssRNA is shown to be present in RA synovial fluid and may thus act as potential endogenous ligand for TLR7 present in $CD8^+$ T cells [31]. Even ssRNA viral infections are known to trigger RA [32]. It thus becomes pertinent to understand and analyse the existence and role of TLR7 in $CD8^+$ T cells of RA patients. Imiquimod, a drug belonging to the imidazoquinolone family of amines mimics ssRNA which is the natural ligand for TLR7 [33].

The ligation of IMQ to the endosomal TLR7 activates the NFκB pathway [34] and leads to the expression of pro-inflammatory mediators and cytokines, leading to an aggravation of inflammatory status [33, 34]. The use of IMQ as a therapeutic agent has been well documented. It has been used as a topical cream to treat several skin diseases including genital warts, perianal warts, basal cell carcinoma and malignant melanoma [33]. IMQ has also been shown to activate apoptotis by activating the intrinsic pathway making it a potent anti-tumor agent [33, 35]. Cytokines including IL-12, TNFα and IFNγ are expressed post-IMQ interaction and increase the cytotoxic fate of T cells. This might have major implications in IMQ being a therapeutic target for patients suffering from RA and hence our study focuses on the effect of IMQ on the $CD8^+$ T cells in patients with RA.

Interestingly, we found out that TLR7 ligation in $CD8^+$ T cells altered the transcriptome profile towards a more inflammatory phenotype. However, these activated $CD8^+$ T cells could not translate these transcripts completely. This intriguing finding led us to analyse different factors responsible for the phenomenon. TTP is an mRNA destabilizing protein encoded by the ZFP36 gene [36]. It binds to the AU rich Element (ARE) present on the 3' UTR of the target mRNA and then destabilize it by shortening of the poly A tail thereby crippling translation [37]. We could see an increased expression of TTP protein post-TLR7 ligation in $CD8^+$ T cells of RA patients thereby destabilizing and blocking the translation of inflammatory genes.

Methods

Blood sample collection

Human blood samples were collected from Rheumatology O.P.D of Pradyumna Bal Memorial Hospital, Bhubaneswar, Odisha, India. The inclusion of participants was based on 2010 ACR/EULAR classification criteria for being diagnosed with RA. The different clinical parameters such as ESR, SGPT, RF and Anti-CCP were taken into consideration and DAS-28 scores were calculated for each patient that participated in this study (Table 1). The participants were informed duly with written informed consent. 5 ml of blood were collected from RA patients and age matched healthy donors in lithium heparin-coated vacutainers (BD Vacutainer, 367886) with the help of the clinicians. The research undertaken was approved by the Institute and Hospital Research ethics committee and the research methods were performed by strictly adhering to the institutional guidelines and norms.

Isolation of $CD8^+$ T cells

The peripheral blood samples from patients and healthy donors were treated with RosetteSep Human $CD8^+$ T Cell

Table 1 Patient characteristics

Clinical and Para clinical Variables	RA patient ($n=80$) Mean ± SEM
Gender (F/M)	50/30
Age (years)	47.86 ± 1.2
Disease duration (years)	4.68 ± 0.5
DAS	7.04 ± 0.1
Swollen joint count	5.23 ± 0.16
Tender joint count	9.67 ± 0.25
ESR (mm/1st h)	54.19 ± 4.4
CRP (mg/L)	33.45 ± 3.1
RF Positive (%)	100
Anti- CCP (%)	100

Seventy-three healthy (female: male: 32:41, average age: 33.08 ± 0.93) controls participated in this study with no symptoms of RA

enrichment cocktail (Stemcell Technologies Inc, BC, Canada) and were then separated over Ficoll-Hypaque density gradient (HiSep TM LSM 1077, HIMEDIA, INDIA). The plasma obtained after separation was stored at − 80 °C for future use. The isolated $CD8^+$ T cells were then evaluated for purity by flow cytometry (BD LSR Fortessa) with anti-CD3-FITC and anti-CD8a-PE antibodies (TONBO biosciences, San Diego, CA). For each sample, a minimum of 10,000 viable events were acquired and their flow cytometric analyses were carried using FlowJo V10 software (Tree Star).

Quantitative PCR analysis

The total RNA was extracted using TRIzol reagent (life technologies Inc, USA) according to the manufacturers' protocol and quantified with Nano drop (Colibri). Single-stranded cDNA was synthesized by cDNA reverse transcription kit (AB Applied biosciences, CA, USA). Real-time PCR reactions were prepared using SYBR green master mix I (Roche, IN, USA) in 96-well plate using Light cycler (Biorad). The primers and their sequences used are mentioned in Table 2. Ct values were obtained for individual gene, were normalized with the housekeeping gene β actin and expressed as ΔCt. The fold change values for each of the target transcripts were then calculated by normalizing with the control samples. The statistical analysis was performed using Graph Pad Prism 5 and the graphs were represented.

Flow cytometric analysis of inflammatory mediators

The isolated $CD8^+$ T cells were washed with FACS buffer (2% FBS in PBS and 2 mM EDTA) and were labelled with anti-CD3 FITC and anti-CD8a PE anti-CD28-PerCpCy5.5 (eBioscience, CA, USA), anti-CD45RO-FITC (eBioscience, CA, USA), anti-CD45RA-PECy7 (TONBO biosciences). After surface staining was completed, the $CD8^+$T cells were fixed using 2% PFA for 30 min, permeabilized with PERM buffer (TONBO Biosciences) and labelled with anti-TLR7-Alexa fluor 488 (R&D Biosciences), anti-Granzyme B-Alexa fluor 647(eBiosciences), anti-Perforin-FITC, anti-TNFα- PE and anti-IFNγ-Alexa fluor 647. The expression levels of these cytokines mentioned above were quantified using BD LSR Fortessa (BD Biosciences), and the data obtained were analyzed using FlowJo software (version 10; Tree Star).

Tristetraprolin (TTP) expression analysis by Western Blot

Total protein of the isolated $CD8^+$ T cells was isolated using RIPA buffer and the concentration of protein was quantified using BCA (Thermo Scientific) according to the manufacturer's protocol. Equal protein from RA and healthy controls (HC) were first run on an SDS-PAGE and then transferred to a nitrocellulose membrane. The Western Blot analysis was performed after incubating the blots with anti-human TTP (SantaCruz Biotechnology) overnight and then treating with anti-rabbit secondary antibody for 2 h. The blots were then developed using Chemi Doc. The densitometry analysis was carried to quantitatively analyze the protein concentrations using ImageJ software.

Ex vivo $CD8^+$T cell activation and analyses

Post isolation the viability of the $CD8^+$ T cells was determined by staining them with Trypan blue (Life technologies, CA, USA) and using an automated cell counter (Countess, Life Technologies). A total of 50,000 cells were cultured in 200 µl of RPMI 1640 medium (GIBCO) containing 10% FBS, 0.1 mg/ml L-Glutamine–Penicillin–Streptomycin and 0.5 mM 2-Mercaptoethanol, in 96-well cell culture plates (NEST) at 37 °C and 5% CO_2. The cells were stimulated with 0.5 µg/ml or 1 µg/ml or 5 µg/ml of IMQ (Sigma-Aldrich) for either 2 h, 6 h or 12 h. Post-stimulation, these cells were washed with PBS and harvested directly in 0.5 ml TRIzol reagent. The mRNA from the cells was extracted and quantified using Nanodrop. 500 ng of RNA was used to prepare cDNA (Applied Biosystems, cDNA reverse transcription kit). A qPCR was performed to analyse the expression of inflammatory mediators using primers and SYBRgreen, as described above. The expression of Granzyme B, Perforin, TNFα and IFNγ proteins was analyzed using flow cytometry, as described above, however, to inhibit the secretion of these cytokines for the flow cytometry analysis, Brefeldin A was used at a concentration of 10 µg/ml. Based on a series of analyses (Supplementary

Table 2 Nucleotide sequence of the primers used for real time PCR

Primer	Forward sequence	Reverse sequence
TLR7	5′CCAGTGTCTAAAGAACCTGGAAAC 3′	5′TCAGGGACAGTGGTCAGTTG 3′
Granzyme B	5′GCAGGAAGATCGAAAGTGCGA3′	5′GCATGCCATTGTTTCGTCCAT3′
Perforin	5′CGCCTACCTCAGGCTTATCTC3′	5′CCTCGACAGTCAGGCAGTC3′
TNFα	5′GGAGAAGGGTGACCGACTCA3′	5′CTGCCCAGACTCGGCAA3′
IFNγ	5′CCAACGCAAAGCAATACATGA3′	5′CCTTTTTCGCTTCCCTGTTTTA3′
ß-actin	5′GCTACGAGCTGCCTGAGC3′	5′GGCTGGAAGAGTGCCTCA3′

Fig. 5), 0.5 µg/ml concentration of IMQ for 12 h was further used for all stimulation experiments.

Statistical analysis

Statistical analyses were carried out using Graph Pad Prism 5. Significant differences were tested by unpaired Student's t test, Spearman correlation analysis and ROC curve analyses. The data were expressed as the mean ± SEM (Standard Error Mean) or mean ± SD (standard deviation). A two-tailed p value of < 0.05 was considered statistically significant. The degree of significance was denoted by * symbol where, mean ± SEM *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Results

Increased expression of TLR7 in CD8$^+$T cells of RA patients is associated with disease severity.

The expression of TLR7 in CD8$^+$T cells of RA patients and healthy controls was analysed. The qPCR and flowcytometry analyses showed that the CD8$^+$T cells of RA patients had a significantly increased expression of TLR7 mRNA transcripts ($p < 0.05$) (Fig. 1a) as well as TLR7 protein ($p < 0.0001$) (Fig. 2a) in comparison to the healthy controls. Furthermore, on the basis of DAS28 scores, the RA patients were categorized into groups of low (DAS28 ≤ 4), moderate (DAS28 between 5 and 7) and high (DAS28 > 7) disease activity states and the differential expression of TLR7 was analysed. The expression of TLR7 mRNA and protein was observed to increase with increasing disease severity ($p < 0.05$ Fig. 1b and $p < 0.01$ Fig. 2b, respectively) hence was found to be directly correlated with the RA disease activity (Spearman non-parametric correlation analysis: $r = 0.97$, $p < 0.0001$ Fig. 1c $r = 0.91$, $p < 0.0001$ Fig. 2 c). The ROC analysis indicated a high specificity and sensitivity of TLR7 expression (mRNA expression: AUC = 0.90, $p < 0.0004$ Fig. 1d; TLR7 protein expression: AUC = 0.99, $p < 0.0001$ Fig. 2d) in CD8$^+$T cells of RA patients in comparison to the healthy controls demonstrating the role of TLR7 in distinguishing between case–control cohort.

The TLR7 engagement with Imiquimod in CD8$^+$T cells of RA patients increases mRNA transcription but reduces protein expression of inflammatory cytokines

The CD8$^+$T cells of RA patients as well as healthy controls were treated with IMQ as described in the method section. The mRNA transcripts and protein expression of different inflammatory mediators was analysed by qPCR and Flowcytometry, respectively. A significant many fold increase was observed in the expression of Granzyme B mRNA (17.5 ± 4.504, $p < 0.01$), Perforin (20.37 ± 3.234, $p < 0.01$), TNFα (19.03 ± 3.433, $p < 0.01$) and IFNγ (21.68 ± 4.496, $p < 0.01$) after 12 h of activation with IMQ in the CD8$^+$T cells of RA patients in comparison to the healthy controls (Fig. 3a–d). However, the same treatment of CD8$^+$T cells led to a reduced expression of inflammatory cytokine proteins in RA patients after 12 h of activation with no effect in the healthy controls (Fig. 3e–h), (Supplementary Fig. 1a–d).

This discrepancy between mRNA and protein abundance post-IMQ treatment led us to believe that there were some unknown factors that were affecting the fate of the mRNA transcripts and were being detrimental to the translation of the transcripts.

TLR7 engagement with Imiquimod activates NFκB and pERK-mediated transcription and induces over expression of TTP

The transciptome analysis post-IMQ-mediated activation revealed an over expression of signature cytokines in CD8$^+$T cells of RA patients. We next sought to identify the transcription factors responsible and hence the mechanism of cytokine expression. The flowcytometry analysis (Supplementary Fig. 2a, b) revealed a significant increased number of cells expressing NFκB- RelA (Fig. 4a $p < 0.01$) and pERK (Fig. 4b $p < 0.05$). Illustrating the role of these transcription factors in increased cytokine expression after IMQ-mediated activation of CD8$^+$T cells of RA patients.

IMQ treatment of CD8$^+$T cells did not allow translation of the increased mRNA transcripts of the cytokines in RA patients which led us to assume a role of mRNA destabilizing proteins. TTP is known to be activated by the NFκB transcription cascade [38] and has a natural feedback response in cases of inflammation [39]. As NFκB was significantly activated in CD8$^+$T cells after IMQ treatment, we supposedly checked the expression of TTP protein by Western blot (Fig. 4c). After normalizing with the expression of β actin housekeeping protein, the mean area densities were plotted (Fig. 4d). The densitometric analysis revealed a significant increase in the expression of TTP protein ($p < 0.001$) in CD8$^+$T cells challenged with IMQ for 12 h. This difference in expression of an mRNA destabilizing enzyme TTP can be the reason behind the decay of the cytokine mRNAs hence a discrepancy in protein abundance.

Imiquimod treatment reduce effector memory sub-population of CD8$^+$T cells in RA patients

We next sought to determine the role of TLR7 ligation in differentiation of CD8$^+$T cells of RA patients. The CD8$^+$T cells were stimulated with IMQ for 12 h and different T cell subset markers were analysed using flow cytometry. The flowcytometry analysis (Fig. 5) revealed no changes in the CD8+ CD45RA+ CD28+ naïve,

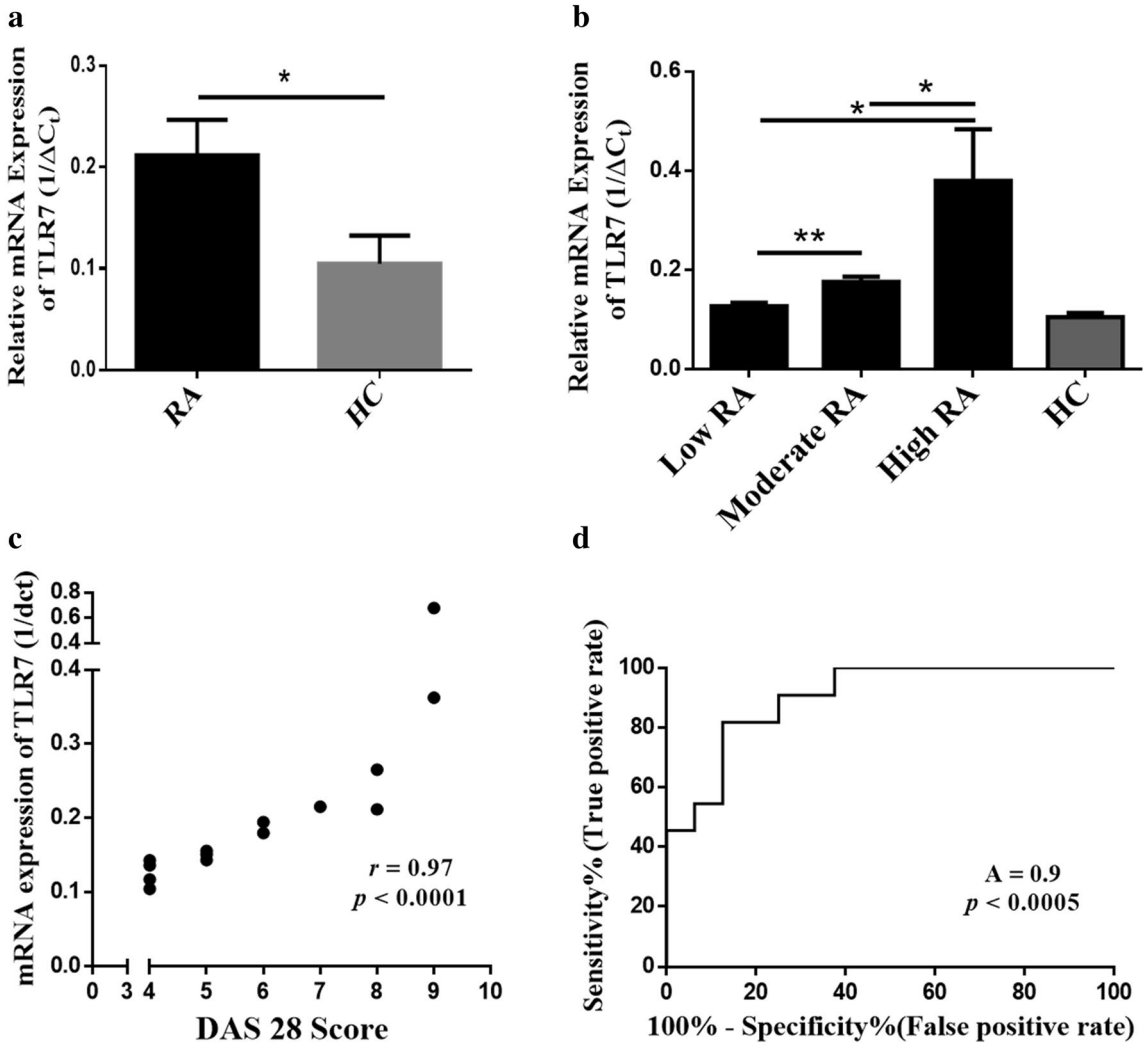

Fig. 1 TLR7 mRNA expression in CD8$^+$T cells of RA patients in relation with different disease activity states. **a** Relative mRNA expression in the CD8$^+$T cells of RA patients ($n=13$) and HC samples ($n=13$) were determined. The Ct values were normalized (ΔCt) by subtracting the Ct values of β-actin from those of TLR7 and 1/ΔCt was plotted. **b** The graph depicts the TLR7 mRNA expression by the CD8$^+$T cells of RA patients and healthy controls according to low, moderate and high DAS28 scores. **c** The Spearman non-parametric correlation analysis of TLR7 mRNA has been represented with respect to DAS28 scores. **d** ROC analysis for CD8$^+$T cells isolated from RA patients and healthy controls was carried out for TLR7 mRNA transcript. Bars represent the mean ± SEM $*p<0.05$, $**p<0.01$, $***p<0.001$

CD8+ CD45RO-CD28- effector and CD8+ CD45RO+ CD28+ central memory CD8$^+$T cells, however, a significant decrease ($p<0.01$) of CD8+ CD45RA-CD28- effector memory sub-population of CD8$^+$T cells after IMQ treatment was observed in the RA patients.

Discussion

RA has been known to be driven by the hyper-activation of CD8$^+$T cells [40, 41]. This activated CD8$^+$T cell repertoire has been known to upregulate the expression of cytolytic enzymes like Granzyme B, Perforin and inflammatory mediators such as TNFα and IFNγ [40]. However, we need to understand if this hyperactivated state of the CD8$^+$T

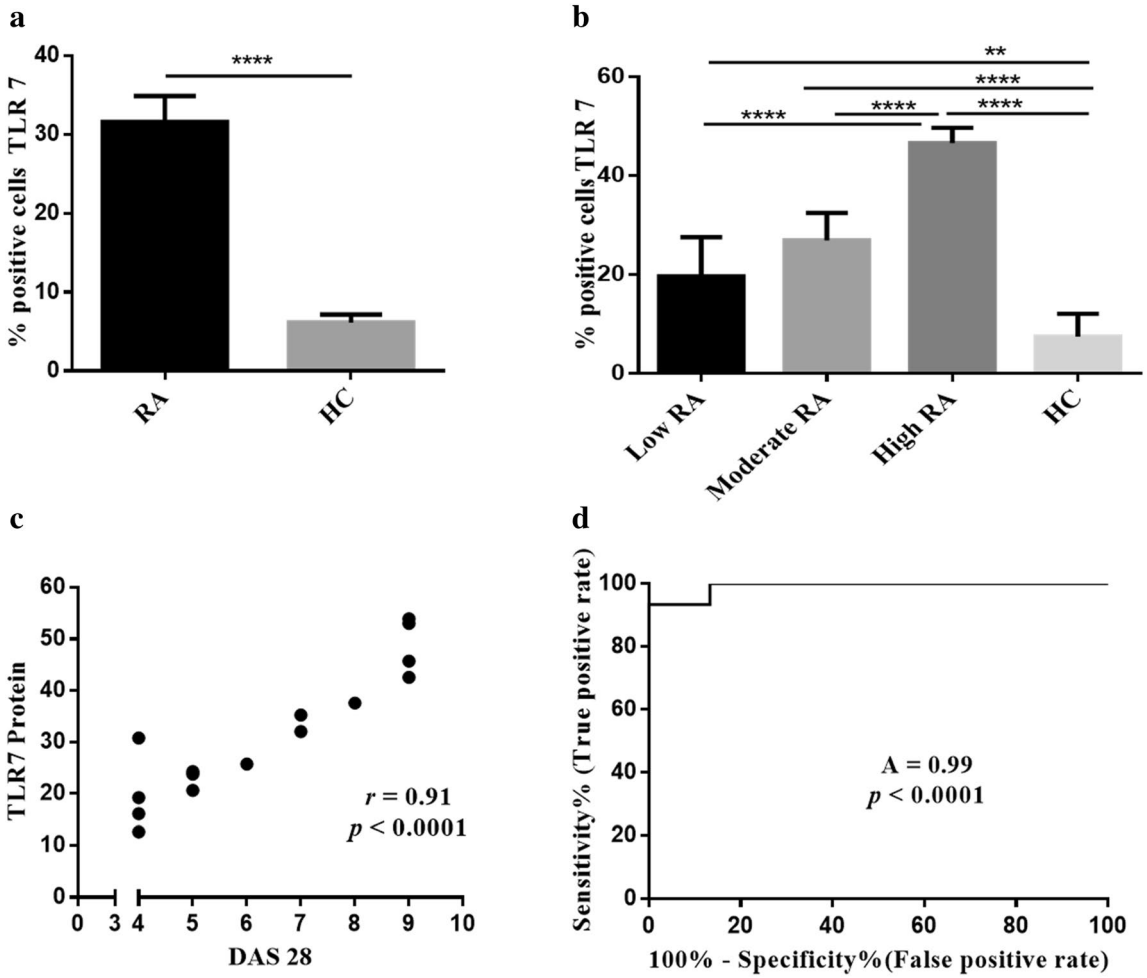

Fig. 2 TLR7 protein expression in CD8$^+$T cells of RA patients in relation with different disease activity states. **a** Relative protein expression in the CD8$^+$T cells of RA patients ($n=15$) and HC samples ($n=15$) were determined. The relative percentage of cells expressing TLR7 has been plotted. **b** The graph depicts the TLR7 protein levels in CD8$^+$T cells of RA patients and healthy controls according to low ($n=4$), moderate ($n=6$) and high ($n=5$) DAS28 scores. **c** The Spearman non-parametric correlation analysis of TLR7 protein expression pattern has been represented with respect to DAS28 scores. **d** ROC analysis for CD8$^+$T cells isolated from RA patients and healthy controls was carried out for TLR7 protein expression. Bars represent the mean ± SEM $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$

cells is only due to its activation through the classical TCR or through any other activation mechanism? We recently [1] reported the presence of TLRs on the CD8$^+$T cells of RA patients especially TLR7. RA patients are home to a number of molecules that may act as ligand to TLR7 thereby activating CD8$^+$T cells and hence disease severity [42, 43]. Also, an unfortunate ssRNA viral infection may also trigger the disease in a predisposed individual. Hence, we initiated this study to understand the presence, role and mechanism of action of TLR7 on CD8$^+$T cells of RA patients.

Interestingly, we found an increased expression of the TLR7 mRNA transcript as well TLR7 protein in the CD8$^+$T cells isolated ex vivo from RA patients in comparison to the healthy controls (Figs. 1 and 2). The significant presence of TLR7 has the potential to activate CD8$^+$T cells of RA patients and may activate the severity of the disease. IMQ was thus used as TLR7 agonist in vitro to activate isolated CD8$^+$T cells of RA patients. Interestingly an increased expression of the mRNA transcripts of cytolytic and pro-inflammatory proteins (Fig. 3) was seen, on the contrary the respective protein expression did not increase rather a depression in cytolytic and pro-inflammatory proteins was observed post 12 h of IMQ treatment (Fig. 3).

TLR7 downstream mechanism is known to be carried out by the activation of the MAPK, NFκB and JAK/STAT pathways [29]. To get an insight into the molecular pathways getting activated post-treatment with IMQ, we looked for the expression of NFκB- RelA and pERK protein (Fig. 4). Upon analysis, we found an increased expression

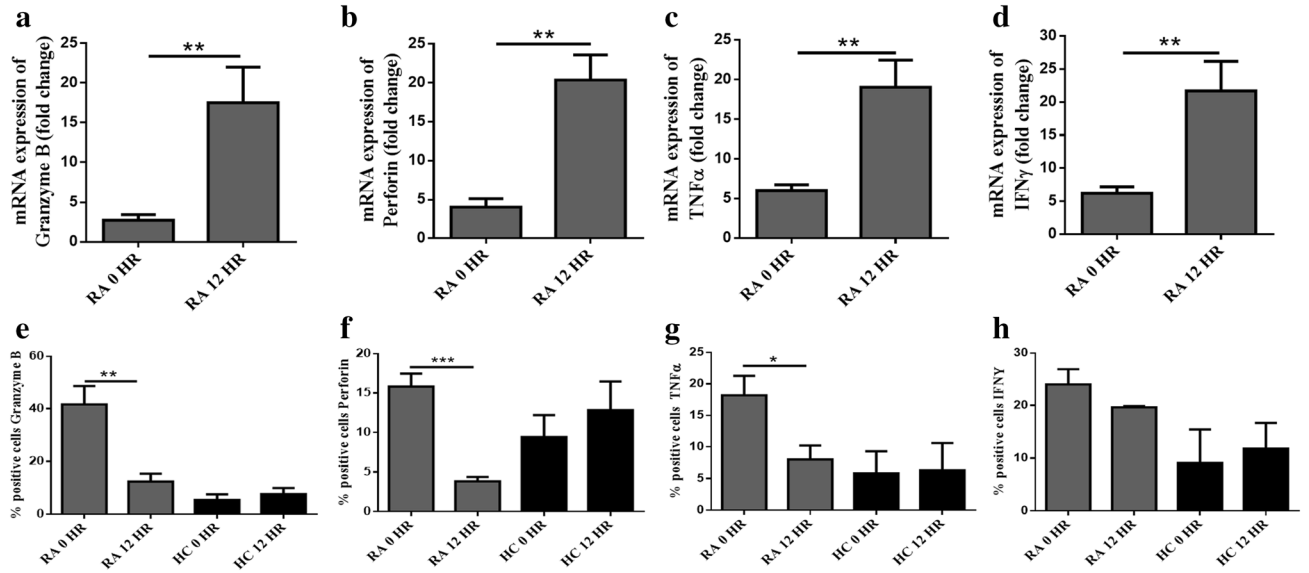

Fig. 3 Cytokine profiling of $CD8^+$T cells of RA patients and healthy controls post stimulation with Imiquimod. The figure represents the fold increase in the relative mRNA expression of **a** Granzyme B, **b** Perforin, **c** TNFα and **d** IFNγ after treatment with IMQ. The Ct values were normalized (ΔCt) by subtracting the Ct values of β-actin from those of the target genes in $CD8^+$T cells of RA patients ($n=12$) and HC samples ($n=12$). The obtained 1/ΔCt values were then normalized with the healthy controls to calculate the fold change and have been represented graphically. Bars represent the mean ± SEM $*p<0.05$, $**p<0.01$, $***p<0.001$. Relative percentage protein expression of **e** Granzyme B, **f** Perforin, **g** TNFα and **h** IFNγ in $CD8^+$T cells at 0 h and 12 h in RA patients ($n=10$) and HC samples ($n=10$). Bars represent the mean ± SEM $*p<0.05$, $**p<0.01$, $***p<0.001$

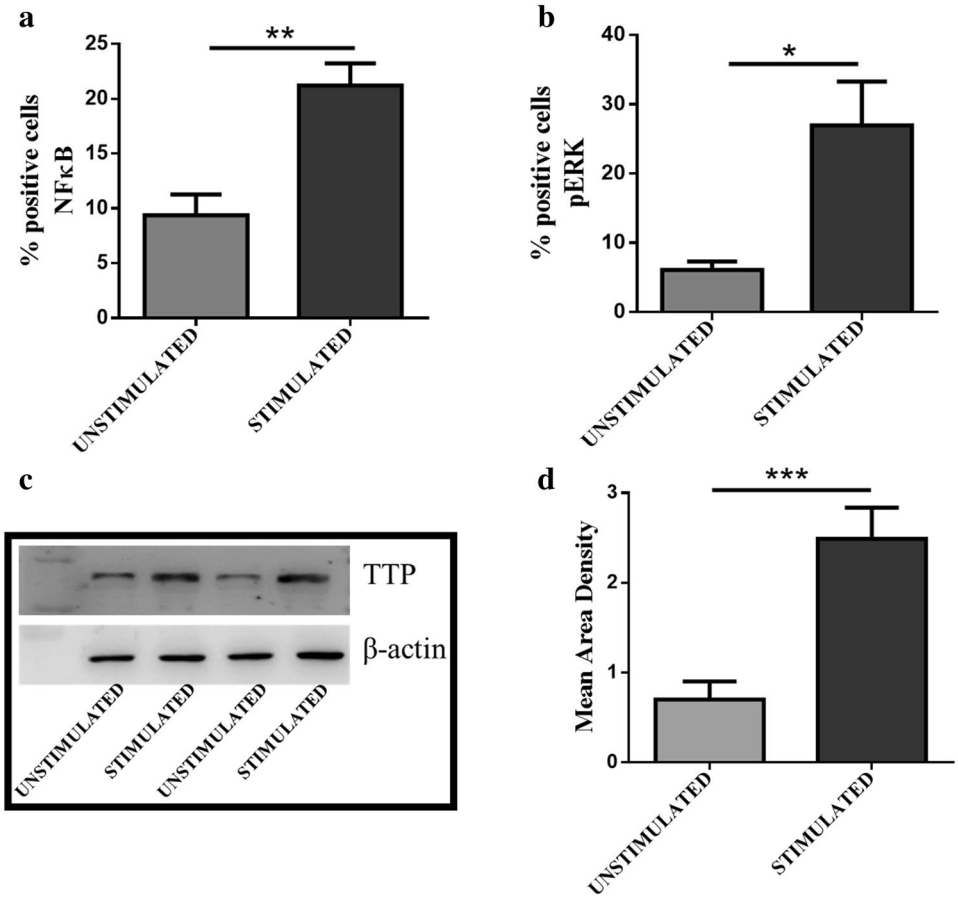

Fig. 4 Activation of inflammatory cytokine transcription factors and mRNA destabilizing protein. The bar graphs and the corresponding images represent the percentage of **a** pERK and **b** NFκB positive $CD8^+$T cells of RA patients ($n=7$), with and without stimulation of IMQ. Figure **c** represents the mean area density of TTP expressed in the stimulated and unstimulated $CD8^+$T cell population of RA patients ($n=25$). Figure **d** depicts the representative bands obtained in the ChemiDoc visualization system. Bars represent the mean ± SEM $*p<0.05$, $**p<0.01$, $***p<0.001$

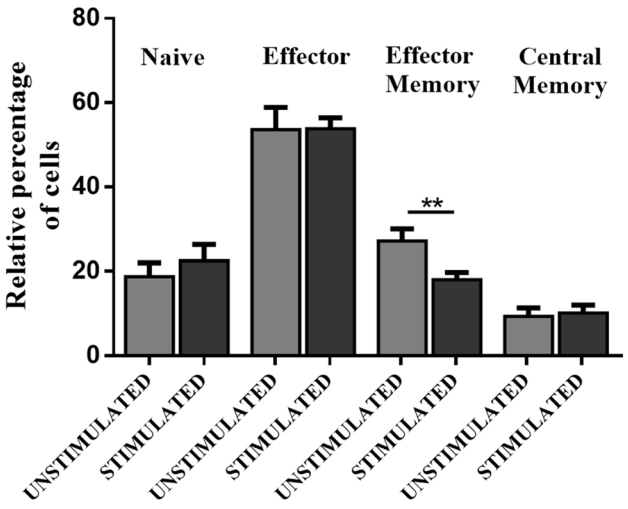

Fig. 5 Altered T cell subset population in the $CD8^+$T cells of RA patients upon Imiquimod treatment. The T cell subsets have been analyzed post stimulation of the $CD8^+$T cells with IMQ. The expression patterns of naïve, effector, effector memory and central memory subsets of $CD8^+$T cells have been visualized. Bars represent the mean ± SEM $*p<0.05$, $**p<0.01$, $***p<0.001$

of both NFκB- RelA and pERK levels. The activation of the cytolytic proteins like Granzyme B and Perforin have been well attributed to the transcription factors NFκB and pERK [41, 44], the activation of pERK and NFκB have also been reported to activate the transcription of inflammatory mediators TNFα, IFNγ [45, 46] and these studies have been reported extensively. Though the changes in transcriptome was inevitably visible, the same could not be seen with respect to the proteins which led us to believe that certain mRNA destabilizing factors might have an essential role in affecting the translation process. Tristetraprolin (TTP), an mRNA destabilizing protein encoded by the ZFP36 gene, gets activated in cases of inflammation and functions in the decay of mRNA of inflammatory molecules [37, 47, 48]. It binds at the AU Rich Element (ARE) site in the 3′ UTR of the target mRNA and hence, we chose TTP as our choice of RNA degrading enzyme because of two main reasons, first, it has been reported that TTP gets activated in inflammatory conditions [49, 50] targeting inflammatory mediators TNFα and IFNγ, second, NFκB is the transcription factor that activates the expression of TTP [38] and the activation of MAPK pathway maintains the expression of TTP [51]. TTP activation depends on the critical equilibrium between the phosphorylation and dephosphorylation of the TTP protein [52]. The phosphorylation of TTP is induced by growth factors and cytokines, these factors lead to the activation of the MAPK-activated protein kinase 2 [48]. This leads to the phosphorylation of the TTP protein in the Ser52 and Ser178 residues, inactivating its function and stabilizing the TTP protein. During the onset of inflammatory responses, the p38 MAPK activity is very potent and this leads to an accumulation of the TTP protein in a dormant form [48]. Post the downregulation of the p38 MAPK activity studies have shown the activation of protein phosphatase-2A (PP2A) which in turn dephosphorylates the TTP protein at the Ser52 and Ser172 residues enabling TTP to bind to the 3′UTR region of the target mRNA and destabilizing it. The cellular levels of TNFα and IFNγ have also been reported to induce expression of TTP to regulate the proinflammatory responses [53].

We deduced using ARESite2 database [54] that TTP binds to a motif having AUUUA sequence and, in Granzyme B, TNFα and IFNγ this motif is extensively present. An immunoblotting analysis revealed a significant increase in the expression of TTP in the $CD8^+$T cells of RA patients when activated with IMQ (Fig. 4). This increase in the expression of TTP post-treatment must be the reason for the decay of the inflammatory mRNA transcripts. The increased expression of NFκB- RelA and pERK, inflammatory conditions and the presence of the AUUUA motifs further support our finding. Moreover, a number of reports suggest activation of TTP post-infection with ssRNA viruses like Influenza A virus [55] and Simian Immunodeficiency virus [56] mediating the activity of the proinflammatory cytokines.

The fate of $CD8^+$T cell population was then analysed after TLR7 engagement with IMQ. The $CD8^+$T cell sublineages were observed to shift towards a less effector memory population (Fig. 5). Effector memory cells are known to carry large amounts of Perforin and are able to secrete significant amounts of IFNγ post-activation by stimuli [57]. An increase in the naïve cell population though not significant also contributes to the factor that the cells that are being treated with IMQ are going into a state where their ability to cause inflammation via different cytokines and inflammatory mediators is being altered and they are attaining a less inflammatory fate. Furthermore, the rates of proliferation of the unstimulated and stimulated group of CD8+ T cells from RA patients were analyzed but they did not seem to have any difference in the rates of proliferation (Supplementary Fig. 3). We also performed flowcytometry analysis to check the expression of FoxP3 and ELISA to check for IL12 expression levels. We did not observe any differential expression of IL-12 and FoxP3 (Supplementary Fig. 4a, b) after IMQ treatment.

In this study, we have reported for the first time the presence of TLR7 in the $CD8^+$T cells of RA patients. The transcription factors that get activated and the subsequent increase in the mRNA transcripts of Granzyme B, Perforin, TNFα and IFNγ have also been addressed. The unique aspect of our study is the downregulation of the inflammatory mediators upon treatment with IMQ. The activation of TTP and the decline in the effector memory population are the two main evidences that support our findings at the

proteome level. In conclusion, we can suggest that IMQ-mediated activation downregulates the expression of inflammatory molecules and due to its immunomodulatory properties would prove beneficial for patients with RA.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00296-021-05050-8.

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Author contribution NS, BG, SR designed and conceptualized the work. NS, AT performed the experiments and drafted the work. NS, PP designed the workflow and recorded clinical data. NS, AT, BG drafted the manuscript. NS, BG, AT, PP, SR critically revised and approved the manuscript.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical statement The study conducted with the human samples were approved by Institutional Ethical Approval Committee, KIIT University and were in accordance to the the Helsinki Declaration of 1975, as revised in 2000. Written informed consent was obtained from all the participants in this study. We would also like to confirm that this work has not been published elsewhere and is not under consideration by another journal. All the authors have approved the submission of the manuscript to Rheumatology International.

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