

## ORIGINAL ARTICLE

## Genetic variation in the serotonin receptor gene affects immune responses in rheumatoid arthritis

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Many genetic variants associate with the risk of developing rheumatoid arthritis (RA); however, their functional roles are largely unknown. Here, we aimed to investigate whether the RA-associated serotonin receptor 2A (*HTR2A*) haplotype affects T-cell and monocyte functions. Patients with established RA ( $n = 379$ ) were genotyped for two single-nucleotide polymorphisms (SNPs) in the *HTR2A* locus, rs6314 and rs1328674, to define presence of the risk haplotype for each individual. Patients with and without the RA-associated TC haplotype were selected and T-cell and monocyte function was monitored following *in vitro* stimulations with staphylococcal enterotoxin B and lipopolysaccharide (LPS) using multiparameter flow cytometry. Within the cohort, 44 patients were heterozygous for the TC haplotype (11.6%) while none were homozygous. Upon stimulation, T cells from TC-carrier patients produced more proinflammatory cytokines (tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-17 (IL-17) and interferon gamma (IFN- $\gamma$ )) and monocytes produced higher levels of TNF- $\alpha$  compared with patients carrying the non-TC haplotype ( $P < 0.05$  and 0.01, respectively). Such cytokine production could be inhibited in the presence of the selective 5-HT2 receptor agonist (2,5-Dimethoxy-4-iodoamphetamine, DOI); interestingly, this effect was more pronounced in TC carriers. Our data demonstrate that association of RA with a distinct serotonin receptor haplotype has functional impact by affecting the immunological phenotype of T cells and monocytes.

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**Keywords:** serotonin receptor gene; rheumatoid arthritis; T cell; monocyte; cytokine

## INTRODUCTION

Detection of association in human complex diseases has been facilitated by the development of new tools for genome-wide genotyping. During the last 5 years, over 1300 genome-wide association studies have been performed,<sup>1</sup> however, most risk alleles have not been translated to specific causal effects. Indeed, due to high abundance of genetic polymorphisms and common linkage disequilibrium in genetic loci, it is an enormous task to identify causality in genetics. The difficulty may also relate to the involvement of less common genetic variants, which calls for an efficient approach to the sample selection for functional study. Two inclusion criteria are most preferable as grounds for experimental mechanistic studies: (i) an association with a combination of single-nucleotide polymorphisms (SNPs) (that is, a haplotype) rather than with a single SNP, which defines a more distinct group of individuals and (ii) availability of data for association of this locus with a disease-related subphenotype.

Serotonin (5-hydroxytryptamine; 5-HT) is an important neurotransmitter that besides its involvement in a large number of central nervous system processes also regulates many physiological functions<sup>2</sup> and displays immunomodulatory effects.<sup>3–8</sup> Several hematopoietic cell lineages such as platelets, monocytes and lymphocytes can store and release 5-HT when stimulated.<sup>9</sup> Seven main groups of 5-HT receptors (5-HT1–7) have been identified; all are G protein-coupled receptors apart from 5-HT3 which is a ligand-gated ion channel.<sup>10</sup> The 5-HT2A receptor is not only expressed by neurons but is also expressed on fibroblasts, platelets and human peripheral blood mononuclear cells

(PBMCs).<sup>11</sup> It has an important role in mediating physiological processes such as smooth muscle contraction,<sup>12</sup> platelet aggregation<sup>13</sup> and the modulation of mood and perception.<sup>14</sup> The expression of 5-HT2A receptor mRNA has been observed in dendritic cells,<sup>4</sup> monocytes<sup>15</sup> and lymphocytes.<sup>16,17</sup> This receptor is encoded by *HTR2A* (MIM 182135) that is localized on the human chromosome 13q14-q21 and consists of three exons with known five non-synonymous and two synonymous variations and two introns with > 200 known variations.

The function of the 5-HT2A receptor in the periphery is still largely unknown, but an increasing body of evidence points to effects of this receptor on immune responses.<sup>17,18</sup> The production of tumor necrosis factor alpha (TNF- $\alpha$ ) from PBMCs following lipopolysaccharide (LPS) stimulation is inhibited by 5-HT via its 5-HT2A receptor<sup>11</sup> and activation of this receptor suppresses TNF- $\alpha$ -induced inflammation in primary aortic smooth muscle cells.<sup>19</sup> The 5-HT2A receptor is also involved in *cis*-urocanic acid-induced immune suppression,<sup>20</sup> and it was recently shown that treatment of cytometric bead array (CBA) mice with a selective serotonin agonist caused immune suppression, an effect that was reversed with a 5-HT2A antagonist.<sup>21</sup>

Rheumatoid arthritis (RA (MIM 180300)) is a common chronic inflammatory disease resulting from the complex interaction between genes and environment. To date, > 30 RA-associated genetic loci have been defined with the HLA-DRB1 being the major one.<sup>22,23</sup> Recently, we demonstrated that a haplotype in *HTR2A* composed of the protective alleles of two SNPs in *HTR2A* is associated with protection against RA.<sup>24</sup> Furthermore, this

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haplotype interacts with HLA-DRB1 shared epitope alleles and the 5-HT<sub>2A</sub> receptor is colocalized with HLA-DR molecules in synovial tissue cells from patients with RA.<sup>24</sup> These findings prompted us to investigate whether this haplotype also associates with the changes in function of the immune system. To this end, we studied the impact of the TC haplotype and the effect of selective modulation of the 5-HT<sub>2A</sub> receptor on effector functions of RA-derived immune cells.

## RESULTS

The TC haplotype of HTR2A affects cytokine secretion from T cells and monocytes

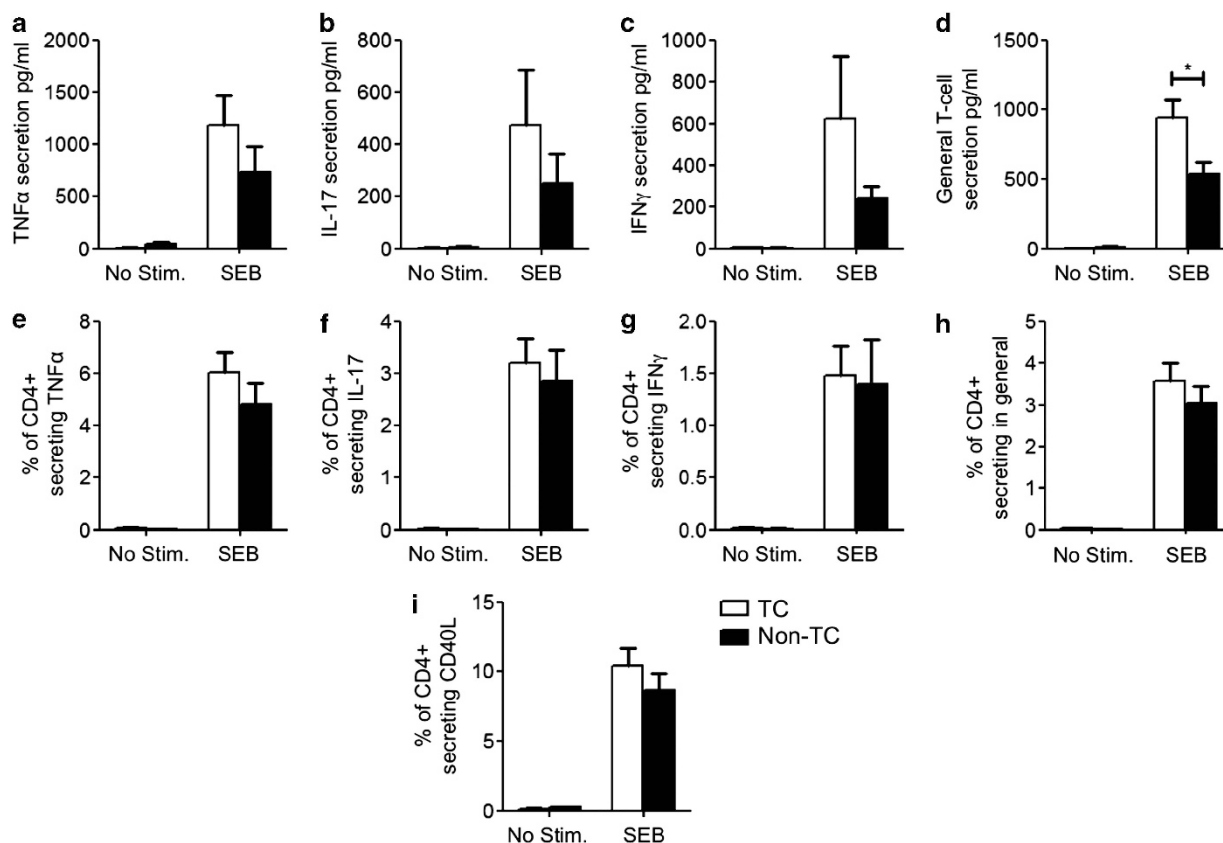
Since the 5-HT<sub>2A</sub> receptor is expressed on PBMC, we first investigated whether the specific TC haplotype in *HTR2A* that associates with RA influences T-cell and/or monocyte functions. The production of TNF- $\alpha$ , interleukin-17 (IL-17) and interferon gamma (IFN- $\gamma$ ) was monitored both in culture supernatants and intracellularly following the short-term stimulation of PBMCs with the superantigen staphylococcal enterotoxin B (SEB) and further compared between TC and non-TC patients. Interestingly, a clear tendency for higher production of TNF- $\alpha$ , IL-17 and IFN- $\gamma$  was shown in patients carrying the TC *HTR2A* haplotype as was monitored both in culture supernatants and intracellularly (Figures 1a–c and e–g, respectively). When considered altogether, these cytokines indicated significantly higher levels of proinflammatory cytokines in culture supernatants from TC patients as compared with non-TC patients (Figure 1d). These data were further corroborated by intracellular cytokine measurements in CD4<sup>+</sup>

T cells, however, this was not statistically significant (Figure 1h). Also, the overall activity levels of CD4 T cells illustrated by the levels of CD40L were similar in the two haplotype groups (Figure 1i). Gating strategy for detection of intracellular cytokine in CD4 T cells is shown in Supplementary Figure 1.

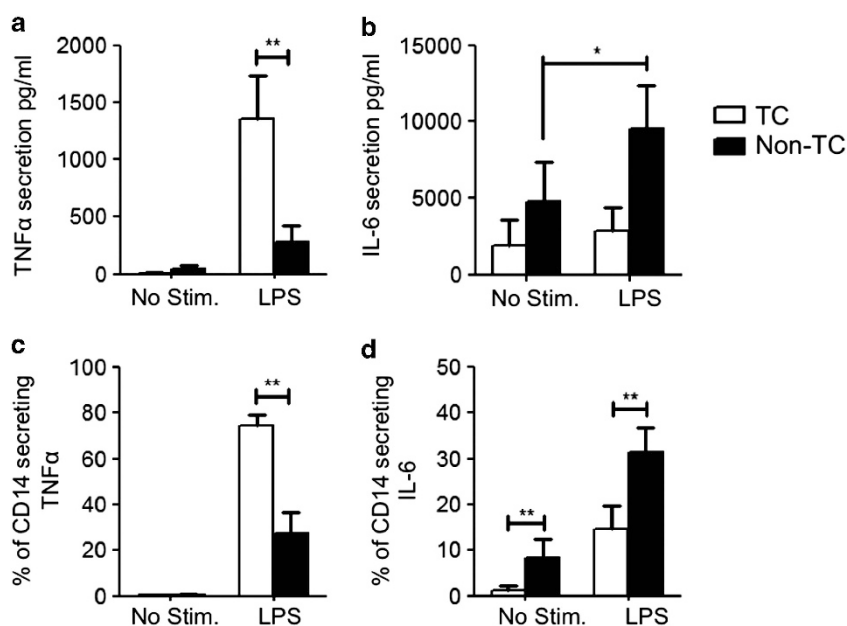
A short-term stimulation of PBMCs with LPS, which specifically stimulates monocytes, resulted in an increase in TNF- $\alpha$  and IL-6 production both in culture supernatants and intracellularly (Figure 2). Similarly to SEB stimulation, the secreted levels of TNF- $\alpha$  following LPS stimulation were significantly higher in the TC group compared with the non-TC group (Figures 2a and c), whereas for IL-6 an opposite effect was observed (Figures 2b and d). Gating strategy for detection of intracellular cytokine in monocyte following stimulation with LPS is shown in Supplementary Figure 2.

T-cell and monocyte cytokine secretion is inhibited via 5-HT<sub>2</sub> receptor

A major aim was to address the question of whether direct activation of 5-HT<sub>2A</sub> using the selective receptor agonist 2,5-dimethoxy-4-iodoamphetamine (DOI)<sup>25</sup> differently affects the function of T cells and monocytes from RA patients with or without the TC haplotype. We first examined whether DOI can induce apoptosis and/or necrosis of CD4<sup>+</sup> T cells and monocytes using Annexin V/7AAD staining. The results demonstrated that the T-cell and monocyte viability was not affected in the presence of up to 75  $\mu$ M of DOI (Supplementary Figure 3). Next, PBMCs were cultured with 50  $\mu$ M of DOI and further stimulated with either SEB or LPS to achieve T-cell and monocyte stimulation, respectively.



**Figure 1.** Increased cytokine production by T cells from RA patients carrying the TC haplotype. PBMCs from RA patients were stimulated with SEB. (a) TNF- $\alpha$ , (b) IL-17 and (c) IFN- $\gamma$  were measured in culture supernatant following SEB stimulation. (d) The mean of the pooled levels of T-cell cytokines in supernatant (TNF- $\alpha$ , IL-17 and IFN- $\gamma$ ). Production of (e) TNF- $\alpha$ , (f) IL-17 and (g) IFN- $\gamma$  was also measured intracellularly using flow cytometry and presented as a percentage of total CD4 T-cell population. (h) Mean of pooled cytokine levels that were monitored by ICS and CD40L levels in CD4 T cells (i). Significance of differences was determined by Mann-Whitney test. \* $P < 0.05$ .



**Figure 2.** Increased cytokine production by monocytes from RA patients carrying the TC haplotype. PBMCs from RA patients were stimulated with LPS. (a) TNF- $\alpha$  and (b) IL-6 production by monocytes was monitored in culture supernatant and intracellularly (c, d), respectively. TC group ( $n = 11-13$ ) and non-TC group ( $n = 10-11$ ). Significance of differences was determined by Mann-Whitney test. \* $P < 0.05$ , \*\* $P < 0.01$ .

DOI inhibited CD4<sup>+</sup> T-cell activation following SEB stimulation as monitored by reduced levels of CD40L in both TC and non-TC RA patients. Of note, the inhibitory effect induced by DOI was significantly stronger in the TC group compared with the non-TC group (Figure 3a). We further examined and compared the effects of DOI on the production of TNF- $\alpha$ , IL-17 and IFN- $\gamma$  by T cells in the two haplotype groups in culture supernatants and intracellularly. Indeed, DOI inhibited the production of these cytokines, however, significant differences were found between the two TC-haplotype groups. The secretion of TNF- $\alpha$  in the TC-patient group was reduced significantly more in comparison with the non-TC group (Figure 3b) and a similar trend was also found for IL-17 and IFN- $\gamma$  (Figures 3c and d). Also, the overall inhibition of T-cell cytokines was significantly stronger in the TC group in comparison with the non-TC group, both in culture supernatants and intracellularly (Figure 3e).

DOI treatment before LPS stimulation compared with LPS stimulation alone resulted in significant inhibition of TNF- $\alpha$  ( $P < 0.0001$ ) and a slight increase in IL-6, both primarily produced by monocytes (Supplementary Figure 4). Also here, the measured concentrations of TNF- $\alpha$  in supernatants were significantly lower in the TC group compared with non-TC group (Figure 3f). This effect, however, could not be detected using intracellular staining (ICS) (Figure 3f). In contrast, IL-6 was less inhibited in the TC group, however, this difference was not significant (Supplementary Figure 5).

To find out whether the inhibition of the cytokine production by DOI is specifically mediated via the 5-HT<sub>2A</sub> receptor or whether 5-HT<sub>2C</sub> receptor also is involved in the cytokine secretion, we pretreated PBMCs from healthy controls with receptor selective antagonists before stimulation with DOI and LPS or SEB. We could see that the inhibition of cytokine production in monocytes and T cells is partly mediated through both 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors (Figures 4a-d).

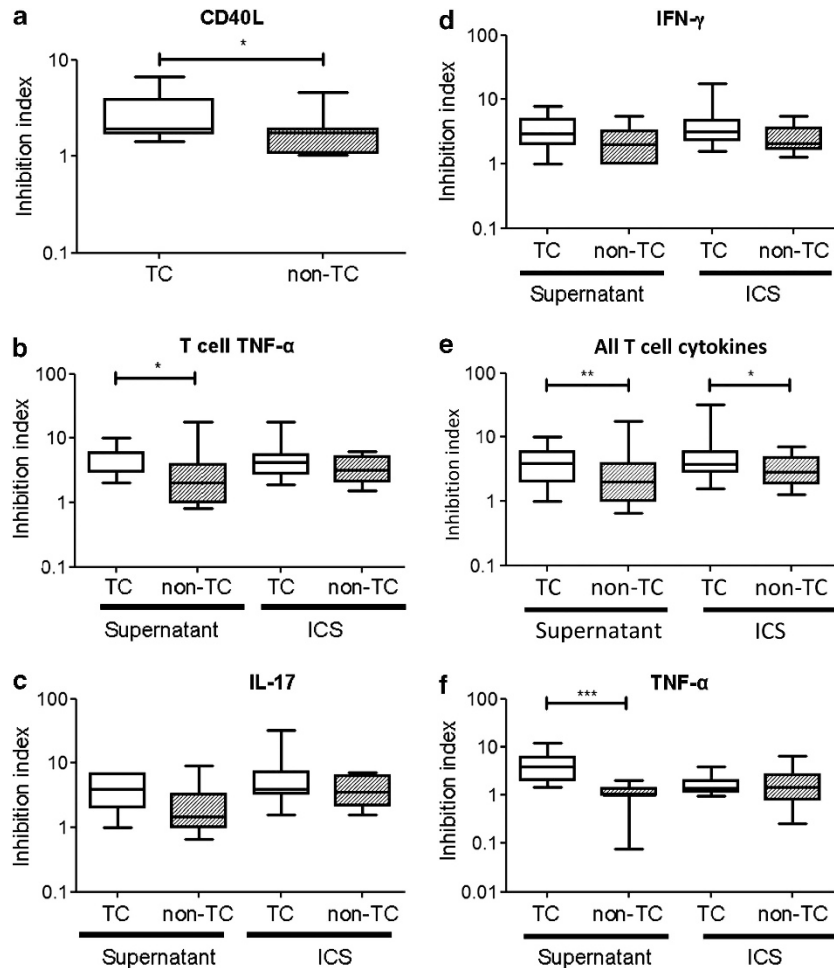
## DISCUSSION

Serotonin (5-HT) is primarily known as a neurotransmitter involved in the regulation of mood, sleep, appetite and cognition.<sup>26</sup>

However, it also influences the immune system and can regulate inflammation and cytokine secretion. Indeed, it has been demonstrated that 5-HT inhibits TNF- $\alpha$  synthesis from human monocytes and PBMCs through several 5-HT receptors.<sup>11,15,27</sup> A specific haplotype in the *HTR2A* was previously demonstrated to be associated with reduced risk for the development of RA, that is, the TC haplotype.<sup>24</sup> We therefore hypothesized that the TC haplotype may control the 5-HT regulatory effects on the immune system and examined its potential effects on T cells and monocytes in RA using a selective 5-HT<sub>2A</sub> receptor agonist (DOI).

Our data demonstrate that upon stimulation, T cells and monocytes from patients carrying the TC haplotype in *HTR2A* produce more proinflammatory cytokines compared with patients who do not. The stimulation, however, was inhibited by the 5-HT<sub>2A</sub> receptor agonist DOI and this effect was more pronounced in the TC group. These findings speak for a regulatory role of 5-HT on cytokines production by T cells and monocytes. Of interest, it further shows the greater inhibitory effect of serotonin in individuals carrying the TC haplotype, which is in agreement with the protective feature of this haplotype in RA,<sup>24</sup> indicating a possible functional explanation for this genetic polymorphism. To strengthen our studies, T-cell and monocyte function was monitored using two different complementary methods for measuring cytokine production: (i) ICS and (ii) CBA. The first allows a precise identification of the cellular source of the cytokine, while CBA gives the cumulative production of cytokines in the culture. CBA appeared to be more sensitive and ICS more specific; however, the data we got from one largely supported the other.

DOI is a selective 5-HT<sub>2A</sub>-receptor agonist; however, it can also react with the 5-HT<sub>2C</sub> receptor to a lesser extent. Previously, both Banging Yu *et al.*<sup>19</sup> and Cloëz-Tayarani *et al.*<sup>11</sup> demonstrated that the repression of TNF- $\alpha$  is mediated through the 5-HT<sub>2A</sub> receptor using DOI. However, the study by Yu *et al.* was performed on rat smooth muscle cells where the 5-HT<sub>2</sub> receptor repertoire may differ from human monocytes and T cells, while in the second study, Ketanserin, a non-selective 5-HT<sub>2A</sub> receptor antagonist was used. Thereby the involvement of the 5-HT<sub>2C</sub> receptor could not be excluded. To find whether in our experimental settings DOI



**Figure 3.** CD4<sup>+</sup> T-cell activation and cytokine secretion by T cells and monocytes are largely inhibited in the TC group of RA patients. PBMCs from RA patients were pretreated with DOI and thereafter stimulated with either (a–e) SEB or (f) LPS and cytokine production was measured in culture supernatant and intracellularly. (a) T-cell activation was monitored using CD40L and (b) TNF- $\alpha$  (c) IL-17 and (d) IFN- $\gamma$  production was measured. The overall effect of DOI on T-cell cytokine secretion is shown in (e) (f) TNF- $\alpha$  produced by monocytes. TC group ( $n = 11–13$ ) and non-TC group ( $n = 10–11$ ). Inhibition index: the ratio of the levels of cytokine secretion following stimulation divided by stimulation response with agonist. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles and the lines within the boxes represent the median. Significance of differences was determined by Mann–Whitney test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

inhibits cytokine production via the 5-HT<sub>2A</sub> and/or 5-HT<sub>2C</sub> receptors, we treated PBMCs with two different antagonists, specific to the 5-HT<sub>2A</sub> or 5-HT<sub>2C</sub> receptor before adding DOI and further stimulated with LPS or SEB. We could see that both antagonists partly reverse the effect of DOI, suggesting that both receptors are involved in the reduction of the cytokine production. Nevertheless, in our study, the involvement of both receptors in the inhibition of cytokine production does not interfere with our main finding, since the associated haplotype is only found in the 5-HT<sub>2A</sub> receptor. Moreover, the two genes are located in different chromosomes, thus, the contribution of the former can be dismissed as an additional source of variation within the two experimental haplotypes groups, rather than as a confounding factor.

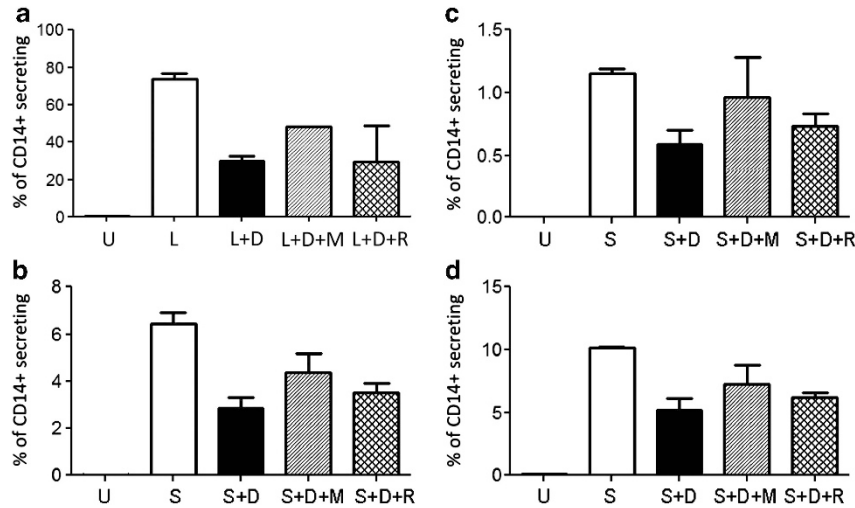
In this work, we demonstrate that an RA-associated *HTR2A* haplotype influences the proinflammatory cytokine response of T cells and monocytes. To our knowledge, this is the first evidence for a functional link between a genetic polymorphism defined by selected haplotype and immunologically important supphenotypes related to development and/or chronicity of RA. Although RA is a heterogeneous disease and performance of functional studies to verify the impact of genetic variance is challenging in

such a setting, we report here the feasibility of dissecting a functional outcome of one genetic association in a setting influenced by multiple genetic influences.

## MATERIALS AND METHODS

### Subjects

In total, 379 RA patients with established disease were recruited for this study. All patients were diagnosed as having RA by a rheumatologist in accordance with the 1987 American College of Rheumatology criteria.<sup>28</sup> Blood samples were obtained from patients following genotyping for TC haplotype (comprising rs6314 allele and rs1328674 allele). Among all patients 44 were heterozygous for the TC haplotype, whereas none of the patients were homozygous. The frequency of the TC haplotype is slightly higher in the current patient cohort (11.4%) compared with what was reported previously.<sup>24</sup> Thirteen patients carrying the specific TC haplotype were selected for functional analysis (that is, TC group). RA patients who do not carry the TC haplotype (non-TC group) were individually matched by sex, treatment and age (within a range of  $\pm 8$  years, apart from one TC patient whose match control was 13 years younger) and used as a control group (Table 1). Selection of TC-haplotype patients was based on the availability of PBMCs for functional studies as well as availability of an appropriate pair. The ethical review board of the Karolinska University



**Figure 4.** Selective 5-HT<sub>2A</sub> receptor and 5-HT<sub>2C</sub> receptor antagonists partially inhibit the effect of DOI. The intracellular TNF- $\alpha$  produced by monocytes was measured in (a) PBMCs from healthy controls treated with 1  $\mu$ M of MDL100907 (selective 5-HT<sub>2A</sub> receptor antagonist) or 1  $\mu$ M RS 102221 (selective 5-HT<sub>2C</sub> receptor antagonist) for 30 min before treatment with 50  $\mu$ M of DOI for 1 h and 50 pg ml<sup>-1</sup> of LPS for 4 h. (b–d) PBMCs from healthy controls were treated with 1  $\mu$ M of MDL100907 or 1  $\mu$ M of RS 102221 for 30 min and thereafter treated with 10  $\mu$ M of DOI for 1 h and with 10 ng ml<sup>-1</sup> of SEB overnight and the intracellular production of (b) TNF- $\alpha$ , (c) IFN- $\gamma$  and (d) CD40L by CD4<sup>+</sup> T cells was measured. These results are representative of two independent experiments. Values are the mean and s.e.m. D, DOI; L, lipopolysaccharide; M, MDL100907; S, SEB; R, RS 102221; U, unstimulated.

**Table 1.** Characteristics and treatment(s) of eligible rheumatoid arthritis patients included in the study

TC patient <sup>a</sup>	Age (years)	Treatment(s) <sup>b</sup>	Non-TC patient <sup>a</sup>	Age (years)	Treatment(s) <sup>b</sup>
1	63	Adalimumab + MTX + Prednisolone	1 <sup>c</sup>	57	Etanercept + MTX + Prednisolone
2	70	Etanercept + MTX + Prednisolone			
3	49	Etanercept + Azathioprine	2	57	Infliximab + Azathioprine
4	43	Etanercept + Metoject	3	41	Etanercept + MTX
5	53	Etanercept + MTX	4	57	Etanercept + MTX
6	58	Etanercept + Salazopyrin	5	51	Infliximab + Salazopyrin
7	49	Etanercept	6	56	Adalimumab
8	78	Untreated	7 <sup>c</sup>	77	Untreated
9	83	NSAID			
10	58	Infliximab + MTX	8	63	Infliximab + MTX
11	70	Infliximab	9	64	Infliximab
12	70	Abatacept + MTX + NSAID	10	65	Abatacept + MTX
13	82	Prednisolone	11	84	Prednisolone

Abbreviations: MTX, methotrexate; NSAID, non-steroidal anti-inflammatory drug. <sup>a</sup>All are women. <sup>b</sup>The non-TC patients were matched for the following treatments; MTX/Metoject, Salazopyrin, Abatacept and Azathioprine (DMARDs), Etanercept, Infliximab and Adalimumab (Biologics), Prednisolone (Corticosteroids). <sup>c</sup>Each of these patients was used as a match for two TC patients.

Hospital approved this study, and all subjects gave informed consent before participation in the study.

### Genotyping

Genomic DNA was extracted from EDTA-treated peripheral white blood cells using salting-out method. The genotyping for rs6314 and rs1328674 was done using TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). The results were analyzed using the Sequence Detection System v 2.2 software (Applied Biosystems). The call rate was above 95% for both SNPs.

### Isolation of PBMCs

PBMCs were isolated from blood of RA patients using Ficoll separation (Ficoll-Paque Plus, GE Healthcare, St Louis, MO, USA) and thereafter frozen in fetal calf serum supplemented with 10% DMSO (Merck KGaA, Darmstadt, Germany) and kept at -150 °C until use.

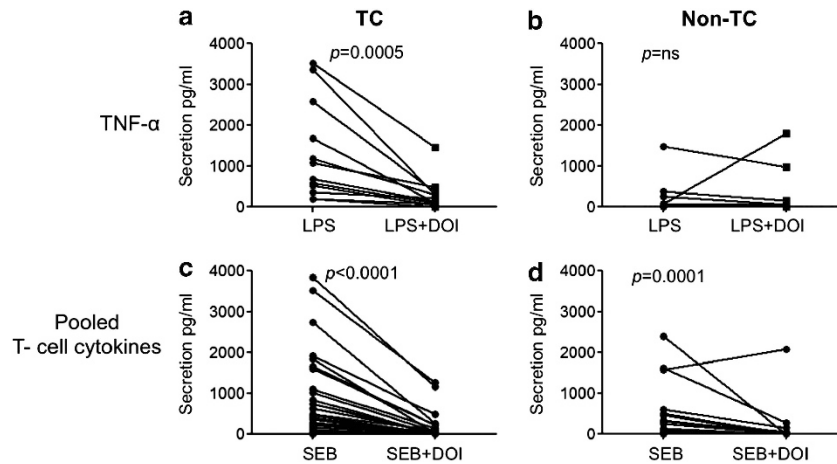
### Antibodies and intracellular flow cytometry

PBMCs were thawed and cultured in flat-bottom 96-well plates, 1.0  $\times$  10<sup>6</sup> cells per well, in the presence or absence of 50  $\mu$ M of ( $\pm$ )DOI

(Sigma-Aldrich, St Louis, MO, USA) for 16 h. Cells were then stimulated with either 50 ng ml<sup>-1</sup> of SEB (Sigma-Aldrich) or 50 pg ml<sup>-1</sup> of LPS (Sigma-Aldrich) for 5 or 4 h, respectively, together with 10  $\mu$ g ml<sup>-1</sup> brefeldin A (Sigma-Aldrich). Following stimulation, cells were treated with LIVE/DEAD Fixable Green Dead Cell Stain (Invitrogen, Carlsbad, CA, USA), washed and permeabilized using a Cytofix/Cytoperm fixation/permeabilization solution kit (BD Biosciences, Franklin Lakes, NJ, USA). SEB-stimulated cells were stained for expression of CD3 (BioLegend, San Diego, CA, USA), CD4, CD14 (BD Biosciences), TNF- $\alpha$ , IFN- $\gamma$ , IL-17A (BioLegend) and CD154 (BD Biosciences), whereas LPS-stimulated cells were stained for expression of IL-6 (BioLegend), CD3, CD14 and TNF- $\alpha$ . Samples were acquired on a CyAn ADP Analyzer (Dako, Glostrup, Denmark) and the data were analyzed by FlowJo software version 7. 6.1 (Tree star, Ashland, OR, USA).

### Detection of cytokines in culture supernatant

In parallel to each cell culture described above, an identical culture was set in the absence of brefeldin A, allowing cytokine secretion. Culture supernatants were collected and the levels of TNF- $\alpha$ , IL-17, IFN- $\gamma$  and IL-6 were determined using CBA according to manufacturer's instructions (BD Biosciences). Samples were acquired using CyAn flow cytometer and



**Figure 5.** DOI causes a greater cytokine inhibition from monocytes and T cells of patients carrying the TC haplotype. The effect of DOI on TNF- $\alpha$  production from LPS-stimulated monocytes isolated from TC patients (a) and non-TC patients (b) Pooled T-cell cytokine levels (that is, TNF- $\alpha$ , IL-17 and IFN- $\gamma$ ) in culture supernatants of patients carrying the TC haplotype (c) in comparison with patients who do not (d) following stimulation with SEB and subsequent treatments with DOI. TC group ( $n = 11-13$ ) and non-TC group ( $n = 10-11$ ). Significance of differences between the groups was determined using Wilcoxon signed rank test. NS, not significant.

the concentrations were determined using FCAP Array v1.0.1 software (BD Biosciences).

Cytokine levels in culture supernatants were monitored in  $\text{pg ml}^{-1}$  and the background levels were subtracted. However, to reduce variability between samples as well as between different cytokines the measured cytokine levels were ranked from 1 to 25, with the highest level being 25 and the lowest being 1. To confirm that this ranking did not introduce an error in our analysis, a pair-wise comparison was performed for cytokine levels measured in supernatant from samples treated with either LPS or SEB following DOI treatment (Figures 5a–d).

### Statistics

The distribution of genotypes was in agreement with Hardy–Weinberg equilibrium ( $P > 0.05$ ). The haplotypes, comprising rs6314 and rs1328674, for each individual were assigned using PHASE software.<sup>29</sup> Whereas inhibition is usually expressed as a percentage of stimulation, in the present study we have inverted this index to circumvent values that lay outside the maximum threshold (100%). Thus, the reduction in cytokine secretion following treatment with DOI was assessed using an inhibition index, calculated as the ratio between an SEB/LPS-stimulated sample and its corresponding DOI-pre-treated one. Due to the limited number of samples that met the criteria to be included in the study, cumulative analysis of proinflammatory T-cell cytokines (TNF- $\alpha$ , IL-17 and IFN- $\gamma$ ) was used to increase the statistical power. Differences in cytokine secretion or inhibition between two groups were analyzed by unpaired, non-Gaussian two-tailed *t*-tests (Mann–Whitney) and values of  $P < 0.05$  were considered as significant. All the graphics were performed using Graphpad Prism (GraphPad Software Inc., La Jolla, CA, USA).

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### ACKNOWLEDGEMENTS

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