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ORIGINAL ARTICLE

T-cell specific upregulation of Sema4A as risk factor for autoimmunity in systemic lupus erythematosus and rheumatoid arthritis

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ABSTRACT

The aim of the present study was to evaluate the impact of *SEMA4A* genetic variants on expression of sema4A protein and its relation to autoimmunity development in Systemic Lupus Erythematosus and Rheumatoid Arthritis patients. A total of 541 SLE patients, 390 RA patients and 607 healthy individuals were genotyped. We also assessed *SEMA4A* mRNA expression from whole blood cells and the in vitro protein production from resting and activated T lymphocytes as well as mature dendritic cells from healthy individuals stratified according to their genotypes for SLE/RA associated *SEMA4A* variants. Our results showed that T/T genotype for rs3738581 SNP is associated with both RA and SLE development ($p = .000053$, OR = 2.35; $p = .0019$, OR = 2.07, respectively; statistical power = 100%) and also to an increased in vitro sema4A production in active T lymphocytes. Our findings are indicative of a T cell-specific upregulation of sema4A in the presence of T/T genotype, being a risk factor for SLE and RA.

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KEYWORDS
SEMA4A; systemic lupus erythematosus; rheumatoid arthritis; polymorphism; T cells

1. Introduction

Semaphorins, first described as guidance factors that assist axon pathfinding during neuronal development [1], have been shown to play important roles in heart morphogenesis [2,3], vascular growth [2,3], tumour progression [4,5], and immunoregulation [6]. This group of proteins has been divided into different subclasses I to VII according to their structure, being the classes III–VII expressed in vertebrates [7].

The Semaphorin 4 A (sema4A) is highly expressed on the surface of mature dendritic cells (DC) and active T lymphocytes, but poorly expressed on B and resting T cells membrane [8]. DC-derived sema4A showed to be essential for T cell priming as well as T-cell derived sema4A for T helper type 1 (Th1) and 17 (Th17) differentiation [9].

Sandrin-Garcia et al. [10] evaluated the expression profile of 4500 genes in patients with active and inactive systemic lupus erythematosus (SLE): a total of 156 genes were differentially expressed between patients and healthy controls. Within them, *SEMA4A* gene was upregulated (+32-fold change) in active SLE patients. Considering the imbalance of T helper cell subsets in SLE pathogenesis and the involvement of sema4A on the immune cell regulation [11], we considered the *SEMA4A* gene as a potential target gene in susceptibility to SLE and autoimmunity development.

In this study, we have investigated the hypothesis that genetic variants in *SEMA4A* result in an altered expression of sema4A and are involved in autoimmunity development. Therefore, we investigated the impact of *SEMA4A* genetic variants in SLE and rheumatoid arthritis (RA) development in Brazilian populations. Following, to functionally understand the impact of the associated genetic variants, we assessed *SEMA4A* mRNA expression in whole blood cells and the in vitro protein production from resting and activated T lymphocytes as well as mature DC cells from healthy individuals stratified according to their genotypes for SLE and RA associated polymorphism.

2. Material and methods

2.1. SEMA4A *genotyping study*

2.1.1. Subjects

For this study, we enrolled patients and controls from three Brazilian cohorts (a Northeastern cohort from state of

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Supplemental data for this article can be accessed here.

Pernambuco, a Southeastern cohort from São Paulo and a South-Brazilian cohort from Rio Grande do Sul), comprising 541 SLE patients, 390 RA patients and 607 healthy controls. The northeastern sample comprised 123 SLE patients, 118 RA patients and 158 healthy individuals from the state of Pernambuco (Northeast of Brazil). The Southeastern sample comprised 158 SLE patients, 89 RA patients and 189 healthy controls from state of São Paulo (Southeast of Brazil) and the Southern sample comprised 260 SLE patients, 183 RA patients and 260 healthy controls from state of Rio Grande do Sul (South of Brazil). Patients from Northeast of Brazil were under care of the Division of Rheumatology of Clinical Hospital from Federal University of Pernambuco, whereas patients from Southeast and South's Brazil were recruited from Division of Clinical Immunology of University Hospital of the School of Medicine of Ribeirão Preto, University of São Paulo and Division of Rheumatology of Clinical Hospital from Federal University of Rio Grande do Sul, respectively. All patients were diagnosed according to the American College of Rheumatology (ACR) criteria for the classification of SLE [12] and RA [13]. As control group, we enrolled healthy blood donors without previous family history of autoimmune diseases, as reported in an appropriate questionnaire. Clinical and laboratory data from all patients were collected from medical records and are shown in Table 1. All the participants provided a written informed consent according to the Declaration of Helsinki and approved by the local Research Ethics Committee (Southeast: CAAE 34636013.9.3001.5440 (SLE and RA), Northeast: CAAE 03065312.3.0000.5208 (SLE and RA) and South: CAAE 01731012.0.0000.5327 (SLE) and HCPA 08-366 (RA)).

Table 1. Controls and patients' characteristics.

	Northeastern	Southeastern	Southern
Controls ($n = 607$)	$n = 158$	$n = 189$	$n = 260$
Females	86.4%	51.6%	35.8%
Males	13.6%	48.4%	64.2%
Age[a]	40.8 ± 14.65	37.4 ± 11.31	42.1 ± 8.9
Rheumatoid arthritis ($n = 390$)	$n = 118$	$n = 89$	$n = 183$
Females	95.3%	92.2%	81.5%
Males	4.7%	7.8%	18.5%
Age[a]	51.2 ± 11.6	55.7 ± 10.8	64 ± 12.7
Age at diagnosis[a]	42.1 ± 11.7	44.8 ± 13.2	46 ± 13.7
Positive rheumatoid factor	71.2%	73.4%	89.7%
Bone erosions	75.8%	89.9%	86.1%
DAS28[b]	4.53 (0–7.84)	5.59 (2.87–7.89)	3.88 (0.84–7.45)
HAQ[b]	1.42 (0–3)	NA	1.22 (0–3)
Systemic lupus erythematosus ($n = 541$)	$n = 123$	$n = 158$	$n = 260$
Females	99%	94.4%	90.8%
Males	1%	5.6%	9.2%
Age[a]	37.5 ± 10.4	39.8 ± 11.9	49.7 ± 15.1
Age at diagnosis[a]	31.2 ± 7.7	NA	33 ± 14
Malar rash	58.9%	55%	56.7%
Discoid rash	17.8%	16%	13.5%
Photosensitivity	67.3%	29.4%	79.3%
Ulcers	19.6%	NA	36.5%
Arthritis	72%	42.6%	83.7%
Serositis	22.4%	25.2%	31.3%
Nephritis	50.5%	55.9%	39.9%
Neurological alterations	7.5%	20.3%	10.6%
Hematological alterations	68.2%	61.8%	70.7%
Positive ANA	93.5%	80.4%	99.5%
Anti-DNA	24.4%	19.6%	46.6%
Anti-Sm	8.42%	NA	17.8%
Antiphospholipid syndrome	5.6%	21%	6.3%
SLEDAI[b]	3.6 (0-16)	NA	3 (0-37)
SLICC[b]	0.9 (0-5)	NA	1 (0-7)

[a]Mean ± SD.
[b]Mean (minimum–maximum).
DAS28: disease activity score in 28 joints; HAQ: health assessment questionnaire; ANA: antinuclear antibody; SLEDAI: systemic lupus erythematosus disease activity index; SLICC: systemic lupus international collaborating clinics; NA: not available.

2.1.2. DNA isolation

Genomic DNA was isolated from peripheral blood samples, using DNA Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the standard protocol from the manufacturer or using a salting out method [14].

2.1.3. SNPs selection and gene genotyping

Polymorphisms were selected crossing and merging data from SNP Browser software 4.0 (Applied Biosystems, Foster City, CA) and HapMap database (hapmap.ncbi.nlm.nih.gov) in order to cover most of the *SEMA4* gene (1q22). The selected tag SNPs - rs3738581 (intronic region, near 3′UTR), rs7695 (3′UTR), rs12401573 (exonic region) and rs3738582 (intronic region)—presented at least 10% minimum allele frequency (MAF) (Figure 1).

Genotyping was performed with fluorogenic allele specific probes (Taqman Probes, Applied Biosystems, Foster City, CA), using an ABI7500 sequence detection system (Applied Biosystems, Foster City, CA). Initially, the four tag SNPs were genotyped in SLE patients and controls and then after analysis, the SLE-associated SNP (rs3738581) was genotyped in RA patients.

2.1.4. Statistical analysis

Genotype frequencies were compared for Hardy–Weinberg (HW) expectations using Genotype Transposer [15]. In a preliminary analysis, the allele and genotype frequencies from healthy controls and RA and SLE patients were compared using chi-square test. Binary logistic regression was used to evaluate the association between the polymorphisms and SLE and RA, adjusting for origin of sample and sex. Bonferroni's correction for multiple comparisons was applied when p-value was significant ($N = 4$). The significance level was set at $\alpha = .05$ (two-tailed). All statistical analyses were performed with SPSS 15.0 (SPSS, Inc., Chicago, IL). The eventual presence of linkage disequilibrium between *SEMA4A* polymorphisms and the association of haplotypes with SLE susceptibility were evaluated by using the online tool SNPStats [16]. The power of the analyses was verified using G*Power software 3.1.9.2 (Kiel University, Germany).

2.2. SEMA4 mRNA and protein expression assays

2.2.1. Subjects

We assessed the *SEMA4A* mRNA expression levels in whole blood cells and the sema4A protein levels from resting and stimulated T cell as well as mature DCs of 28 healthy

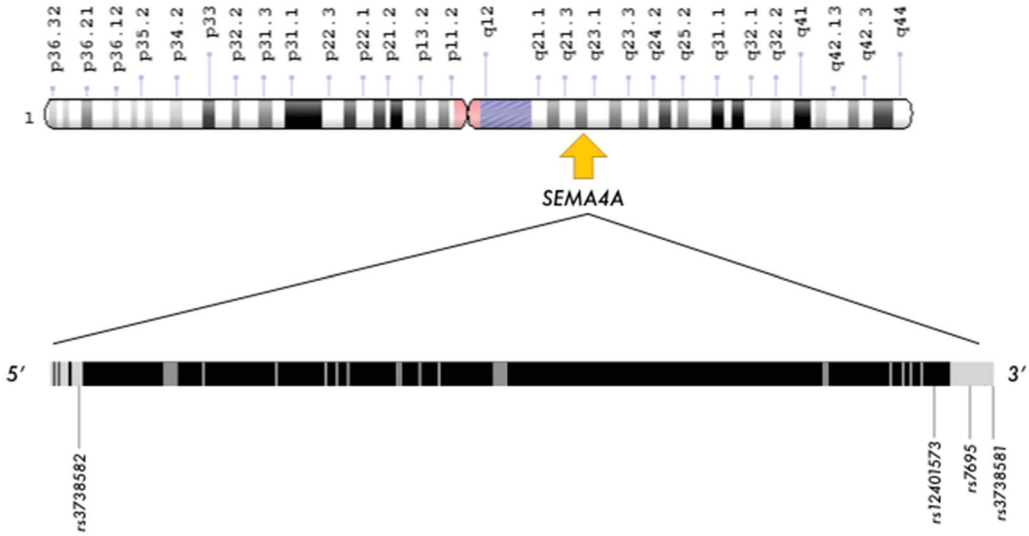

Figure 1. *SEMA4* gene (1q22) structure and studied Tag SNPs; rs3738581 (intron, near 3′UTR), rs7695 (3′UTR), rs12401573 (exon 15) and rs3738582 (intron, near 5′ UTR).

individuals stratified according to their genotypes for the SLE/RA associated *SEMA4A* polymorphism.

2.2.2. Whole blood RNA extraction and cDNA synthesis

Peripheral blood samples were collected and immediately used for RNA isolation, which was performed using the Qiagen Whole Blood RNAse kit, following the manufacturer's instructions. The samples were stored at −80 °C until used and RNA integrity analysis was performed by gel electrophoresis and quantification by Nanodrop 2000 (Thermo Scientific USA).

For cDNA synthesis, we followed the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen, USA) standard protocol, employing for each sample a standard RNA input of 500 ng for each reaction of 20 µL of cDNA. Oligo(dT) was used as primers in all samples. cDNA was stored at −20 °C until qPCR assays.

2.2.3. mRNA expression analysis

Gene expression assays for *SEMA4A* cDNA was performed using the ABI 7500 SDS platform (Applied Biosystems, USA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* was the reference gene used for normalization (*SEMA4A*: Hs00223617_m1 and *GAPDH*: Hs02758991_g1). Gene expression analyses were performed with technical triplicates. The calculation of $2^{-\Delta Ct}$ was performed for each subject [17]. Student's *t*-test was applied to compare the quantitative expression between different genotypes.

2.2.4. Cells culture and immunophenotype analysis

Peripheral blood (10 mL) was collected in EDTA tube by venipuncture and isolated by density gradient using Ficoll-Paque (Sigma-Aldrich, USA). After centrifugation, the PBMCs ring was collected and washed twice in 1× PBS saline. The cell pellet was suspended in 1 mL of RPMI-1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% of FBS (Fetal Bovine Serum, Sigma-Aldrich). Cells were counted by means of microscopy in a Neubauer Chamber and the viability always exceeded 95%, as judged from their ability to exclude Trypan Blue (Sigma-Aldrich, St. Louis, MO). PBMCs were culture on 96-well plate "U bottom" at a concentration of the 200,000 cells/well for subsequent staining with antibodies.

T lymphocytes were cultured in nonstimulated and stimulated conditions. For stimulation, T cells were cultured with phytohemaglutinin 1%. Following 18 h, cells were harvested and analyzed by flow cytometry.

DCs were generated from monocytes isolated by adherence after 7 days of culture. The following cytokines were used: 50 ng/mL of human granulocyte-macrophage colony stimulating factor (GM-CSF) and recombinant human IL-4 (Sigma-Aldrich, USA) for 5 days. On day 5, the DCs were maturated through 500 ng/ml of lipopolysaccharide (LPS; Sigma-Aldrich). Following two days, DCs were harvested and analyzed by light microscopy and by flow cytometry. The phenotype of the cultured DC was confirmed by the expression of HLA-DR, CD11c, CD80 and CD86, and low levels of CD14 [18,19].

Cells were incubated with conjugated mAb or isotype controls for 30 min protected from light at room temperature. The monoclonal antibodies used were: SEMA4A-FITC, CD80—PE-CY7, CD86—APC, CD14—Texas Red, CD11c—PE and HLA-DR—PerCP-Cy5.5 for DCs; CD3—APCH7 and CD4—PerCP-Cy5.5 for resting T cells; and CD3, CD4 and CD25—FITC for stimulated T lymphocytes. All purchased from BD Biosciences, San Diego, CA. The stained cells were washed twice with PBS, fixed with 2% paraformaldehyde in PBS. Cells were kept at 4 °C and protected from light until analysis by flow cytometry. The frequency of positive cells was calculated from lymphocyte gates from PBMCs and 10,000 events were acquired by size (FSC) and granularity (SSC) using the BD *FACSAria*™ III cell-sorter flow cytometer (BD Biosciences, San Diego, CA). For DC, at least 20,000 events were acquired and analyzed. Compensation was made with non-stained and single

Table 2. *SEMA4A* rs3738581 SNP allele and genotype frequencies in controls and SLE and RA patients.

SNPs	Controls *N* (%)	SLE *N* (%)	*p*	OR	95% CI	RA *N* (%)	*p*	OR	95% CI
rs3738581									
C	780 (64.3)	596 (55.1)		OR = 1		434 (55.6)		OR = 1	
T	434 (35.7)	486 (44.9)	.000009	OR = 1.47	1.23–1.73	346 (44.4)	.00014	OR = 1.43	1.19–1.72
C/C	252 (41.5)	156 (28.8)		OR = 1		121 (31.1)		OR = 1	
C/T	276 (45.5)	284 (52.5)	.0001[a]	OR = 1.66	1.27–2.17	192 (49.2)	.011[c]	OR = 1.45	1.1–1.95
T/T	79 (13)	101 (18.7)	.0007[b]	OR = 2.06	1.42–2.99	77 (19.7)	.0003[d]	OR = 2.03	1.36–3.03
HWE	0.80	0.16				0.95			

[a]Binary logistic regression, adjusted by gender and origin of sample: $p = .00018$, OR = 1.76, 95% CI = 1.31–2.36; *p*-value after Bonferroni correction = .0007.
[b]Binary logistic regression, adjusted by gender and origin of sample: $p = .000053$, OR = 2.35, 95% CI = 1.55–3.55; *p*-value after Bonferroni correction = .0002.
[c]Binary logistic regression, adjusted by gender and origin of sample: $p = .019$, OR = 1.49, 95% CI = 1.1–2.09; *p*-value after Bonferroni correction = .076.
[d]Binary logistic regression, adjusted by gender and origin of sample: $p = .0019$, OR = 2.07, 95% CI = 1.31–3.27; *p*-value after Bonferroni correction = .0076.
SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; *p*: chi-square test *p*-value; OR: odds ratio; CI: confidence interval; HWE: Hardy–Weinberg equilibrium.

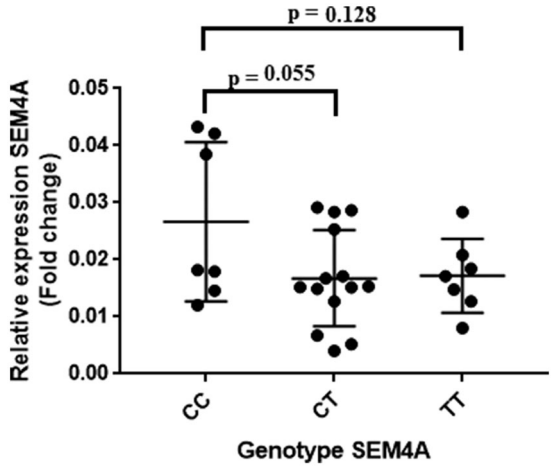

Figure 2. *SEMA4A* gene expression in 28 healthy controls stratified by genotypes (T/T genotype: 7 individuals; C/T: 14; C/C:7). The results were normalized to *GAPDH* expression. The calculation of $2^{-\Delta Ct}$ was performed for each subject. Student's *t*-test was applied to compare the quantitative expression between different genotypes.

stained controls. All analyzes were performed by FlowJo 7.5.5 (Tree Star Corporation, Ashland, OR).

All variables were tested for normality of distribution by means with the Kolmogorov-Smirnov test. Statistical analysis of data was performed using One-way ANOVA. Bonferroni's correction was applied for multiple comparisons among individuals stratified by genotypes. Data are presented as mean fluorescence intensity (MFI) of SEMA4A. The significance level was set at $\alpha = 0.05$ (two-tailed). Analyses of data were performed through the SPSS 15.0 Software Inc. (Chicago, IL).

3. Results

3.1. Genotyping study

Genotype distributions were in Hardy–Weinberg equilibrium for the assessed SNPs with exception of *SEMA4A* rs12401573 in SLE group ($p = .04$) (Supplementary Table 1).

Considering patients and controls independently of their geographical origin, we found a significant association of *SEMA4A* rs3738581 SNP [C > T] with RA and SLE susceptibility with a statistical power = 1.0 for both groups. The T allele and C/T and T/T genotypes were significantly more frequent in SLE (44.9%, *p* value = .000009, OR = 1.47, 95% CI = 1.23–1.73; 52.5%, *p*-value = .0001, OR = 1.66, 95% CI = 1.27–2.17; and 18.7%, *p*-value = .0007, OR = 2.06, 95% CI = 1.42–2.99, respectively) and RA patients (44.4%, *p*-value = .00014, OR = 1.43, 95% CI = 1.19–1.72; 49.2%, *p*-value = .011, OR = 1.45, 95% CI = 1.1–1.95; and 19.7%, *p*-value = .0003, OR = 2.03, 95% CI = 1.36–3.03, respectively) than in controls (35.7%, 45.5%, and 13%, respectively). When we performed a binary logistic regression adjusting for gender and geographical origin of sample, the association between C/T and T/T genotypes and susceptibility to SLE (*p*-value = .00018, OR = 1.76, 95% CI = 1.31–2.36; *p*-value = .000053, OR = 2.35, 95% CI = 1.55–3.55, respectively) and RA (*p*-value = .019, OR = 1.49, 95% CI = 1.1–2.09; *p*-value = .0019, OR = 2.07, 95% CI = 1.31–3.27, respectively) remained statistically significant. After applying Bonferroni's correction, the associations still remained statistically significant ($p\text{Bonf} < .05$) with exception of C/T genotype for RA group ($p\text{Bonf} = .076$). *SEMA4A* rs3738581 genotype and allele frequencies are shown in Table 2.

When we assessed the influence of *SEMA4A* polymorphisms on SLE and RA clinical and laboratorial features, the association between T/C and C/C genotypes for rs12401573 SNP [T > C] and hematological alterations in SLE patients was observed (*p*-value = .007, OR = 2.01, 95% CI = 1.21–3.32; *p*-value = .018, OR = 2.09, 95% CI = 1.14–3.84, respectively) (Supplementary Table 2). Linkage disequilibrium was not observed for the studied *SEMA4A* polymorphisms.

3.2. SEMA4A expression analyses

To determine whether SLE and RA associated *SEMA4A* rs3738581 SNP differentially affects gene expression, we compared *SEMA4A* mRNA levels of C/C healthy individuals with heterozygous (C/T) and homozygous (T/T) individuals for the risk allele in whole blood cells. Our findings indicated C/T and T/T genotypes do not impact *SEMA4A* gene regulation (*p*-value > .05) (Figure 2). To explore if any genotype-associated down or upregulation of *SEMA4A* could be cell type or stimulus dependent, we evaluated the in vitro sema4A expression in mature DCs as well as resting and active T lymphocytes. We found a significantly increased expression of sema4A on the surface of stimulated T cells ($CD3^+CD4^+CD25^+$) from individuals homozygous for the risk allele when compared to the expression of C/C

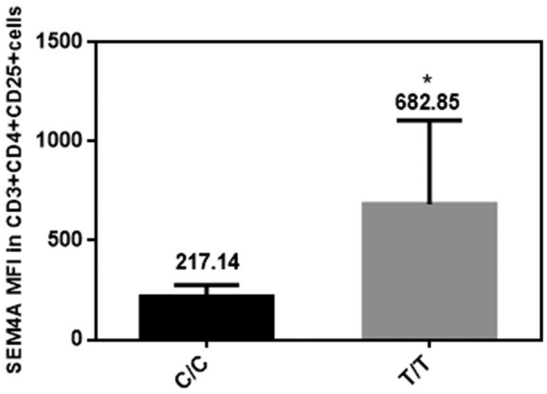

Figure 3. Sema4A production in 28 healthy controls stratified by genotypes (T/T genotype: 7 individuals; C/T: 14; C/C:7). Data are presented as mean fluorescence intensity (MFI) of sema4A. *p-value $< .05$.

T cells (MFI 682.85 ± 25.03 *vs.* MFI 217.14 ± 14.05 respectively, p-value $= .04$) (Figure 3).

4. Discussion

The development of SLE and RA autoimmunity is related to both environmental and genetic factors, the latter involving immune response–related genes variants and differential expression [20,21]. In this study we evidenced for the first time an increased sema4A expression in active T cells related to the SLE and RA-associated polymorphism rs3738581 [C > T]. The *SEMA4A* rs3738581 T allele seems to confer a similar augmented risk for the development of both RA and SLE when in homozygous condition (OR = 2.07 and 2.35, respectively) with a power of 100%. When considering *SEMA4A* SNPs in SLE clinical and laboratorial manifestations we observed the association between T/C and C/C genotypes for rs12401573 SNP [T > C] and hematological alterations.

The polymorphism rs3738581 is located within an intronic region near 3′ UTR of *SEMA4A* gene, which could interfere with the binding of miRNAs. In whole blood cells, we did not observe statistically significant changes in *SEMA4A* mRNA expression in heterozygous or homozygous healthy individuals for rs3738581 risk allele. When we evaluated the in vitro sema4A production in mature DCs and T cells, in nonstimulated and stimulated conditions, to further explore if any genotype-associated down or upregulation of *SEMA4A* could be cell type or stimulus dependent, an increased sema4A production was observed in active T lymphocytes of risk allele homozygous subjects.

Our results are indicative of a T cell-specific upregulation of sema4A after activation in the presence of T/T genotype. The risk allele in homozygous individuals may have an impact on amplified sema4A production in T cells, being a susceptibility factor for the autoimmunity development. The specific mechanism by which the sema4A manifests greater production due to T/T genotype remains unknown.

Studies on the roles of sema4A have observed this molecule as critical immune regulators in Th1 and Th17 differentiation [9]. SLE and RA are inherited as complex traits, in which no single gene variant is sufficient to cause disease. Due to the moderate value of OR, the high risk *SEMA4A* allele is not sufficient by itself to result in disease development. Rather, increased sema4A levels may contribute to the pathogenesis of autoimmunity, perhaps by functioning as an adjuvant, exacerbating Th1 and Th17 response.

It is important to point out that increased levels of sema4A have already been observed in the synovial tissue and serum of RA patients compared to osteoarthritis. Further, increased sema4A levels were also correlated with RA activity [22]. Moreover, as before mentioned, Sandrin-Garcia et al. [10] observed the upregulation of *SEMA4A* gene in active SLE patients. Wang et al. [22] observed the in vitro upregulated expression of matrix metalloproteinases and proinflammatory cytokines interleukin-1β and tumour necrosis factor (TNF)-α through the treatment with human recombinant sema4A. High levels of metalloproteinases as well as the pro-inflammatory cytokines IL-1β and TNF-α have been involved in SLE and RA pathogenesis, revealing an important role in autoimmunity and inflammation [23–27]. Sema4A is also considered a player in experimental autoimmune encephalomyelitis as increased expression of sema4A was shown to cause neuroinflammation that leads to demyelination [28]. These findings together with our results indicate a relationship between altered expression of sema4A and the beginning of autoimmunity, which may culminate in the development of autoimmune diseases such as SLE and RA.

5. Conclusion

In conclusion, our results support a role of *SEMA4A* gene in the susceptibility to RA and SLE. To the best of our knowledge, this is the first association study between *SEMA4A* SNPs and RA and SLE. We also showed an impact of rs3738581 SNP on increased sema4A expression levels and their potential correlation with SLE and RA development and worsening. Further studies in different cohorts and mechanistic studies are required to confirm and fully understand the role of SEMA4A in autoimmunity development.

Disclosure statement

The authors have declared no conflicts of interest.

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