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Πτυχιακή Εργασία

«Διερεύνηση του ρόλου της πρωτεΐνης SMC1A (Structural Maintenance of Chromosomes 1A) στην παθογένεια του Συστηματικού Ερυθηματώδους Λύκου.»

"Delineating the impact of deregulated SMC1A (Structural Maintenance of Chromosomes 1A) in the pathogenesis of Systemic Lupus Erythematosus."

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Σύνοψη

Ο Συστηματικός Ερυθηματώδης Λύκος (ΣΕΛ) αποτελεί το πρωτότυπο συστηματικό αυτοάνοσο νόσημα, που προσβάλλει πολλαπλά όργανα των ασθενών, όπως το δέρμα , οι νεφροί και το κεντρικό νευρικό σύστημα. Ένα ιδιαίτερο χαρακτηριστικό αυτού του νοσήματος είναι ότι εμφανίζεται σε πολύ μεγαλύτερη συχνότητα στις γυναίκες σε σύγκριση με τους άντρες. Αντίθετα, οι άντρες, παρότι προσβάλλονται λιγότερο συχνά, εμφανίζουν συνήθως πιο σοβαρές εκδηλώσεις της νόσου (πχ. νεφρίτιδα).

Στο εργαστήριό μας πραγματοποιήθηκε RNA sequencing σε ολικό αίμα από υγιείς και ασθενείς με ΣΕΛ προκειμένου να εντοπίσουμε γονίδια και μοριακούς μηχανισμούς που εμπλέκονται στη νόσο. Ακολούθησε βιοπληροφορική επεξεργασία των αποτελεσμάτων για την ανίχνευση γονιδίων που εκφράζονται διαφορετικά στα δύο φύλα. Η ανάλυση αυτών των δεδομένων οδήγησε στην ανακάλυψη μιας φυλο-εξαρτώμενης (sex-biased) μοριακής «υπογραφής», η οποία αποτελείται από 6 γονίδια που εκφράζονται διαφορετικά στα δύο φύλα και είναι συγκεκριμένη για το ΣΕΛ. Μεταξύ αυτών των γονιδίων, το SMC1A (Structural Maintenance of Chromosomes 1A) βρέθηκε να έχει την μεγαλύτερη αλλά και πιο σημαντική στατιστικά διαφορά μεταξύ ανδρών και γυναικών ασθενών. Σύμφωνα με τα αποτελέσματα της ανάλυσης, το SMC1A ήταν σημαντικά μειωμένο στους άνδρες ασθενείς συγκριτικά με τις γυναίκες ασθενείς και τους αντίστοιχους υγιείς.

Το γονίδιο SMC1A κωδικοποιεί για μια πρωτεΐνη που αποτελεί βασικό δομικό συστατικό του συμπλέγματος της κοχεσίνης (cohesin complex) (SMC1A, SMC3, RAD21. STAG1/2). Η πρωτεΐνη SMC1A, ως μέλος αυτού του συμπλέγματος, συμμετέχει σε ποικίλες λειτουργίες που σχετίζονται με την βιολογία του χρωμοσώματος και εν γένει των κυττάρων. Αρχικά, είναι απαραίτητη για την σύζευξη των αδελφών χρωματίδων και τον σωστό διαχωρισμό των χρωμοσωμάτων κατά τη διάρκεια του κυτταρικού κύκλου ενώ παράλληλα συμμετέχει στην γονιδιακή ρύθμιση μέσω αναδιαμόρφωσης της αρχιτεκτονικής της χρωματίνης. Επιπλέον, εμπλέκεται στο δίκτυο επιδιόρθωσης των βλαβών του DNA καθώς έχει δειχθεί ότι η φωσφορυλίωσή της, από τον παράγοντα που αντιλαμβάνεται τις βλάβες στο DNA, ATM (Ataxia Telangiectasia Mutated) , είναι αναγκαία για την επιβίωση του κυττάρου μετά από την εμφάνιση βλάβης στο DNA.

Πρόσφατες μελέτες υποστηρίζουν ότι τα λεμφοκύτταρα από ασθενείς με ΣΕΛ δεν μπορούν να επιδιορθώσουν αποτελεσματικά τις βλάβες του DNA με αποτέλεσμα να οδηγούνται σε απόπτωση. Επιπλέον, τα Τ λεμφοκύτταρα των ασθενών εμφανίζουν μεγάλο αριθμό από θραύσματα στην διπλή έλικα του DNA (double-strand DNA breaks).

Λαμβάνοντας υπόψιν τα παραπάνω, ο σκοπός της παρούσας επιστημονικής μελέτης είναι να διερευνηθεί ο ρόλος του απορυθμισμένου γονιδίου SMC1A στην παθογένεια του ΣΕΛ καθώς και η πιθανή εμπλοκή του στον φυλετικό διμορφισμό που χαρακτηρίζει την ασθένεια. Στην συγκεκριμένη μελέτη εστιάσαμε στον ρόλο που έχει η πρωτεΐνη στην επιδιόρθωση των βλαβών του DNA. Με πειράματα real-time PCR που μπορούμε να μετρήσουμε την μεταγραφική ικανότητα του γονιδίου, βρέθηκε ότι τα mRNA επίπεδα του SMC1A είναι σημαντικά μειωμένα σε $CD4^+$ T λεμφοκύτταρα που απομονώθηκαν από άνδρες ασθενείς με ΣΕΛ, ενώ εμφανίζονται μειωμένα και στις γυναίκες ασθενείς. Επιπλέον, σε πειράματα ανοσοφθορισμού φάνηκε ότι τα $CD4^+$ T λεμφοκύτταρα των ανδρών ασθενών έχουν αυξημένη βλάβη του DNA όπως εκτιμήθηκε με τα επίπεδα φωσφορυλιωμένης γH2Ax και μειωμένα επίπεδα φωσφορυλιωμένου SMC1A (pSMC1A), που δεν συνάδει με το υψηλό επίπεδο βλάβης στο DNA, συγκριτικά με τις γυναίκες ασθενείς και τους αντίστοιχους υγιείς. Αξιοσημείωτο είναι, επίσης, ότι η χορήγηση ορού από ασθενή με ΣΕΛ σε καλλιέργεια $CD4^+$ T λεμφοκυττάρων, με σκοπό να εκθέσουμε τα κύτταρα στο περιβάλλον της ασθένειας, οδήγησε σε σημαντική αύξηση των επιπέδων μεταγραφής και φωσφορυλίωσης του SMC1A. Συνεπώς, συμπεραίνουμε ότι το περιβάλλον του ΣΕΛ φυσιολογικά τείνει να επάγει την έκφραση και την δράση του SMC1A, ωστόσο τα λεμφοκύτταρα των ασθενών εμφανίζουν σημαντικά χαμηλή έκφραση και δράση του.

Τα αποτελέσματα της παρούσας μελέτης δείχνουν ότι στο ΣΕΛ η έκφραση του SMC1A είναι σημαντικά μειωμένη και επιπλέον, επηρεάζεται από το φύλο των ασθενών. Επίσης, υποδηλώνουν μια πιθανώς ελλιπή δράση του SMC1A στην επιδιόρθωση των βλαβών του DNA που εμφανίζουν τα λεμφοκύτταρα των ανδρών ασθενών. Η περαιτέρω διερεύνηση του ρόλου του SMC1A στην παθογένεια της ασθένειας θα μπορούσε να βοηθήσει στην καλύτερη κατανόηση του ρόλου που παίζει η εμφάνιση υψηλών επιπέδων βλάβης του DNA των ανοσοποιητικών κυττάρων στην σοβαρότητα ή στην διαιώνιση της ασθένειας. Τέλος, θα μπορούσε ενδεχομένως να οδηγήσει σε νέους στόχους και καινοφανείς θεραπείες προσαρμοσμένες στο φύλο του κάθε ασθενούς.

Abstract

A striking characteristic of Systemic Lupus Erythematosus (SLE), the prototype systemic autoimmune disease, is that it features a substantially greater frequency in females than in males (female:male ratio ranging from 7:1 to 15:1). By contrast, males – although less frequently afflicted – tend to suffer from more severe disease.

We have recently completed a whole-blood RNA sequencing in SLE patients and matched healthy individuals in order to delineate the molecular basis of the disease. Further bioinformatics analysis was carried out to detect differentially expressed genes between the two genders in SLE versus healthy controls. A sex-biased molecular signature was unraveled, which was specifically associated with the disease. Among these genes, SMC1A (Structural Maintenance of Chromosomes 1A) exhibited the strongest gender bias with the greatest statistical significance. Specifically, SMC1A expression was significantly reduced in male SLE patients compared to female SLE patients and their healthy counterparts.

SMC1A encodes for a structural component of cohesin complex and participates in sister chromatid cohesion and gene regulation via chromatin architecture remodeling. Importantly, SMC1A is also implicated in DNA damage repair network as its phosphorylation constitutes a critical downstream event for cell survival and chromosomal stability after DNA damage. Notably, it has been recently demonstrated that SLE lymphocytes are less efficient in repairing DNA damage leading to increased cell apoptosis and additionally have increased levels of DNA double-strand breaks (DSBs).

Taking these into consideration, our aim was to delineate the role of deregulated SMC1A in SLE susceptibility or severity and its potential implication in lupus sexual dimorphism by focusing on its role in DNA damage repair. SMC1A mRNA levels were found significantly reduced in purified $CD4^+$ T lymphocytes from male SLE patients but they were also moderately reduced in female SLE patients. Additionally, SLE male $CD4^+$ T cells appeared to have diminished phosphorylated SMC1A (pSMC1a) levels, in spite of increased levels of DSBs, compared to female SLE patients and their healthy counterparts.

These results indicate a gender-biased (i.e. reduced in males) SMC1A expression in SLE and imply a defective activity of this protein in repairing DNA damage of male lupus $CD4^+$ T cells. Further delineating the role of SMC1A in SLE pathogenesis could improve our understanding of how DNA damage is associated with the disease. Finally, it could potentially lead to novel targets of treatment tailored for males and females.

Introduction

1. Systemic Lupus Erythematosus (SLE)

Systemic Lupus Erythematosus (SLE), or lupus, is the prototype chronic progressive autoimmune disorder that affects multiple organ systems, such as skin, kidneys and/or central nervous system [1]. The heterogeneity of the disease is impressive and some patients can suffer from a mild illness with skin or joint involvement whereas others from a life-threatening condition with renal or cardiovascular involvement [2]. In addition, SLE is characterized by an undulating course of exacerbations and remissions. The fundamental characteristics of SLE are the impaired clearance of dead cells and the loss of B- and T- cell tolerance to nuclear antigens, resulting in the production of autoantibodies directed against nuclear antigens, double-stranded DNA (dsDNA) and histones proteins. Finally, immune complexes are formed, deposited in tissues and together with other cellular and soluble mediators of inflammation contribute to end-organ damage [1, 3]. Also, one of the most prominent feature in patients with SLE is the overactivation of the Type I Interferon system [4]. It has been considered that SLE is a result of a complex interaction of genetics, epigenetics, environment, cellular effectors and hormones leading to immune deregulation and breakdown of tolerance to self-antigens [5]. (Fig. 1)

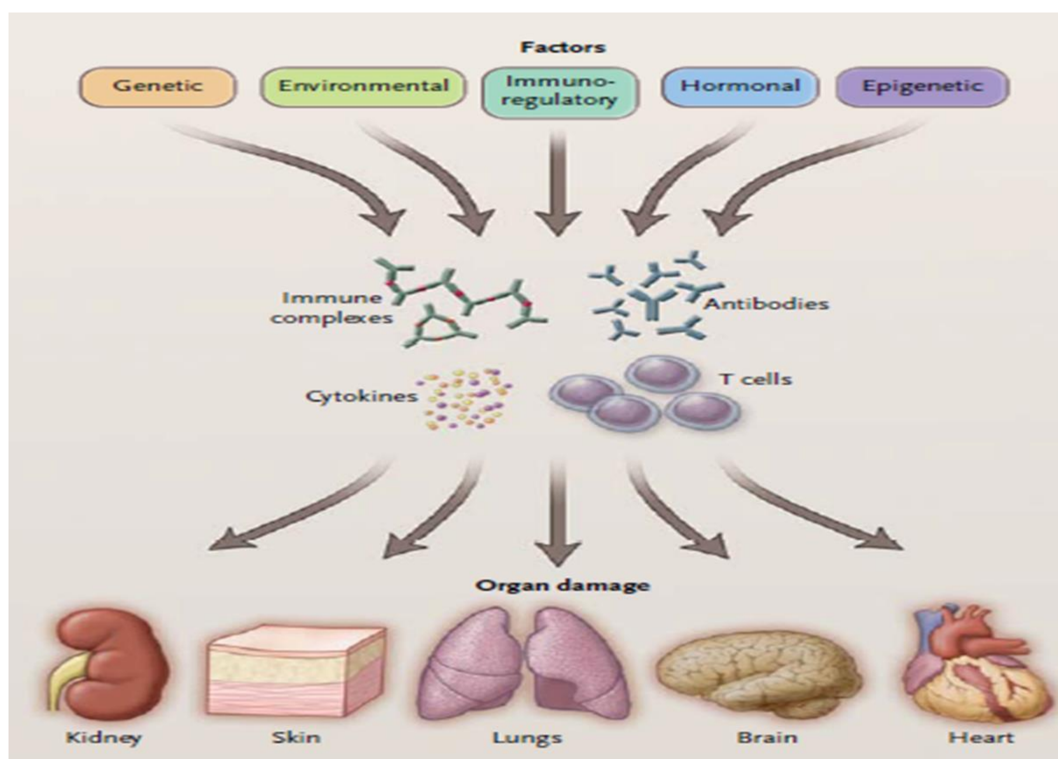


Figure 1. Etiopathogenesis of Systemic Lupus Erythematosus. *Tsokos GC, N Engl J Med, 2011*

2. The gender bias issue in SLE

A striking characteristic of SLE is that it features a substantially greater frequency in females than in males (female:male ration ranging from 7:1 to 15:1). By contrast, males, although less frequently afflicted, tend to suffer from more severe disease manifestations and increased rates of organ damage [6-8]. The basis for the gender bias in SLE is poorly understood. Hormonal differences, changes in microbiota and X-linked gene dosage are all being investigated as potential driving factors. Interestingly, estradiol has been postulated to favors Th2 immune response and stimulates autoreactive B cells in SLE [9]. Additionally, in a recent study, Nadine Dragin and his colleagues demonstrated that estradiol is able to downregulate the thymic autoimmune regulator AIRE (Autoimmune Regulator), leading to inefficient immune tolerance and subsequently production of autoreactive T cells in females during pregnancy [10]. On the other hand, gene dosage differences and partial X-reactivation might lead to overexpression of immunity-associated X-linked genes [11]. Delineation of molecular mechanisms which contribute to the sexual dimorphism in SLE may advance our understanding of disease pathogenesis and assist the development of more effective treatments tailored for males and females.

To delineate the molecular basis of the disease, whole blood RNA sequencing have been recently completed in SLE patients and matched healthy controls [12]. Thorough bioinformatics analysis identified a sex-biased molecular signature, which was specifically associated with SLE. This molecular signature includes 6 genes which were significantly differentially expressed in SLE males versus females: **SMC1A** (Structural Maintenance of Chromosomes 1A), **APOE** (Apolipoprotein E), **MTCO2 pseudogene 12** (Mitochondrially Encoded Cytochrome C Oxidase II Pseudogene 12), **OPLAH** [5-Oxoprolinase (ATP-Hydrolysing)], **FRG1B** (FSHD Region Gene 1 Family Member B) and **ARSD** (Arylsulfatase D). Among these genes, SMC1A exhibited the strongest gender bias with the greatest statistical significance. SMC1A is ubiquitously expressed and the protein encoded by this gene has diverse roles in many aspects of chromosome biology.

3. SMC1A, a member of cohesin complex

SMC1A (Structural Maintenance of Chromosomes 1A) belongs to SMC family of proteins which are highly conserved from bacteria to humans. SMC1A is the first SMC gene that was identified in Saccharomyces cerevisiae and was shown to be required for proper chromosome segregation during cell cycle [13]. Later, it was discovered that SMC1A gene encodes for a structural component of the cohesin

complex, which is required for sister chromatid cohesion and cell viability [14, 15]. The core of this complex composed of a SMC1A/SMC3 heterodimer and two non-SMC components, RAD21 (Double-Strand-Break Repair Protein Rad21 Homolog) and STAG1/2 (Stromal Antigen 1/2), in humans. The SMC1A gene maps to Xp11.22–Xp11.21 in a region that escapes X inactivation and consists of 25 exons [16].

SMC proteins are large polypeptides with their molecular mass ranging from 110 to 170kDa. These proteins contain about 1000 to 1300 amino acids. They consist of five distinct motifs: N- and C- terminal domains, two coiled-coil domains, and the hinge domain. The two terminal domains are highly conserved among SMC proteins and each contains approximately 100 to 150 amino acids. The N-terminus contains a nucleoside triphosphate binding motif (NTP) and binds ATP. The C-terminus contains an ATP hydrolysis domain and a DA box and binds DNA. Between the N- and C-terminal domains are two long coiled-coil motifs (200-450 amino acids) and the hinge domain (about 150 amino acids) which is positioned at the center of protein and joins the two coiled-coil domains [17].

SMC1A and SMC3 proteins fold back upon themselves into anti-parallel intramolecular coiled coils, with the N- and C-terminal regions of the protein folding together into a globular “head” domain. The SMC1A and SMC3 heads dimerize in the presence of ATP and are capable of ATP hydrolysis. SMC1A and SMC3 hinge domains also dimerize, thus completing the complex. The final structure of cohesin complex looks like a ring, which is large enough to capture a double-stranded DNA inside it. The cohesin core complex interacts with several other proteins, which contribute to its function, including NIPBL, required for the loading of cohesin into chromatin, WAPL, required for complex release, and PDS5A/B, which have been proposed to modulate the dynamic association of cohesin with chromatin [18-21]. (Fig. 2)

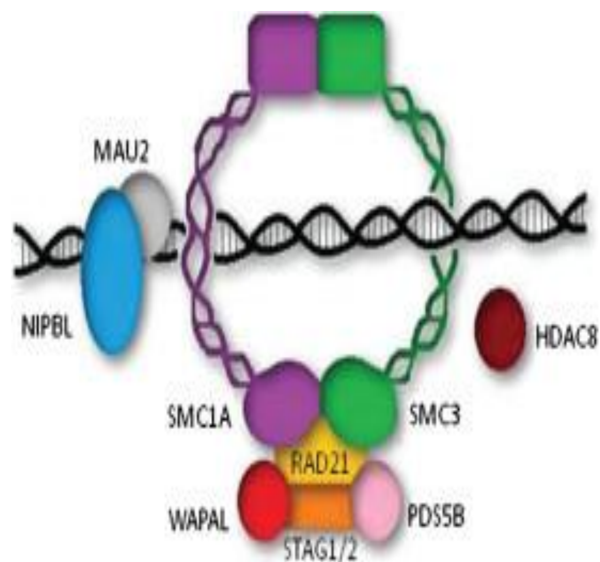


Figure 2. The structure of cohesin complex.

4. SMC1A's role in diverse aspects of chromosome biology

SMC1A as the critical subunit of the cohesin complex was initially known only for its role in sister chromatid cohesion. However, it is now known to be involved in various vital cellular functions, such as transcriptional regulation, cell cycle checkpoint and DNA damage repair.

4.1. Sister chromatid cohesion

Under normal conditions, SMC1A, as part of cohesin, is recruited to chromatin in G1 phase and holds sister chromatids together from the time of replication in S phase until they part to opposite spindle poles at the metaphase-to-anaphase transition in order to ensure proper chromosome segregation during mitosis. Sister chromatids cohesion is critical for the accurate chromosome segregation during cell division and constitutes a fundamental physiological process for the life of every mammalian cell [22, 23].

4.2. Transcriptional regulation

Besides its canonical role in sister chromatids cohesion, cohesin complex has been recently implicated in gene regulation. New published data proposed that cohesin complex implicates in the loop extrusion model. According to this model, cohesin slides along DNA until it hits a CTCF (CCCTC-binding factor) motif and creates a loop. By binding to specific sequences, CTCF defines contact points for cohesin-mediated long-range chromosomal interactions. Transcription-mediated cohesin translocation could contribute to the relative movement of cohesin complex versus DNA in order to form chromatin loops. The loop extrusion model is able to explain how certain regions of chromosomes stay close together and how regulatory elements come in close proximity with the genes that control [24-26]. (Fig. 3)

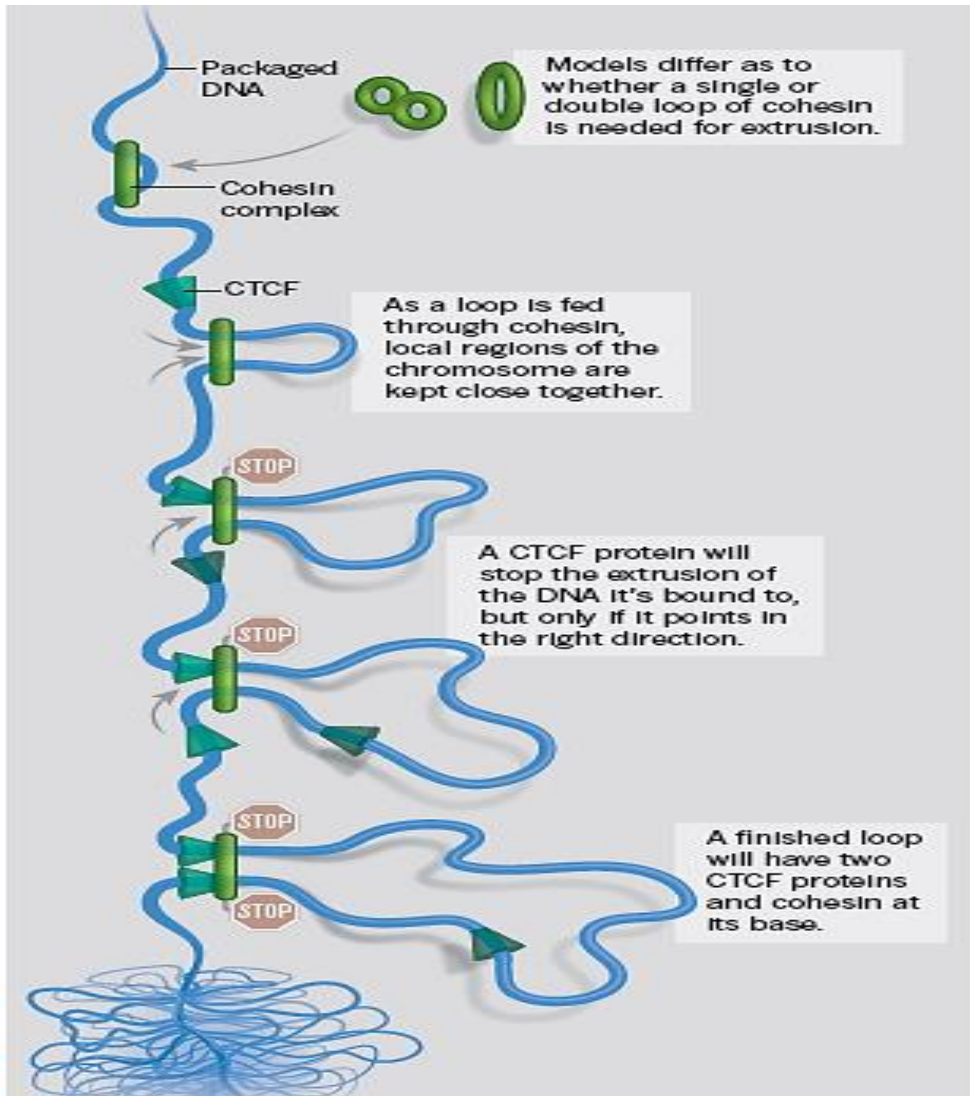


Figure 3. The loop extrusion model. *Dolgin E, Nature, 2017*

4.3. DNA damage repair

SMC1A constitutes also a core component of the tetrameric cohesin complex, which facilitates the homologous recombination (HR) pathways for DNA double-strand breaks (DSBs) repair. It has been demonstrated that SMC1A is phosphorylated by the DNA damage sensor ATM (Ataxia Telangiectasia Mutated) in serine 957 and 966 residues and acts as a downstream effector of the S-phase checkpoint ATM-NBS1-SMC1 pathway in response to ionizing radiation (IR), hypoxia and reoxygenation (H/R) or other DNA damage stimulus.

After DNA damage has occurred, DSBs are induced, and many DNA damage response proteins are recruited to the damaged site. BRCA1 (Breast Cancer 1), cohesin (SMC1A, SMC3 and RAD21) and MRN complex (MRE11, Rad50 and NBS1) constitute the major representatives of these proteins. Afterwards, ATM dimmers will be autophophorylated leading to release of active ATM monomers. That active ATM monomers can phosphorylate substrates such as BRCA1, NBS1 (Nijmegen Breakage Syndrome 1) and SMC1A. Both BRCA1 and NBS1 are required for the phosphorylation of SMC1A, when it is fully assembled as part of cohesin complex, by activated ATM, thus inducing S phase arrest. The phosphorylation of SMC1A is required for S-phase checkpoint activation. The exact role of cohesin after SMC1A phosphorylation is to hold the DSB and undamaged chromatid at close proximity, promoting strand invasion and sister-chromatid homologous recombination [27-29]. (Fig. 4)

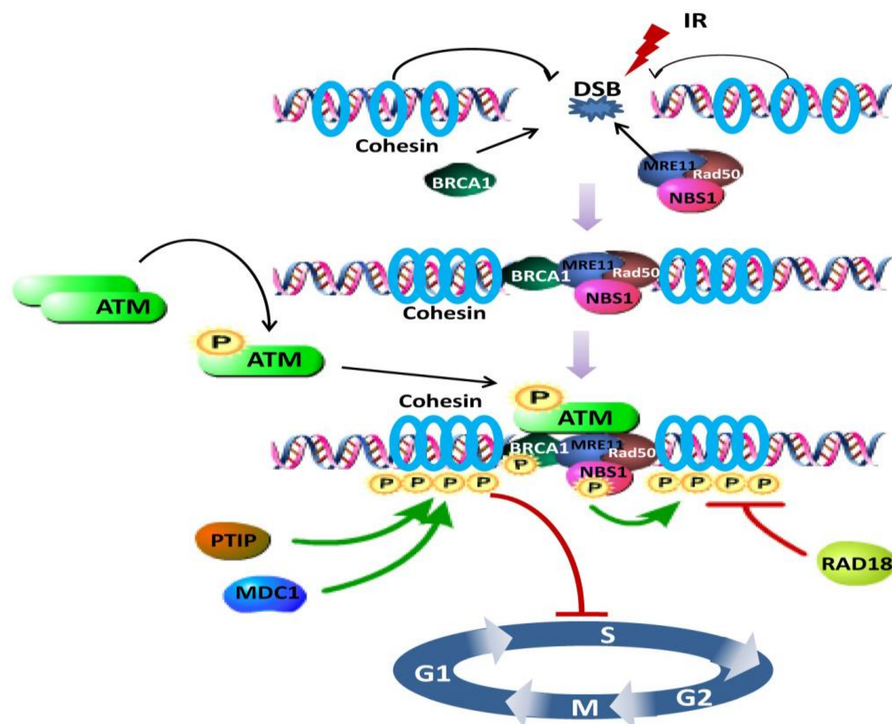


Figure 4. Model for function of cohesin in DNA damage response. *Yi F et al, Int J Biol, 2017*

5. Cornelia de Lange Syndrome and its immunologic features

Mutations in cohesion pathways (i.e. structural components of cohesin complex or auxiliary genes) are responsible for multispectral developmental abnormalities termed “Cohesinopathies”. These include Warsaw Breakage Syndrome (WABS), Roberts Syndrome (RBS), and Cornelia de Lange Syndrome (CdLS) [30].

CdLS is an autosomal dominant disorder that arises from either heterozygous or X-linked mutations in the cohesin subunits SMC1A, SMC3, and RAD21, as well as auxiliary factors, NIPBL and HDAC8. CdLS occurs in approximately 1 in 10,000 live births and around 5% of the reported cases arise from mutations in the SMC1A gene. [17, 31]

CdLS constitutes a developmental disorder, is associated with malformations affecting multiple organ systems and is characterized by recurrent infections, which result in significant morbidity. It has been reported that CdLS patients with severe disease have defects in humoral immunity in high frequency and suffer from antibody deficiency syndrome. Also, disease is characterized by impaired proportions of T cell subsets. It has been demonstrated that patients have lower percentages of a $CD4^+$ T cell subset (Tfh), which is able to stimulate B cells to differentiate into antibody-producing cells, as well as significantly lower T reg cell percentages, which are critical for tolerance maintenance and homeostasis. In addition, the gene expression of NFATc2 (Nuclear Factor of Activated T-Cells 2), a key transcription factor in T cell activation, has been proposed to be decreased in CdLS patients [32, 33]. Therefore, it is obvious that CdLS syndrome exhibits various immunologic features.

Finally, recent studies suggest that the dominant mutations in genes, predominantly coding for structural components of the cohesin ring, lead to a modest loss of cohesion pathway activity in CdLS patients. It is considered that this modest loss is sufficient for the proper progress of mitosis but insufficient for cis-DNA tethering leading mainly to transcriptional deregulation and gene expression defects [30].

6. The role of SMC1A in SLE

Preliminary RNA sequencing (RNA-seq) data from our lab suggest that sex-biased SMC1A expression in $CD4^+$ T cells might confer susceptibility to SLE. SMC1A was found significantly deregulated in SLE patients and especially in males compared to females.

There are two potential hypotheses, which can explain how deregulated SMC1A can contribute to SLE susceptibility or severity:

A. The first hypothesis is based on the role of SMC1A, as part of cohesin complex, in chromatin organization and gene regulation. Aberrant DNA looping might lead to deregulation of key immune-related genes in SLE. So far, various data have been published, which correlate cohesin with regulation of various cytokines expression. For instance, it has been proposed that cohesins form chromosomal interactions at IFNG locus regulating IFNγ expression [34] as well as that they regulate expression of Th2 cytokines (IL-4, IL-5, IL-13) by a similar mechanism [35].
B. The second hypothesis is based on the role of SMC1A in DNA damage repair pathway. As mentioned before, SMC1A phosphorylation is the critical downstream event for cell survival and chromosomal stability after DNA damage [28]. Lupus lymphocytes are less efficient in repairing DNA damage leading to increased apoptosis. It is also speculated that defective DNA repair in SLE may play a role in chronicity and progression of disease [36, 37]. In addition, recent data revealed that DNA damage primes the type I interferon system via the cytosolic DNA sensor STING to promote anti-microbial innate immunity [38]. Consequently, deregulated SMC1A in SLE might lead to defective response to DNA damage and DNA repair contributing to disease severity or disease perpetuation.

7. Aim of study

This scientific project aims to delineate the role of SMC1A in SLE pathogenesis and its potential implication in disease sexual dimorphism. For that purpose, we study the expression profile of SLE patients versus healthy counterparts according to gender. Consequently, we aim to investigate the functional implications of deregulated SMC1A in lupus disease attending to its role in DNA damage repair. Our experiments are focused on $CD4^+$ T lymphocytes, which constitute the critical cell type for SLE pathogenesis and are recently found to have significantly increased levels of DSBs and DNA damage. Finally, our goal is to discern potential differences in DNA damage repair capacity of $CD4^+$ T cells between male and female SLE patients and their healthy counterparts.

Material & Methods

Human subjects

Peripheral blood samples were obtained from male and female patients diagnosed with Systemic Lupus Erythematosus (SLE) and healthy individuals. Active SLE was defined as a SLE Disease Activity Index score (SLEDAI) higher than 8. The samples were recruited from the University Hospital of Heraklion (Crete, Greece). The study was approved by the Institutional Review Committee, and all subjects gave written informed consent.

Serum collection

Healthy and/or SLE peripheral blood sera was added in a collection tube without anticoagulants. The tube was centrifuged at 2500 rpm for 15mins and the serum was collected under sterile conditions.

Reagents

RPMI-1640 (Gibco), fetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100μg/ml), were all from Gibco, Carlsbad, CA. H_20_2, DAPI, Ficol Histopaque 1077 and Ficol Histopaque 1119 were from Sigma-Aldrich. Fluorescent-conjugated monoclonal antibodies to CD4, CD14 were from R&D systems. Rabbit SMC1 (p Ser966) antibody purchased from Novus. Mouse anti-phospho-Histone H2AX (Ser139) purchased from Millipore. Secondary antibodies anti-rabbit CF488 and anti-mouse CF555 were purchased from biotium.

Cells

Total peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained by Ficol-Histopaque 1077 density-gradient centrifugation at 1800 rpm for 30 minutes. Cells were washed once at 1500 rpm for 10 min using sterile PBS. Erythrocytes were lysed if needed. The lysis was performed by resuspending the cell lysate in water for injection (WFI) for 35 sec. Then, equal amount of 1,8% w/v NaCl solution was added in order to reduce the osmotic shock. The PBMCs were magnetically sorted using the CD4 and CD14 human MicroBead kit (Miltenyi Biotec) in order to positive select $CD4^+$ or $CD14^+$ cell subsets, respectively. $CD4^+$, $CD14^+$ cells were cultured in RPMI-1640 supplemented with 10% FBS and 1%penicillin/ streptomycin mix.

Polymorphonuclear cells (PMNs) were isolated using Ficoll-Histopaque 1077, 1119 double density gradient centrifugation at 650g for 30 min. Then, their erythrocytes were washed (300g, 10 min) and lysed as previously described. PMNs were cultured in RPMI-1640 supplemented with 2% FBS and 10mM Hepes (Gibco).

Flow Cytometry (FACS)

The purity of magnetically isolated cells was evaluated by flow cytometry. Cells were stained for extracellular markers for 20 min at 4°C in PBS/5% FBS. Monoclonal antibodies specific for CD4 and CD14 markers were used. Cells were acquired on a FACS Calibur (BD Biosciences) and analyzed using the FlowJo software (Tree Star).

RNA extraction

Total RNA from cultured cells was collected using the TRIZOL extraction protocol. Total RNA was treated with DNAse in order to eliminate any genomic DNA contaminations. Turbo DNAse kit (Ambion) was used according to manufacturer's protocol.

Real Time PCR (RT-PCR)

cDNA was prepared from isolated RNA using Perfect Real time cDNA Synthesis Kit (Takara) according to manufacturer's protocol. 200ng of RNA were used as a template for every reaction. RNAse H (2U/reaction) was added in order to clean the resulting cDNA from any RNA and incomplete cDNA products. cDNA was stored at -20°C. PCR amplification of the resulting cDNA samples was performed using appropriate volumes of KAPA SYBR® FAST Universal 2x qPCR Master Mix and specific for each gene primers at a CFX Connect™, Real-Time System. Total volume of each PCR reaction was 20μl. Expression was normalized to GAPDH and calculated by the change-in-threshold method [$2^{(-\Delta\Delta C_T)}$]. Specific primers were as follows:

(5'→ 3'):
SMC1A forward human
CAT CAA AGC TCG TAA CTT CCT CG
SMC1A reverse human
CCC CAG AAC GAC TAA TCT CTT CA

GAPDH forward human
CAT GTT CCA ATA TGA TTC CAC C
GAPDH reverse human
GAT GGG ATT TCC ATT GAT GAC

Treatment of cells with hydrogen peroxide (H_2O_2) or SLE serum

CD4^{+} T cells were isolated and treated with 100μM H_20_2 (Sigma-Aldrich) or 20% SLE serum for 4 hours at 37°C in a 5% CO_2 atmosphere.

Immunofluorescence (IF)

For IF microscopy cells were plated on poly-L-lysine coverslips (100.000 cells /slide). When the appropriate treatments were finished, slides were washed once with PBS, fixed with 4% FA for 20mins (RT) re-washed twice with PBS, and blocked with 5% BSA, 0.1% Triton at RT. Primary antibodies were left for 1hr at RT or O/N at 4°C. Slides were washed several times with PBS and the secondary antibodies were incubated for 1hr at RT. Cell nucleus was counterstained with DAPI (200nM). After staining, coverslips were mounted on Mowiol 4-88 (Sigma-Aldrich) and were observed under confocal microscope (Leica SP8).

RNA sequencing (RNA-Seq)

Whole blood mRNA was extracted from 142 SLE patients with varying levels of disease activity/severity and 58 matched healthy individuals. Paired-end RNA sequencing was performed using the Illumina HiSeq 2000 platform [12].

Results

RNA-Seq analysis revealed a sex-biased molecular signature in SLE.

Analysis of the whole-blood RNA sequencing data was performed to address the gender bias issue in SLE via an unsupervised manner. The ultimate aim was to monitor for differentially expressed genes between male and female SLE patients that are not differentially expressed between male and female healthy subjects. To this end, a list of differentially expressed genes in SLE males versus females were generated using a cut-off of 5% false discovery rate (5%FDR). To increase the specificity of our results, we generated a similar list of differentially expressed genes in healthy males versus females by using a 50% and 90% FDR cut-off. The intersection of these two lists identified a gender-biased molecular signature specifically associated with the disease. Thus, out of 39 genes which were differentially expressed in SLE males versus females, 6 were not differentially expressed in healthy males versus females at either 50 or 90% FDR: SMC1A, APOE, MTCO2 pseudogene 12, OPLAH, FRG1B and ARSD. (Fig. 5)

After detailed literature search in order to find the most relevant gene for SLE disease, we decide to focus on SMC1A (Table 1). SMC1A is the most statistical significant differentially expressed gene between the two genders of SLE patients according to RNA-Seq results and it has also diverse roles in many aspects of chromosome biology.

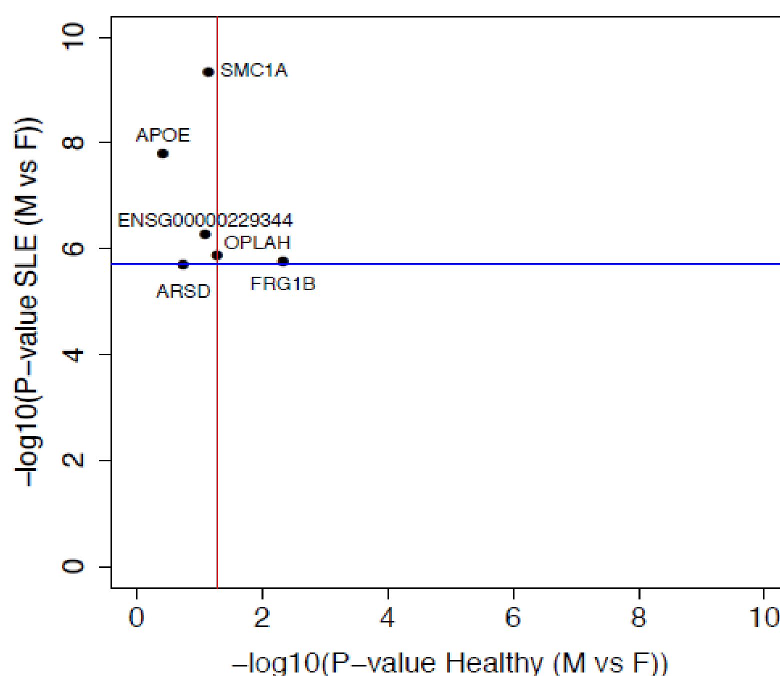


Figure 5. *Sex-biased molecular signature in SLE: differentially expressed genes which are specific to gender in SLE patients.*

Table 1. Main characteristics of the 6 differentially expressed genes between male and female SLE patients emerged by stringent bioinformatics analysis.

Gene Symbol	Full name	Gene locus	Protein Function	Disorders
SMC1A	Structural Maintenance of Chromosomes 1A	Xp11.22	sister chromatid cohesion, transcriptional regulation, DNA damage repair	Cornelia de Lange syndrome
APOE	Apolipoprotein E	19q13.32	binding, internalization and catabolism of lipoprotein particles	Type III Hyperlipoprteinemia, Alzheimer disease 2, Age-related macular degeneration, *putative susceptibility factor for aggressive Multiple Sclerosis (E4 allele)
MTCO2 Pseudogene 12	Mitochondrially encoded Cytochrome C Oxidase II pseudogene 12	1p36.33	No protein coding gene. MT-CO2 like sequence embeded in nuclear DNA	None
OPLAH	5-Oxoprolinase ATP Hydrolyzing	8q24.3	cleavage of 5-oxo-L-proline to form L-glutamate coupled to the hydrolysis of ATP to ADP and inorganic phosphate	5-Oxoprolinase Deficiency , Glutathione Synthetase Deficiency
FRG1B	FSHD Region Gene 1 Family Member B	20q11.1	No protein coding gene	None
ARSD	Arylsulfatase D	Xp22.33	correct composition of bone and cartilage matrix	Chondrodysplasia punctate *in newborns has linked with maternal SLE

SMC1A is downregulated in male SLE compared to female SLE patients and healthy counterparts.

The whole-blood RNA-Seq analysis indicated that SMC1A gene is significantly reduced in male SLE patients compared to female SLE patients and their healthy counterparts. On the other hand, it retains almost the same in female SLE patients compared to female healthy individuals. In order to validate the RNA-Seq results, we performed RT-PCR by measuring SMC1A mRNA levels in a small number of the original data set. This experiment gave us roughly the same pattern. (Fig. 6)

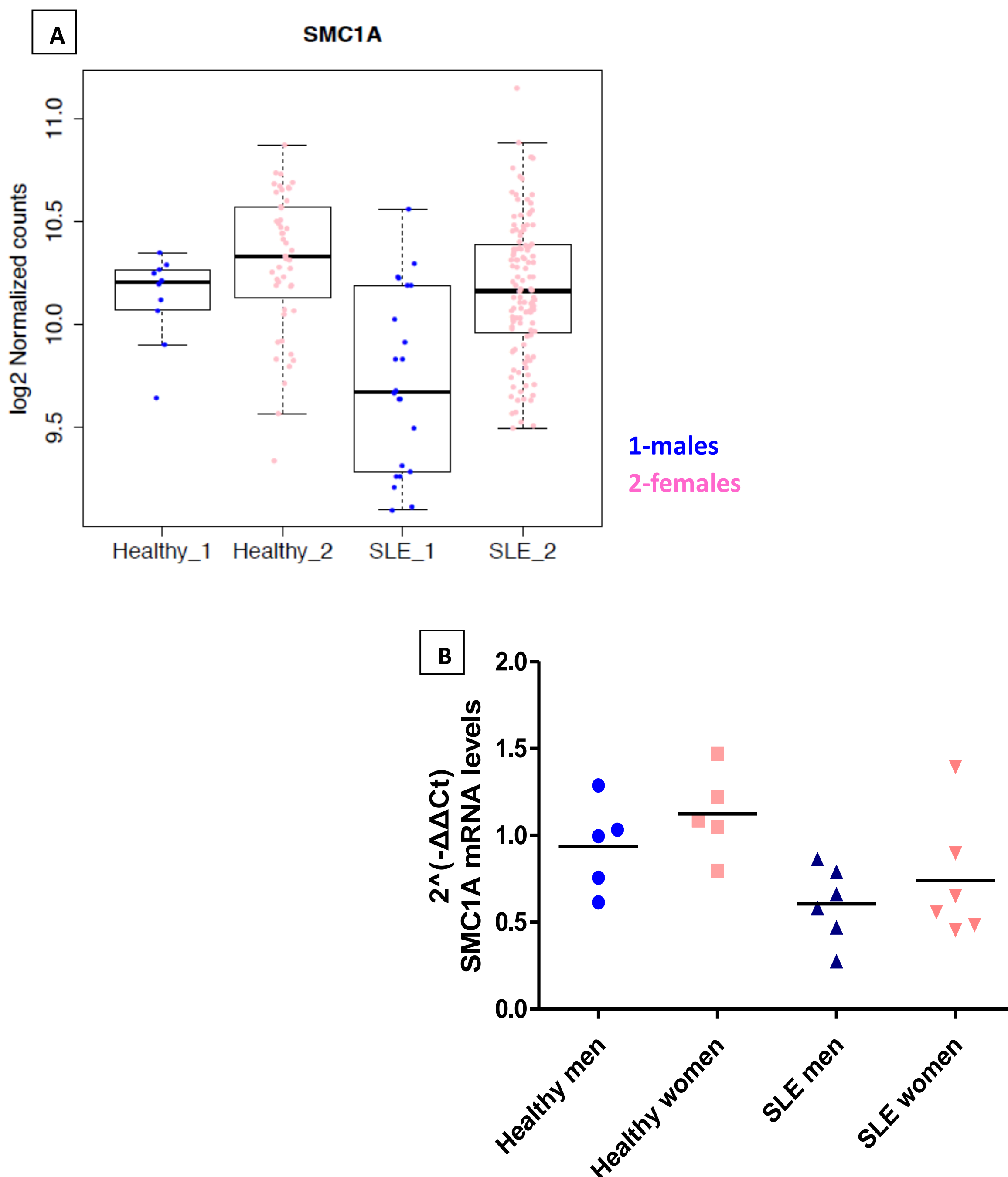


***Figure 6**. **(A)** RNA-Seq results for SMC1A in whole blood cells. **(B)** RT-PCR results for SMC1A in a small part of the original dataset (whole blood cells).*

Lymphocytes have elevated SMC1A mRNA levels compared to monocytes and PMNs.

Next, we asked which immune cell type is more relevant in order to address the functional role of SMC1A and its implication in SLE pathogenesis. RNA-Seq analysis was performed in whole blood cells and for that reason it was difficult to discern which immune cell types accounted for the deregulated phenotype of SMC1A in SLE. Nevertheless, since RNA-Seq took place in the whole blood, the signature must derive from a relevant cell population. We, therefore, fractionated lymphocytes, monocytes and polymorphonuclear cells (PMNs) from 5 SLE patients (only women) and 6 healthy individuals and measured the SMC1A mRNA levels in order to get a hint for the most relevant cell type. RT-PCR analysis indicated that SMC1A mRNA levels are elevated in lymphocytes of healthy individuals and SLE patients. Therefore, in order to be able to distinguish strong differences in the comparison with males, we decided to focus on lymphocytes. Finally, based on the literature for $CD4^+$ T lymphocytes and their critical role in lupus pathogenesis, we considered $CD4^+$ T cells as an appropriate cell type to continue working with it. (Fig. 7)

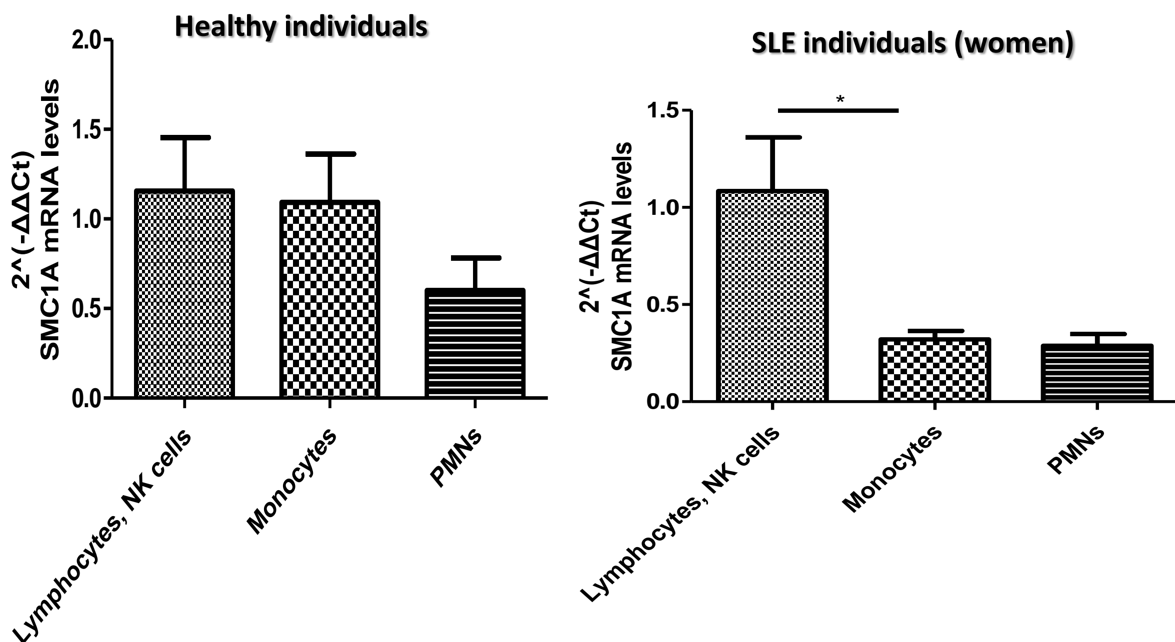


Figure 7. *SMC1A mRNA levels in fractionated immune cells from healthy individuals and SLE patients (women).*

SMC1A mRNA levels are reduced in lupus CD4⁺ T cells compared to healthy.

In order to confirm the deregulated phenotype of SMC1A in $CD4^+$ T cells from SLE patients, we measured the SMC1A mRNA levels in $CD4^+$ T cells from healthy male/female individuals compared with the levels in $CD4^+$ T cells from male/female SLE patients. RT-PCR results confirmed the downregulated SMC1A pattern in male SLE patients compared to healthy individuals but also revealed an overall reduced phenotype of SMC1A in SLE disease state, both in male and female patients, compared to healthy state. (Fig. 8)

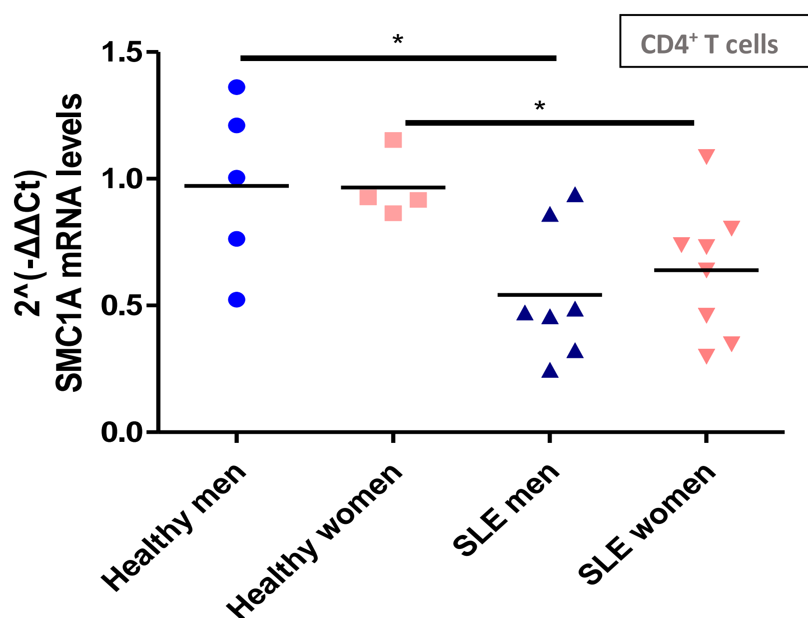


Figure 8. *SMC1A mRNA levels in $CD4^+$ T cells of male/female healthy individuals and SLE patients.*

SMC1A seems to be implicated in DNA damage repair pathways of CD4⁺ T cells through its phosphorylation on Ser966.

It is known that SMC1A participates in DNA damage repair network through its phosphorylation in two serines 957 and 966 by the DNA damage sensor ATM and accumulates in sites of DNA double-strand breaks (DSBs). Based on these, we aimed to delineate the role of SMC1A in DNA damage repair of $CD4^+$ T cells. Therefore, we induced DNA damage in healthy $CD4^+$ T cells with hydrogen peroxide (H_2O_2) through oxidative stress and performed immunofluorescence(IF) for γH2Ax (phosphorylated H2Ax), which constitutes an indicator for the DSBs. Additionally, we tested for colocalization between γH2Ax and p-SMC1A (phosphorylated SMC1A). The confocal microscopy images implied that SMC1A is implicated in DNA damage repair pathways of $CD4^+$ T cells while its phosphorylated form increases dramatically after the

treatment of cells with H_2O_2. The levels of γH2Ax were also increased compared to untreated cells, as it was expected due to DNA damage induction. (Fig. 9)

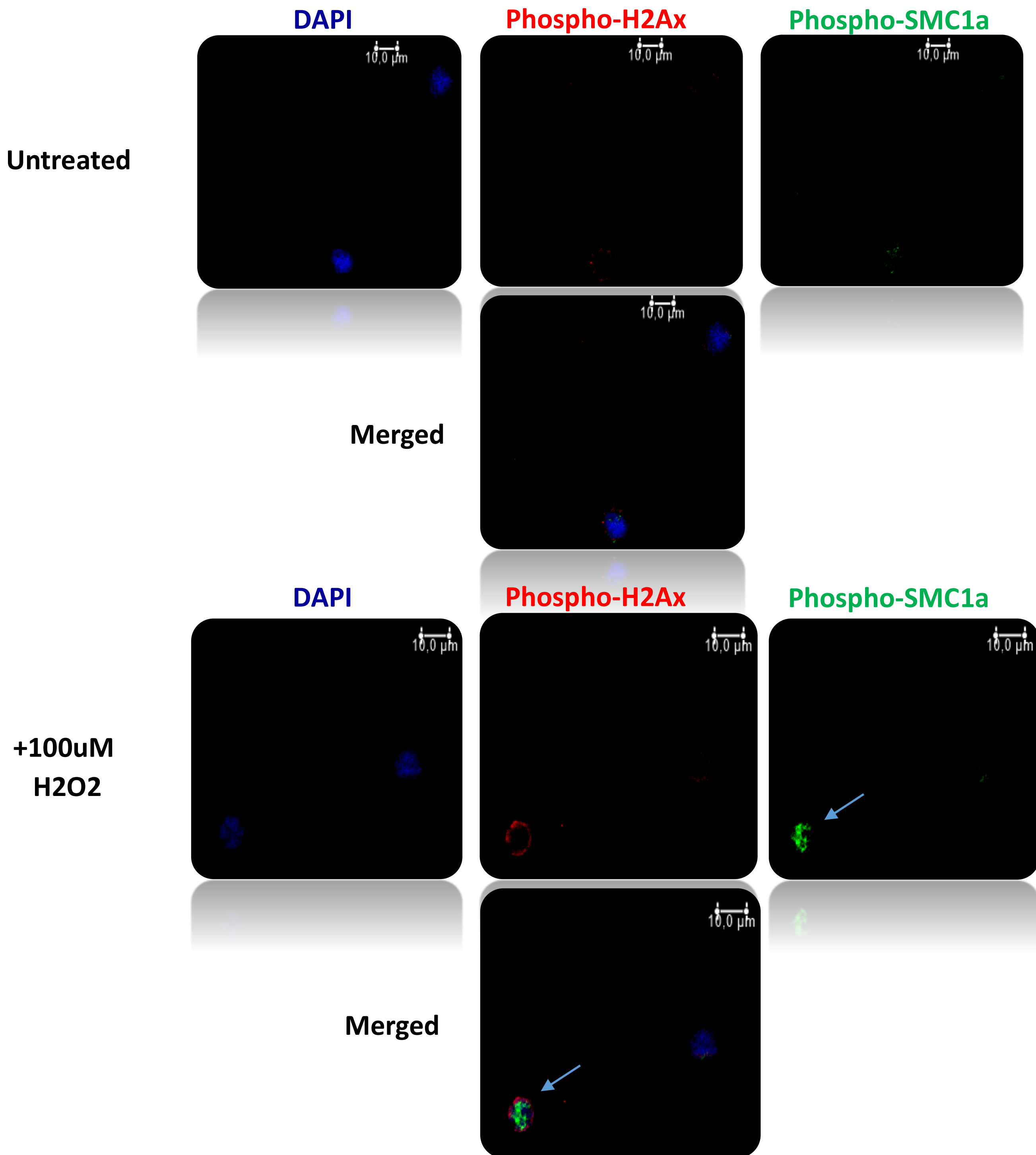


Figure 9. *Immunofluorescence of healthy CD4⁺T cells for γH2Ax (DSBs indicator) and pSMC1A after treatment with H_2O_2. DAPI was used for nuclear staining. Images were analyzed using LAS AF Lite software.*

Phosphorylated SMC1A is diminished in CD4⁺ T cells of male SLE patients, in spite of increased DNA damage levels.

Previous studies have shown that lupus lymphocytes exhibit increased levels of DNA DSBs. In our results, we detected reduced transcriptional levels of SMC1A in lupus $CD4^+$ T cells. Based on the above, we hypothesized that increased levels of DNA damage in lupus T cells might be a result of impaired SMC1A role in DNA damage repair. To test this hypothesis, we isolated $CD4^+$ T cells from healthy individuals and SLE patients and performed IF assay for the detection of DSBs levels (γH2Ax) and pSMC1A. The IF images indicate that $CD4^+$ T cells of SLE patients have indeed more double-strand DNA breaks compared to healthy individuals. Interestingly, phosphorylated SMC1A seems to be slightly or not at all increased in male SLE patients compared to healthy males, irrespectively with the DSBs levels. (Fig.10)

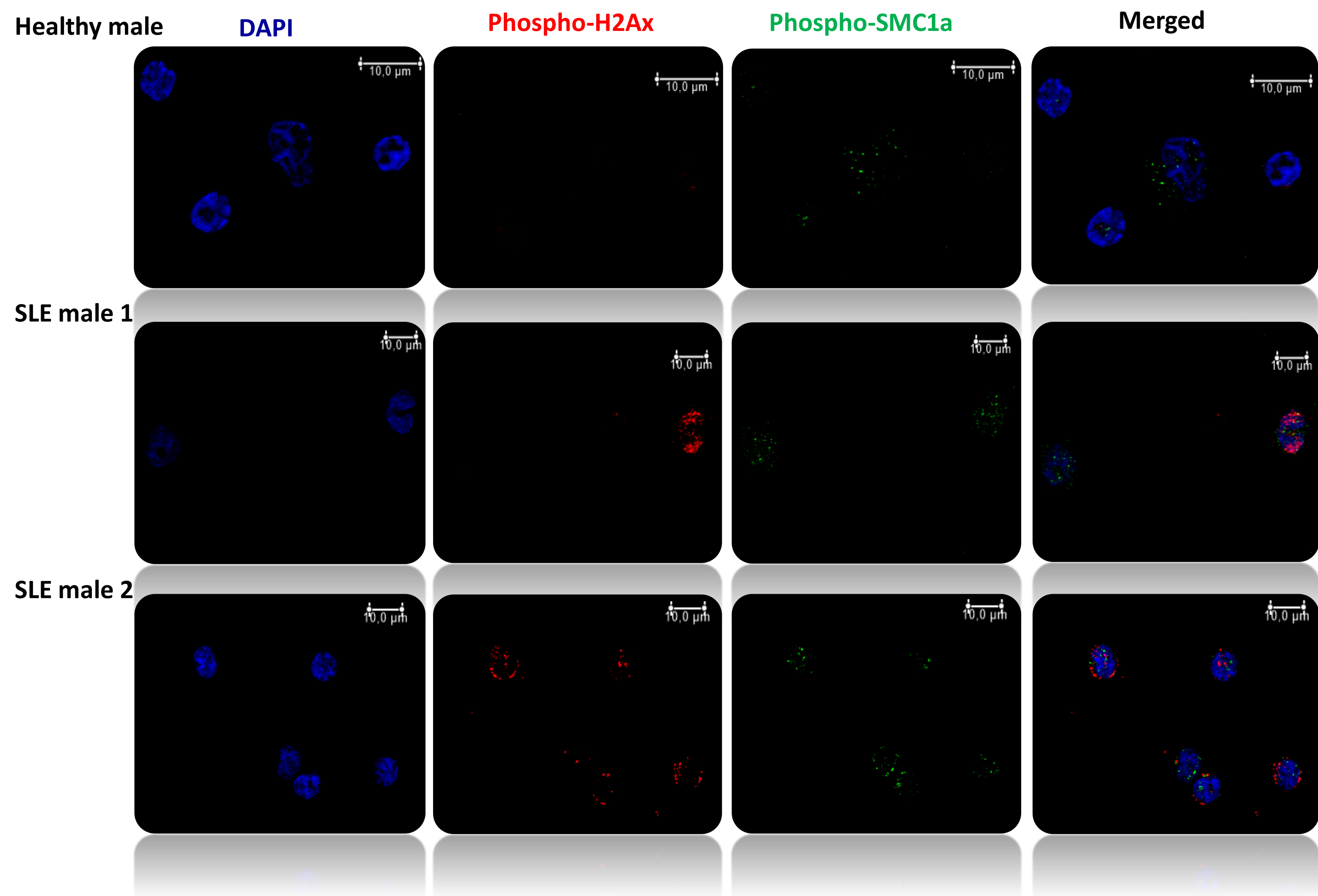
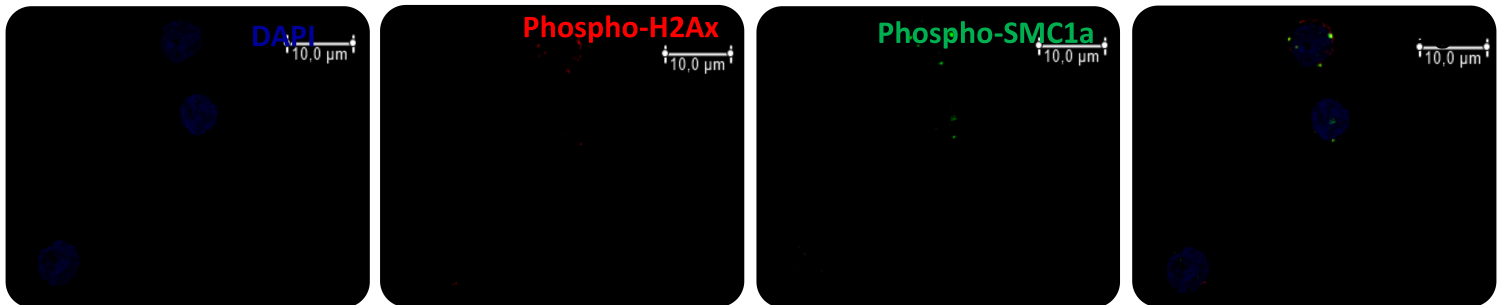


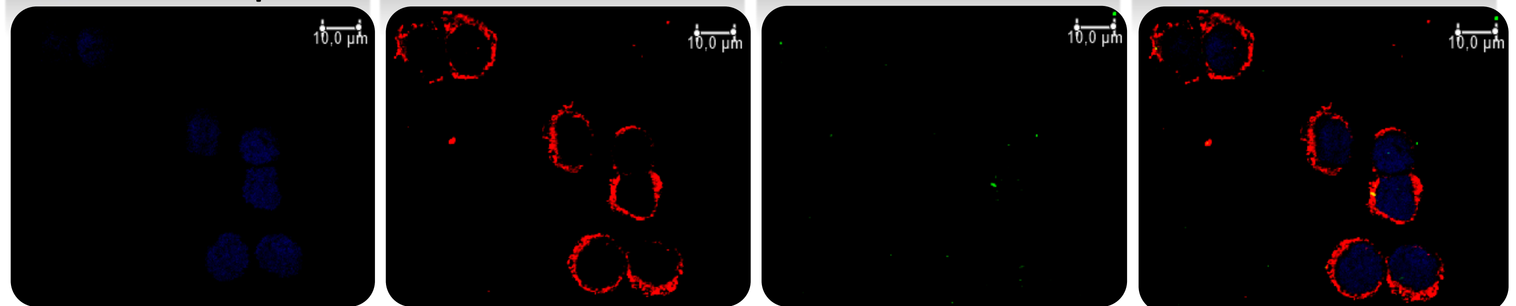
Figure 10. *Immunofluorescence for γH2Ax and pSMC1A in CD4 T cells from healthy male and SLE male patients. DAPI was used for nuclear staining. Images were analyzed using LAS AF Lite software.*

In another experiment, in which we compared CD4+ T cells isolated from healthy male, SLE male with nephritis, SLE female and healthy female, we also observed minimal levels of pSMC1A despite the increased levels of DNA breaks in CD4+ T cells of male SLE patient. On the other hand, female SLE patient had increased levels of pSMC1A and less DNA breaks compared to male SLE patient. (Fig. 11) Therefore, male SLE patients seem to exhibit impaired phosphorylation of SMC1A, in spite of increased DNA damage levels.

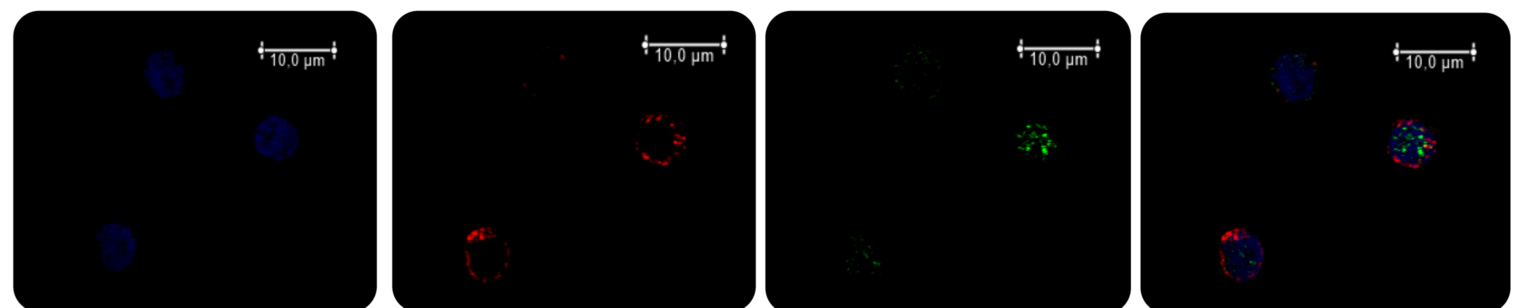
Healthy male



SLE male with *nephritis*



SLE female



Healthy female

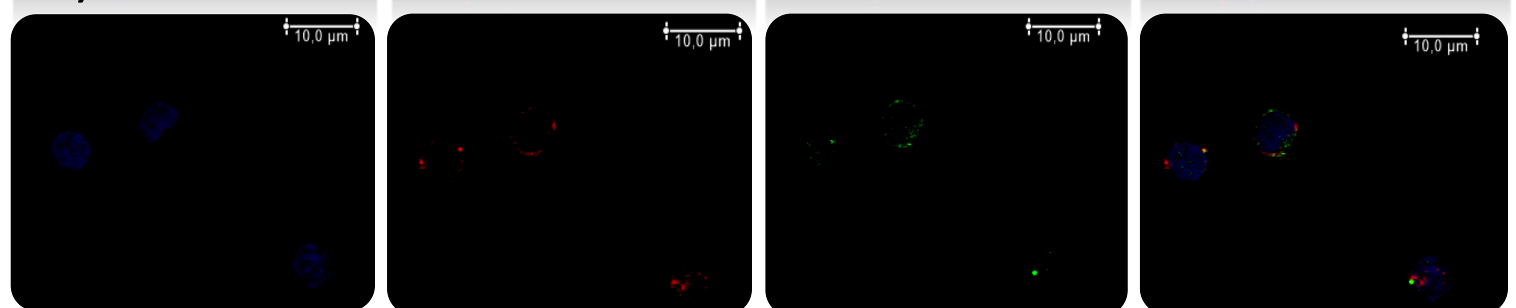


Figure 11. Immunofluorescence for γH2Ax and pSMC1A in CD4⁺ T cells from one healthy & one SLE patient male, one healthy & one SLE patient female. DAPI was used for nuclear staining. Images were analyzed using LAS AF Lite software.

SLE serum induces the transcriptional and phosphorylation levels of SMC1A in healthy CD4⁺ T cells.

The serum from SLE patients contains various pro-inflammatory cytokines, immune complexes, autoantibodies and other factors which mimic the lupus milieu. Therefore, we wondered which would be the effect on SMC1A transcription and phosphorylation after the exposure of healthy cells in SLE disease milieu. For that purpose, we isolated CD4⁺ T cells from healthy individuals and treated them with SLE serum. The results demonstrated that SLE milieu exposure of healthy CD4⁺ T cells induces the transcriptional and phosphorylation levels of SMC1A. (Fig 12, 13)

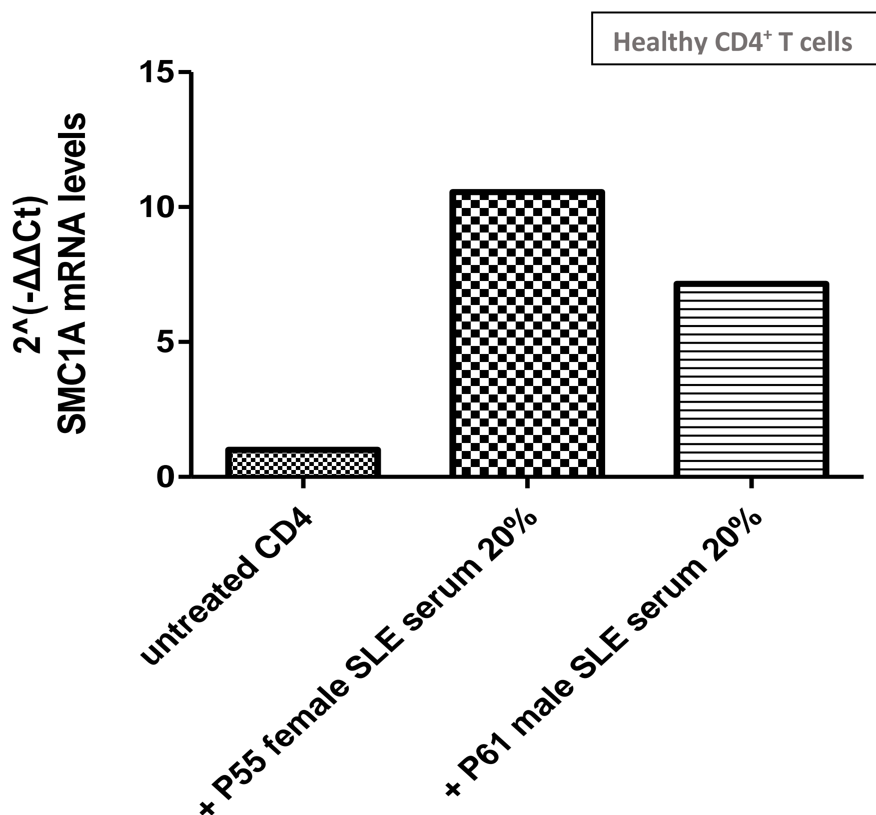


Figure 12. *SMC1A mRNA levels of healthy CD4+ T cells after SLE serum treatment.*

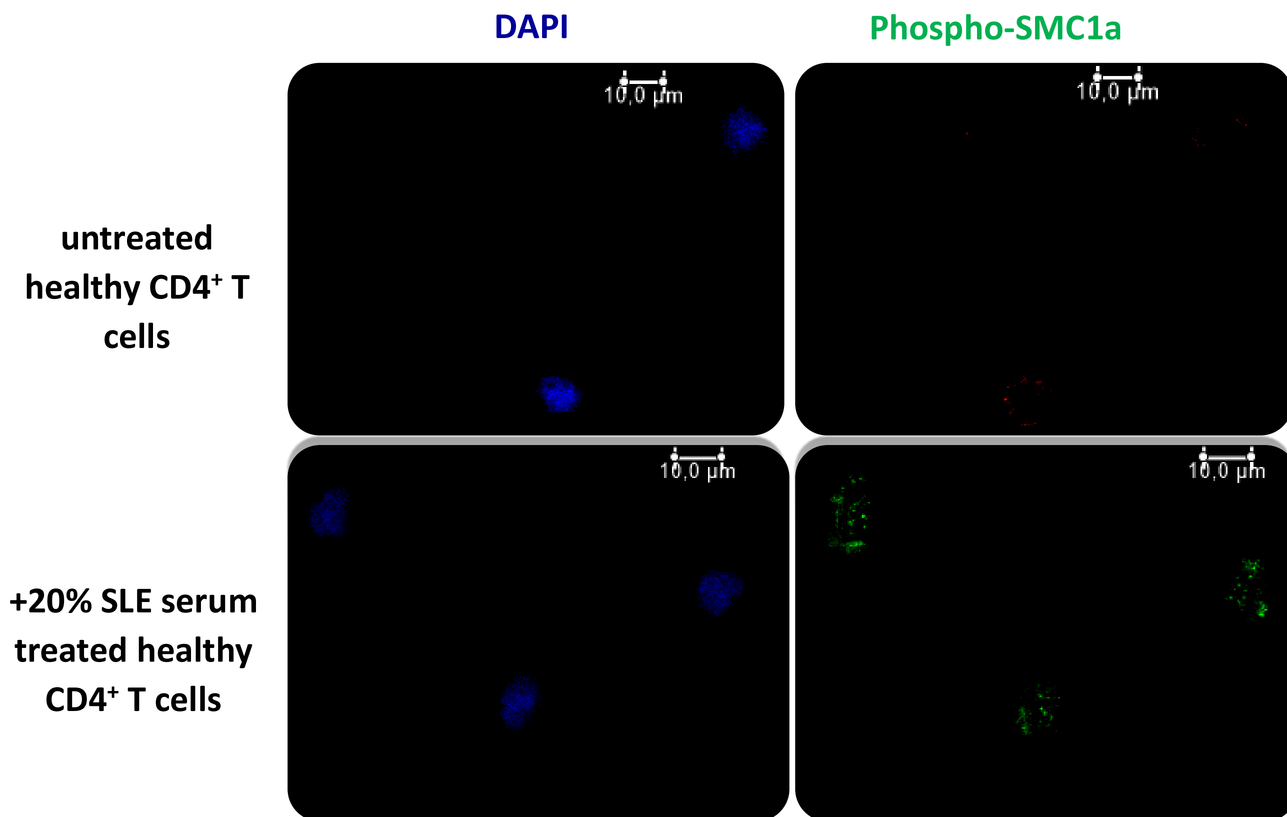


Figure 13. *Immunofluorescence for pSMC1A in healthy CD4$^+$ T cells after treatment with SLE serum. DAPI was used for nuclear staining. Images were analyzed using LAS AF Lite software.*

Discussion

SLE is the prototype systemic autoimmune disease which is characterized by impaired clearance of dead cells, loss of B- and T-cell tolerance to nuclear antigens and subsequently overproduction of autoantibodies against these antigens. $CD4^+$ T cells constitute a critical cell type for SLE pathogenesis as they regulate B cell responses. Autoreactive B cells, which produce the autoantibodies, infiltrate to target tissues and lead to tissue damage [1].

A striking feature of SLE is that it afflicts females in a substantially greater prevalence than males. Intriguingly, males – although less frequently affected- suffer from more severe disease manifestations [27-29]. Delineation of the mechanisms contributing to sexual dimorphism in SLE may advance our understanding of the disease pathogenesis and assist the development of targeted therapies tailored for males and females. Based on these, the initial aim in our lab was to investigate the gender bias issue in SLE via an unsupervised manner detecting the differential expressed genes between the two genders. For that purpose, we have recently performed whole-blood RNA sequencing in a large cohort of 148 SLE patients and 52 healthy individuals [33]. Analysis of the RNA-Seq data lead to a sex-biased molecular signature specifically associated with the disease. Among these genes, SMC1A, which encodes for the cohesin complex protein, exhibited the strongest gender bias with the greatest statistical significance.

SMC1A has three important roles in chromosome biology as part of the cohesin complex. First, its canonical role is the sister chromatids cohesion ensuring proper chromosome segregation during cell cycle [10-11]. Second, it participates in gene regulation through loop extrusion and formation of transcriptionally associated domains (TADs) [12-14]. And third, it is implicated in DNA damage repair network, while its phosphorylation seems to be a critical event for cell survival after DNA damage [15-17].

An important link between DNA damage repair and the activation of the type I interferon (IFN), which characterizes SLE, emerges [25]. Recently it has been demonstrated that some rare variants of the genome surveillance enzyme ribonuclease H2 gene are associated with systemic autoimmunity and lead to defective ribonucleotide removal. In addition, the embedded ribonucleotides enhanced type I IFN signaling, linking mutations in a DNA repair enzyme with systemic autoimmunity [39]. Furthermore, in another recent study they observed accumulation of spontaneous DNA breaks which stimulate the type I IFN signaling via the activation of STING pathway by self-DNA released into the cytoplasm [38]. In addition, the DNA repair nuclease MRE11A was found downregulated in $CD4^+$ T cells of patients with Rheumatoid Arthritis and this reduction was connected with damaged telomeres and premature aging of the cells [40]. All these recently published data lead to the

conclusion that DNA repair network emerges as an important new player in the pathogenesis of autoimmunity.

In the given scientific project, we proposed to delineate the role of deregulated SMC1A in SLE susceptibility or severity focusing on its role in DNA damage repair. Additionally, we wanted to investigate its potential implication in disease sexual dimorphism.

Whole-blood RNA-Seq data analysis indicated significantly decreased mRNA levels of SMC1A in male SLE patients compared to female SLE patients and their healthy counterparts. On the other hand, it retained almost the same in SLE females compared to healthy females. Based on these, we first hypothesized that SMC1A might be a severity and not susceptibility gene while it is downregulated in male patients, who have often more severe disease. Nevertheless, RT-PCR which performed in $CD4^+$ T cells isolated from male/female healthy individuals compared to $CD4^+$ T cells from male/female SLE patients did not reproduce exactly the same pattern of SMC1A. In $CD4^+$ T cells, SMC1A was found to be significantly reduced in male SLE patients compared to healthy individuals but also displayed an overall downregulated pattern of SMC1A in SLE disease state, both in male and female patients. One limitation, which could explain this pattern, is that we included women with active SLE compared to men with not active disease. Another limitation could be the small sample size of male and female patients; increasing the number of patients would increase confidence in the data generated. However, the main conclusion of our data was that SMC1A is reduced in SLE patients and especially males.

According to previous studies, lupus lymphocytes exhibit elevated levels of DNA double-strand breaks (DSBs), increased overall DNA damage and less efficient DNA repairing mechanism and all these have been correlated with the disease chronicity and progression [36-37]. We hypothesized that increased levels of DNA damage in lupus $CD4^+$ T cells might provoked by impaired SMC1A activity in DNA damage repair. First, we confirmed its implication in DNA damage repair network of $CD4^+$ T cells as we observed increased phosphorylated SMC1A levels after the treatment of cells with hydrogen peroxide, which can induce DNA damage through oxidative stress. DNA DSBs were similarly increased in $CD4^+$ T cells after hydrogen peroxide exposure compared to untreated cells. Afterwards, we aimed to delineate the role of SMC1A in DNA damage repair of lupus $CD4^+$ T cells. Interestingly, we observed diminished phosphorylated levels of this protein, in spite of increased DSBs in $CD4^+$ T cells from male SLE patients compared to female SLE patients and their healthy counterparts. This result may imply an impaired role of SMC1A in DNA damage repair in $CD4^+$ T cells of male SLE patients, explaining somehow the great levels of DNA damage. Last but not least, our results demonstrated that exposure of healthy $CD4^+$ T cells in SLE disease milieu induces the transcriptional and phosphorylated levels of SMC1A. This might mean that, whereas in normal state the SLE milieu induces SMC1A expression and

activity, the lupus $CD4^+$ T cells tend to have diminished SMC1A expression and activity. Therefore, all these data give some evidence that deregulated SMC1A in SLE is possible to be implicated with disease pathogenesis.

Conclusively, the results of our study could aid us to better understand lupus disease, but also potentially lead to identification of novel targets of treatment. Additionally, as gender differences may affect drug action, tailored treatments for males and females would improve outcomes and overall prognosis for both genders.

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