UCTD and SLE patients show increased levels of oxidative and DNA damage together with an altered kinetics of DSB repair

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Abstract

Immunological tolerance is a critical feature of the immune system; its loss might lead to an abnormal response of lymphocytes causing autoimmune diseases. One of the most important groups belonging to autoimmune disorders is the connective tissue diseases (CTD). CTD are classified among systemic rheumatic diseases and include pathologies such as systemic lupus erythematosus (SLE), and undifferentiated CTD (UCTD).

In this study, we evaluated oxidative and genome damage in peripheral blood lymphocytes from patients with SLE and UCTD, further classified on the basis of disease activity and the presence/absence of a serological profile. Oxidative damage was evaluated in cell membrane using the fluorescent fatty acid analogue BODIPY$^{581/591}$ C11. The percentage of oxidised lymphocytes in both SLE and UCTD patients was higher than in the control group, and the oxidative stress correlated positively with both disease activity and autoantibody profile. The γH2AX focus assay was used to quantify the presence of spontaneous double strand breaks (DSBs), and to assess the abilities of DSBs repair system after T cells were treated with mitomycin C (MMC). Subjects with these autoimmune disorders showed a higher number of γH2AX foci than healthy controls, but no correlation with diseases activity and presence of serological profile was observed. In addition, patients displayed an altered response to MMC-induced DSBs, which led their peripheral cells to greatly increase apoptosis. Taken together our results confirmed an interplay among oxidative stress, DNA damage and impaired DNA repair, which are directly correlated to the aggressiveness and clinical progression of the diseases. We propose the evaluation of these molecular markers to better characterize SLE and UCTD, aiming to improve the treatment plan and the quality of the patients' life.

Introduction

The immune system represents the natural defence of our body: it is responsible for the identification of non-self antigens and for preventing the harmful reaction against self antigens, the latter is referred as immunological tolerance. This phenomenon is important for the maintenance of the normal immune balance and its failure can lead to autoimmune diseases (1). Genetic susceptibility, epigenetics and environmental factors (diet, infections, etc) are implicated in the pathogenesis of these disorders (2,3).

Undifferentiated connective tissue disease (UCTD) and systemic lupus erythematosus (SLE) are two chronic autoimmune diseases characterized by an inflammation of connective tissue, which is supported by an activation of the immune system towards self-epitopes expressed on cell surfaces or on the matrix (4). The clinical course of these diseases is defined by periods of remission alternating with exacerbation. Because of the heterogeneous nature of connective tissue diseases (CTD), the symptoms and the immunological features are often shared among the various conditions (3,5).

Under the term UCTD can be grouped transitional forms, incomplete defined CTD and the stable UCTD, which will not change over time. The stable UCTD are not easy to identify since they are very similar to the incomplete/transitional forms, for this reason some inclusion and exclusion (4) criteria were suggested in order to make the diagnosis easier. However, reliable epidemiological data are not available for UCTD due to the absence of a widely accepted definition of these disorders (6).

On the contrary, SLE is a defined CTD described by clinical and serological heterogeneity (7,8). The pathogenetic mechanism is still unclear but it is known that the aetiology is multifactorial (9). While UCTD patients generally present a monogenic autoantibody profile, in SLE it is multiple and typically directed towards the nuclear components of cells (7).

These illnesses show an increase of radical species during the inflammatory response, which can result in tissue damage (10,11). The high levels of reactive oxygen/nitrogen species (ROS/RNS) and/or the impairment of protection systems can stimulate tissue inflammation, immunological disorders and also cellular death (12,13). Furthermore, chronic inflammation and joint damage, supported by stress conditions, might lead to the degradation of connective tissue causing immunomodulation and autoimmune diseases (14). In addition, in SLE, it was observed that oxidative stress also boosts serum protein modifications, which are related to the disease activity: in fact, patients develop autoantibodies towards these oxidised proteins (15). Oxidative stress can also induce damage at DNA level, as single and double strand breaks (SSBs, DSBs), thus leading to genomic instability and the activation of apoptosis. Defects in the clearance of apoptotic debris, typical of CTD diseases, can foster the production of autoantibodies and their expansion (4,10,15-17).

DSBs represent the most serious injuries that involve the DNA. Once DNA has undergone a DSB, following the activation of the the so-called DNA damage response (DDR) all molecules of H2AX histone variant at the site of DSBs, a region of several Mbp, are phosphorylated on Ser-139 (namely γH2AX) (18-21). Several studies have demonstrated that the use of the γH2AX assay is a reliable method to quantify DSBs formation inside the nucleus of a cell population from patients with chronic inflammation-based pathologies (22-27). The induction and the disappearance of γH2AX foci can also be used for evaluating the kinetics of DSBs repair (25,28).

Since oxidative stress and genomic damage are interconnected, they can create a supportive environment to the development of autoreactivity and then worsen the patient's clinical course. To clarify this interplay in CTD pathogenesis, using the γH2AX assay, we quantified in peripheral lymphocytes of UCTD and SLE women the spontaneous and mutagen-induced

DNA lesions as well as the presence of oxidative stress in the same cell system analysing the fluorescence shift of a fatty acid structural analogue.

Materials and Methods

Study population

Since SLE and UCTD are much more frequent in women than in men, the study was conducted on 86 women, aged 20-70, recruited from the Unit of Rheumatology (Pisa, Italy) and divided into three groups: 26 UCTD patients, 35 SLE and 25 healthy controls. The demographic and clinical characteristics of the study population are summarized in Table 1. The SLE diagnosis was based on the Systemic Lupus International Collaborating Clinics classification criteria and the disease activity was defined following the SLEDAI index (29-31). UCTD disease was confirmed taking into account the preliminary criteria suggested in 1999 and in 2005 by Bombardieri's group, while the activity status was determined according to clinical judgment (4,6). Serological profiles were provided by the Unit of Rheumatology (Pisa, Italy). Patients who took drugs known to induce genome damage were excluded. The study was approved by the ethical committee of Pisa University.

➔ Table I

Cell cultures

At least 9 ml of whole blood sample was collected from each woman by venepuncture, placed in tubes containing lithium heparin and processed within 24 h.

Peripheral blood lymphocytes cultures were started by adding 150 μl of whole blood to 2.35 ml of RPMI-1640 (Life Technologies, Monza, Italy) supplemented by 15% FBS (Life Technologies, Monza, Italy), 1.5% PHA (Life Technologies, Monza, Italy) and 1% antibiotics and antimycotics (Euroclone, Milano, Italy). Cultures were kept at 37°C for variable time

depending on the specific method applied, as described in the following sections. For each subject two independent culture *per* assay were set up.

Oxidative damage analysis

A specific structural analogue of membrane fatty acid was used to detect the lipid bilayer oxidation. This probe, BODIPY$^{581/591}$ C11 (Life Technologies, Monza, Italy), is characterized by fluorescent properties: its emission can arrive up to 595 nm, but when it is exposed to oxidative environment, it changes its range of fluorescence as a result of the oxidation of its structure. At 47.30 h from the start of culturing 2.5 μl of this probe were added to the samples and the tubes were placed at 37°C for a further 30 min in order to promote the fluorescent molecule incorporation inside the membrane of lymphocytes. The samples were then harvested according to standard procedure (32). After the addition of hypotonic KCl solution (0.0075 M), cells were pre-fixed with acetic acid:methanol at 5:3 ratio. Subsequently, lymphocytes were fixed in 100% methanol and washed twice in a solution of acetic acid: methanol in variable ratio depending on the percentage of humidity. After that, they were dropped onto clean glass slides, allowed to dry, counterstained with 4 μl of propidium iodide (PI) and then observed through a fluorescent microscope (Nikon-Optiphot 2) equipped with two specific filters, TRICT and FICT, at 1000x magnification. At FICT filter, undamaged cells presented a light brown cytoplasm, while lymphocytes with an oxidised membrane showed a green fluorescence (see Figure 1A); TRICT filter was used to detect cells regardless of the oxidation status. Oxidative damage was quantified as a percentage of cells with green cytoplasm on the total of 1000 cells scored *per* culture. Data were expressed as the average value of the two cultures.

Genomic damage analysis

Spontaneous DNA injury was determined in unstimulated cells while the reparative ability was carried out in proliferating lymphocytes treated with the chemical mutagen mitomycin C

(MMC, Sigma-Aldrich, Milano, Italy). In this last analysis, quantification of γH2AX foci was assessed at three different time points as follows: lymphocyte cultures were incubated for 20 h at 37°C, then one-third of samples was immediately harvested in order to have information about the basal DNA damage due to proliferation (time-point 0), while the remaining cultures were or not exposed to MMC 1 μM and harvested after 2 and 4 h in order to quantify the MMC-induced damage and the amount of DNA lesions not yet repaired, respectively. For both analysis samples were processed as described elsewhere (32).

Detection of γH2AX foci was performed using phospho-histone H2AX (Ser139) rabbit polyclonal primary antibody (Cell Signaling, Euroclone, Milano, Italy) and DyLight™ 488-conjguated anti-rabbit secondary antibody (Pierce, Euroclone, Milano, Italy). Briefly, dry slides were washed twice in PBS (1X) and left for 30 min in blocking solution composed by inactivated foetal bovine serum (FBS, 10% Life Technologies, Monza, Italy), TRITON® X-100 (0.3% Sigma-Aldrich, Milano, Italy) in H_2O. After that, slides were incubated overnight at 4°C with primary antibody diluted 1:50 in blocking solution, they were washed 3 times in PBS (1X), and incubated for 2 h at room temperature with secondary antibody diluted 1:200 in blocking solution. Once the incubation period ended, slides were washed 3 times in PBS (1X), counterstained with 4 μl of PI and observed using FITC and TRICT filters (see Figure 2A). 53BP1 (p53 binding protein 1), which plays a pivotal role in the initial phases of the DDR and in the DSBs repair system choice, is considered another suitable marker of DSB formation (18). Thus, the simultaneous detection of both γH2AX and 53BP1 by a dual immunofluorescence was also performed (see Supplemental File for experimental details). However, as the two markers were expected to co-localise at the site of DSBs (see Figure 1 of Supplemental File), quantification of DSBs formation was reported for γH2AX foci alone.

Spontaneous genomic damage was evaluated scoring 1000 nuclei *per* culture and recording the number of nuclei showing at least one γH2AX signal and the total number of signals in each nucleus. The level of γH2AX for each sample was expressed as percentage of γH2AX positive nuclei or as average number of γH2AX foci *per* positive nucleus. For the analysis of MMC-induced DSBs, 400 nuclei were examined as outlined above, and the results were expressed as the average number of γH2AX foci *per* nucleus.

Statistical analysis

All statistical analyses were carried out with the Statgraphic Plus 5.1 (Statistical Graphics Corporation, 2001, Rockville, MD, USA) software package. Concerning the investigated parameters, the presence of variation was evaluated using multifactorial ANOVA including age as covariate and smoking habit, diseases activity or autoantibody profile as categorical variables. Differences between the analysed groups were performed using Multiple Range Test. Results were reported as mean ± standard error (SE), and the difference between groups were considered significant for $P < 0.05$.

Results

Oxidative damage

We observed higher frequency of oxidation in the UCTD and SLE group (89.6 ± 12.03 and (98.8 ± 12.10, respectively) than in controls (7.20 ± 14.68; Figure 1B). Since these two diseases are characterized by alternated cycles of exacerbation and remission, the oxidative damage was evaluated accordingly. The level of damaged lymphocytes in patients with active disease status was significantly ($P = 0.0198$) higher than those in remission. Taking into account the disease activity and the type of illness, it was possible to observe that the average of oxidised cells in patients with the illness in activity was higher than those in remission (SLE: 139.78 ± 18.09 *vs*. 76.52 ± 18.14; UCTD: 108.89 ± 18.09 *vs*. 88.40 ± 13.96; Figure

1C). The interaction plot shown in Figure 1D also underlines this specific trend: high oxidative stress in people with active disease and less oxidised cells in the remission group. Concerning the autoantibody profile, it is monospecific in UCTD and multiple in SLE patients. The analysis showed a significant difference ($P = 0.084$) between patients with positive profile and those that did not have any autoantibodies. Confirming as already stated above, Figure 1E indicates that patients with autoreactivity (SLE 121.91 ± 13.36; UCTD 116.18 ± 18.85) have more oxidised lymphocytes than subjects with a negative profile (SLE 76.70 ± 19.69; UCTD 72.06 ± 17.55).

Spontaneous genomic damage

As shown in Figure 2B, UCTD and SLE patients showed comparable genomic damage levels, expressed as percentage of positive γH2AX nuclei, (67.57 ± 12.88; 69.99 ± 12.56, respectively), which were significantly higher ($P = 0.0130$) than those of controls (22.12 ± 13.95). When H2AX phosphorylation was evaluated in relation to smoking, we observed that smokers had more damage (72.61 ± 14.74) than nonsmokers (33.62 ± 8.19; Figure 2C). Both diseases activity and autoantibody profile did not influence the expression of DNA damage. Furthermore, the presence of pathological conditions and smoking habit induced significant increases in the average number of γH2AX foci per positive nuclei ($P = 0.04$ and $P = 0.024$, respectively). Specifically, both connective tissue diseases showed a higher level of DSBs (SLE 2.89 ± 0.39; UCTD 3.37 ± 0.40) than healthy subjects (1.77 ± 0.43; Figure 2D). Interestingly, despite the percentage of positive nuclei in both diseases was the same, the UCTD group presented more DNA damage at single nucleus level than SLE patients, even though the difference was not statistically significant.

Assessment of repair kinetics in mitomycin C-treated peripheral cells

Following a previous study (36), assessment of the kinetics of repair was carried out considering three time points. This allowed us to have information on the basal DNA damage

(time = 0 h), the maximum level of MMC-induced DSBs (time = 2 h) and the ability of the DSBs repair system to solve the damage (time = 4 h). The results, expressed using the number of γH2AX foci *per* nucleus, indicated a significant difference among either the three time points ($P = 0.0038$) or the pathological conditions ($P = 0.0001$). Moreover, the interaction between the response to the induced damage and the three groups of subjects showed a strong variability ($P = 0.0115$). In the group of the healthy controls, the kinetics of repair described an increase of DSBs after 2 h (4.12 ± 0.53) with respect to the time 0 (1.48 ± 0.57) and a consistent reduction of the damage at 4 h (2.00 ± 0.38). On the contrary, no difference was observed at 2 and 4h compared to the basal level (0 h) in both SLE and UCTD groups (Figure 3A). In support of these data, Figure 3B confirms that only the control group shows the expected kinetics of repair, while patients, independently of the disease, exhibit values of γH2AX comparable to those obtained at time 0 h. As during the scoring of DSBs levels in the patients, we observed quite frequently the presence of apoptotic-like cells, the possibility was investigated that apoptosis had played a relevant role during mutagen treatment. Thus, a morphological analysis of nuclei together with a TUNEL assay were performed in MMC-treated lymphocytes from a subgroup of patients and controls (experimental details and complete results are provided in Supplemental File). In summary, the main findings we found indicate a significantly higher level ($P = 0.0003$) of apoptotic nuclei in the disease groups as compared to the healthy subjects (see Figure 2C of Supplemental File).

Discussion

Autoimmune diseases are characterized by a chronic inflammation that promotes, over time, alterations of cellular metabolism. This variation will determine a cascade of events which will end with damage to the biological macromolecules.

The presence of an excess of oxidative stress, which is a specific feature of these disorders, due to high levels of ROS and RNS, can cause further damage to DNA and modulate the expression of inflammatory molecules. In autoimmune diseases, the sum of these events will lead to the exacerbation of chronic inflammation and, consequently, to tissue damage (33). In this study, we evaluated oxidative and genome lesions in T cells from patients affected by UCTD and SLE. Our results indicated that women with autoimmune disease showed oxidative damage, here assessed as membrane oxidation, more pronounced than the healthy controls. This supports the notion that increased levels of reactive species is a specific feature of UCTD and SLE, as confirmed by other authors (34,35).

Concerning the UCTD patients, we found that the level of oxidative damage was slightly less than in SLE patients. This might be due to the fact that chronic inflammation is more localized and without additional complications. Furthermore, dividing the affected subjects according to the disease activity, we observed that people with high disease activity had a 1.5 fold increase in oxidised cells than the remission group. The same trend was confirmed within each disease group (SLE and UCTD patients with high disease activity showed oxidative damage 1.6 and 1.4 fold more elevated than the remission group, respectively).

In this context, several works about SLE support the hypothesis that lipidic peroxidation is strongly linked to the disease activity (33,34,36,37). Another interesting aspect emerging from our study is the correlation between serological activity and oxidative stress. SLE patients with an active antibody profile showed a damage 1.4 fold higher than UCTD. Previous work showed that the presence of serological activity correlates with oxidative stress, and it is well known that people with SLE have a greater autoantibody profile than UCTD patients (37). The multiple autoreactivity of SLE patients can promote a strong inflammation which, in turn, leads to a greater production of ROS by phagocytes. These reactive species can modify, in the cell environment, proteins through oxidation, and

subsequently the acquired immune system would not recognize them as self, causing the production of autoantibodies toward these proteins (35,38,39).

The lower oxidation levels shown by the UCTD group compared to SLE patients can be explained by the fact that patients with an undifferentiated framework express positive autoantibodies titres without showing severe organ involvement. This would promote only a mild-to-moderate inflammation that in turn stimulates ROS production by phagocytes and lead, over time, to an increase in cell oxidation.

In autoimmune diseases, since chronic inflammation and oxidative stress fuel each other in a feedback circle, genomic damage occurs frequently that, if not repaired, can promote apoptosis. Peculiarity of autoimmune diseases, is the delay in the clearance of cellular debris, this time-lag promotes the immunocomplex formations that will increase inflammation and consequently the oxidative stress, which in turn will continue to damage the macromolecules. In SLE patients, high levels of DSBs and crosslinking between DNA and protein were observed due to the presence of chronic inflammation and oxidative stress (12,15,40), as well as increased H2AX phosphorylation was detected in other inflammatory-based diseases (22-24).

We expressed the presence of spontaneous DSBs in T cells of people with SLE and UCTD as either a percentage of γH2AX-positive nuclei, which gives us a general view of the damage in a cell population, or the average number of γH2AX foci *per* positive nucleus, the latter pointing out the intensity of the damage at single cell level.

Both UCTD and SLE showed a nuclear damage 2-3 fold higher than healthy controls. The presence of DSBs using the phosphorylated histone variant H2AX has already been evaluated in mononuclear cells isolated from SLE patients (28). Our results agree with this study since UCTD and SLE groups show a strong occurrence of DSBs. Although there was no significant difference between the two autoimmune diseases, it is worth dwelling on the fact that

unexpectedly the frequency of γH2AX-positive nuclei in UCTD group resulted 30% higher than in the SLE patients. However, SLE patients, despite having a multiple autoantibody profile and high level of oxidative stress, might fail to repair these lesions properly, promoting apoptosis. In support of this hypothesis, there are several scientific papers which affirm that the presence of DNA damage accumulation subsequently lead to an increase of apoptotic cells in these patients (33,41,42).

Unlike oxidative damage, DSBs levels does not seem influenced by the disease activity status or the presence of an autoantibody profile (28,43). Smoking is known to be a source of DNA damage and it can promote the increase of cellular metabolism, leading to high ROS levels (44). Independently of the pathological condition, we observed that smokers had more DSBs than non-smokers.

The genome damage represented by DSBs, is associated to genomic instability which might promote the development of neoplastic diseases over time. A further aim of this work was to give information about a possible predisposition towards carcinogenesis. In the autoimmune disease, the combination of chronic inflammation, oxidative stress, DNA lesions and an impaired DNA repair system, inevitably leads to the accumulation of DSBs which, in turn, promotes genomic instability as the formation of micronuclei or chromosomic rearrangement (45). The DNA repair system plays an important role in preventing genomic instability, but this activity can be delayed or annulled, causing genomic rearrangement or cell death, as observed in colon and oesophageal cancer or in neurodegenerative diseases such as Alzheimer (46-49). In this context, several works analysing autoimmune diseases describe the production of autoantibodies against enzymes involved in the nuclear damage repair (50,51).

In this work, we also assessed the ability of peripheral white blood cells to repair the MMC-induced damage. Following a previous study, an optimal time point of the damage induction was found at 2h post MMC treatment, and a significant decrease of these lesions was

observed after 4h (32). The results obtained in the present study show that peripheral cells of patients have an impaired response to MMC treatment, as they did not even increase the level of γH2AX over the expected time. We thought the observed trend could be attributable to activation of the apoptosis pathway rather than to a delay in the proper signalling to the DDR machinery (*i.e.* γH2AX formation). Indeed, some works have already described an increase in apoptosis level in the cells of SLE patients (17,28,42,52). This feature might be expected to occur also in UCTD, since UCTD cells presented high level of oxidative stress and high number of DSBs. The analysis carried out in the peripheral cells of a subgroup of the study population following MMC treatment, indicates that peripheral cells of patients are forced to resolve the surplus of DSBs induced by the mutagenic stimulus via apoptosis. The presence of apoptotic nuclei might therefore explain the lack of γH2AX foci observed in the overall patient groups treated with MMC, suggesting that these lesions cannot be properly counteracted by the DSBs repair system.

In conclusion, this work indicates that UCTD and SLE patients show, in their peripheral cells, the presence of oxidative stress, DNA damage and an altered DSBs repair system, especially against mutagenic insults. Collectively, these features can influence the aggressiveness and the progression of autoimmune diseases. Our findings are in line with previous observation carried out on SLE patients and, to our knowledge, also represent the first attempt to describe part of the molecular mechanisms underlying UCTD pathogenesis, thereby allowing a better comprehension of this group of autoimmune diseases which is still poorly studied. Our work also offers cues for further investigations concerning the role of apoptosis in these two illnesses: since this phenomenon boosts the new epitopes availability and consequently the production of autoantibodies, it might increase the inflammation and lead to a worsening of the patient's conditions. Besides, from a clinical point of view, the evaluation of oxidative and

genomic damage might represent valuable molecular markers to predict the progression of SLE and UCTD, aiming to improve the treatment plan and the quality of the patients' life.

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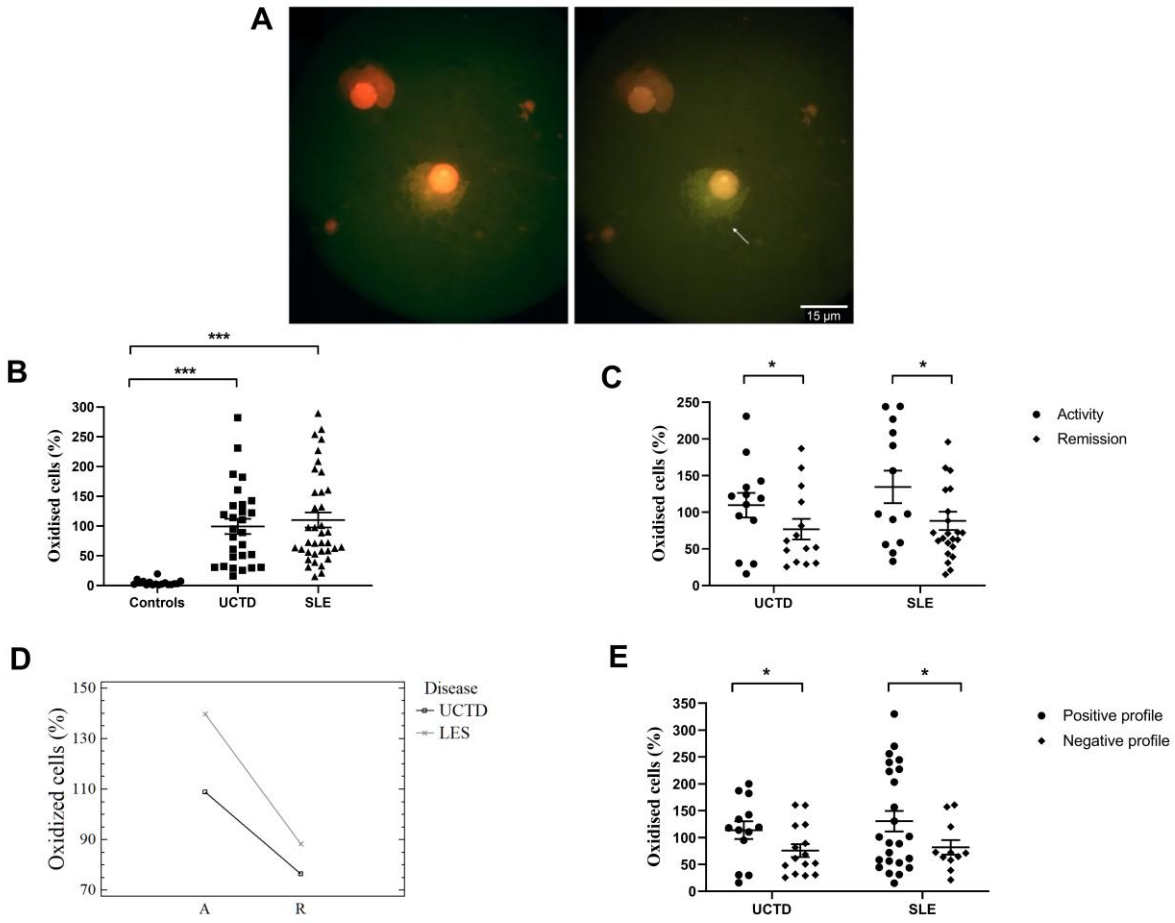
Legend to figures

Figure 1. Analysis of spontaneous oxidative damage in peripheral blood lymphocytes. **(A)** A non-oxidised cell visualised under a TRITC (top left) or FITC (top right) filter; an oxidised cell visualised under a TRITC (bottom left) or FITC (bottom right) filter (white arrow indicates the green fluorescence of the cell membrane). Average percentage of oxidised cells **(B)** in patients (UCTD and SLE) and controls; **(C)** in UCTD and SLE patients divided according to the disease status. **(D)** Interaction plot between disease group and disease activity: UCTD and SLE show the same trend (A, Activity; R, Remission). **(E)** Average percentage of oxidised cells in UCTD and SLE patients divided on the basis of the presence of autoantibody profile. Bars represent mean ± S.E. *** indicates a significant difference at $P < 0.001$); * indicates a significant difference at $P < 0.05$.

Figure 1. Analysis of spontaneous genomic damage in peripheral blood lymphocytes. **(A)** γH2AX foci visualised as green-yellow fluorescence spots in nuclei of lymphocytes analysed with a TRITC (top and bottom left) or FITC (top and bottom center) filter; merged images (top and bottom right). Percentage of γH2AX positive nuclei **(B)** in the study population according to pathological conditions, **(C)** in the study population divided into smokers and non-smokers. **(D)** Average number of γH2AX foci *per* positive nucleus in the study population according to pathological conditions. Bars represent mean ± S.E. *** indicates a significant difference at $P < 0.001$); * indicates a significant difference at $P < 0.05$.

Figure 3. Repair kinetics Time-course of γH2AX foci in MMC-treated lymphocytes. **(A)** Number of γH2AX foci *per* nucleus in UCTD, SLE and controls analysed after 0, 2 and 4h from the start of MMC treatment. Bars represent mean ± S.E. *** indicates a significant

difference at $P < 0.001$); ** indicates a significant difference at $P < 0.01$. **(B)** Interaction plot between disease groups at different MMC exposure times.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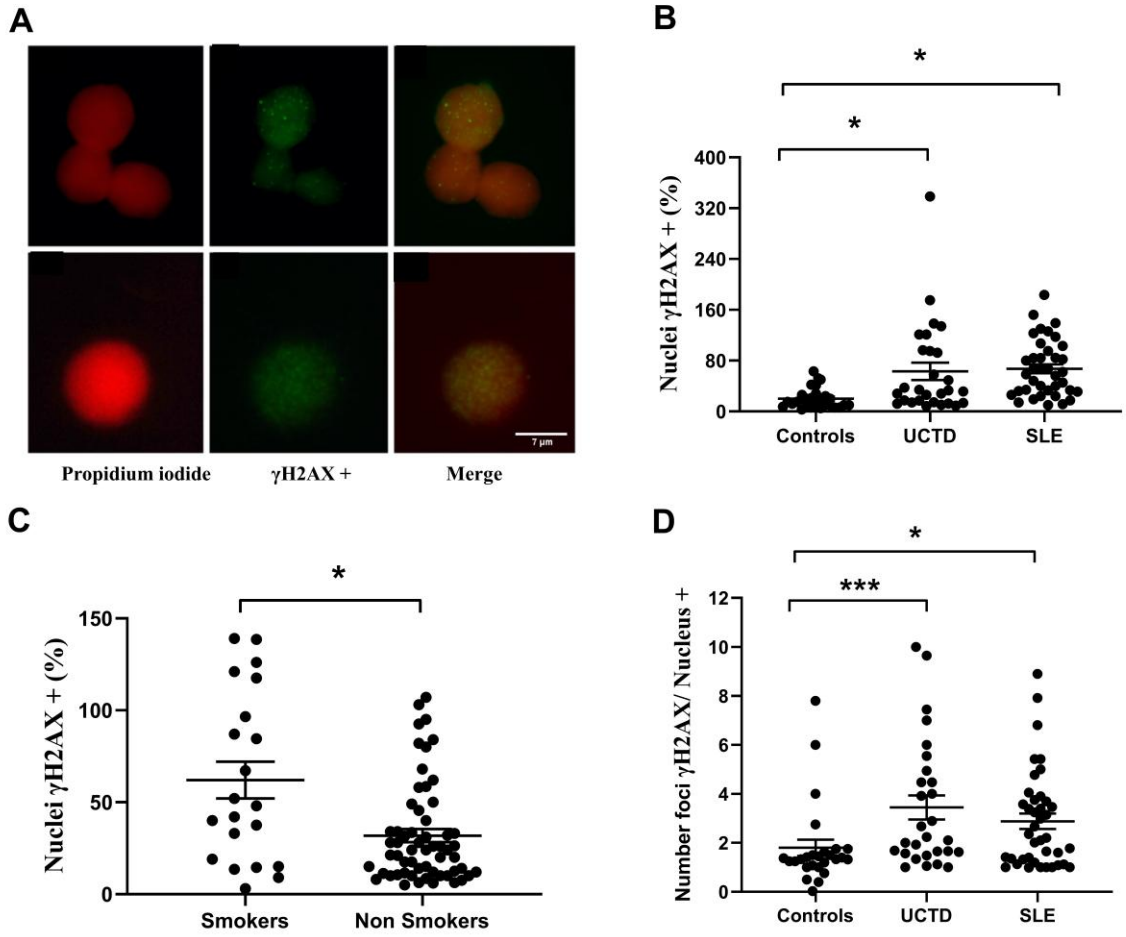
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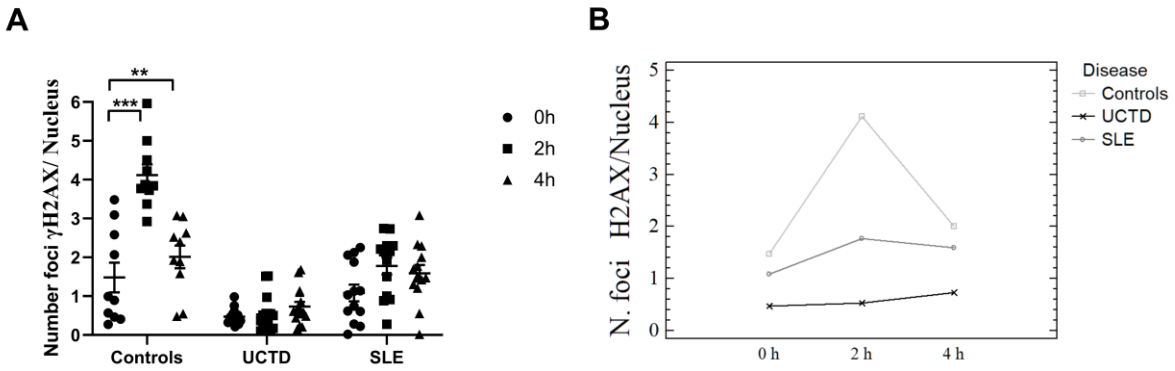
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