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DNA damage accumulation, defective chromatin organization and deficient DNA repair capacity in patients with rheumatoid arthritis

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ABSTRACT

We investigated the DNA damage response and repair network in 18 patients with active rheumatoid arthritis and tested the hypothesis that treatment influences this network. A 3-fold increase of endogenous DNA damage (single- and double-strand breaks) was detected in patient-derived peripheral blood mononuclear cells than controls (alkaline comet assay; mean ± SD Olive Tail Moment of 11.8 ± 7.3 versus 4.3 ± 2.2, $p < .001$). Patients exhibited significantly higher formation of DNA damage (oxidative stress and abasic sites), deficient global genome repair and more condensed chromatin structure than controls. Twelve weeks following treatment, chromatin structure loosened, global genome repair capacity was restored, oxidative stress and abasic sites decreased and levels of endogenous DNA damage reached control values in all 8 patients examined. We conclude that deregulated chromatin organization, deficient DNA repair capacity and augmented formation of DNA damage, which are reversible after treatment, contribute to the accumulation of endogenous DNA damage in rheumatoid arthritis.

1. Introduction

Each human cell is subjected to a daily load of approximately 7×10^4 genomic lesions, most of which are of endogenous origin [1,2]. Major sources of endogenous DNA damage include, among others, reactive oxygen and nitrogen species [3,4]. A significant portion of DNA lesions that are commonly associated with reactive oxygen species (ROS) are single-strand DNA breaks (SSBs), which can be converted to DNA double-strand breaks (DSBs) when replication forks come across SSBs. Although less frequent, DSBs are far more dangerous and difficult to repair as they represent a complete physical break in the DNA backbone [5]. Another critical source of endogenous DNA damage is the abasic (apurinic/apyrimidinic, AP) site, which constitutes an intermediate/product of most DNA damage processing pathways and occurs with high frequencies in malignant tumors and/or following ionizing irradiation [6]. Cleavage of AP sites by AP endonucleases or by DNA N-glycosylases/AP lyases results in the formation of DNA SSBs with 3′- or 5′-blocked ends, that cannot be used as substrates by DNA polymerases or DNA ligases [7] and can also be converted into highly toxic DSBs after DNA replication [8].

In general, protection against genotoxic insults is secured by a complex network of DNA damage response and repair (DDR/R) pathways triggered by the detection of DNA lesions through specific sensors [9]. The subsequent step is the initiation of a signal transduction cascade including molecules which activate various genome-protection pathways, i.e. DNA repair, cell cycle control, apoptosis, transcription and chromatin remodeling. Aberrations of DDR/R have been linked to human disease including cancer, hematologic and neurodegenerative diseases [10–12]. Moreover, polymorphisms in central DDR/R molecules such as the exonuclease TREX1 have been linked to syndromes characterized by systemic inflammation like Aicardi-Goutières Syndrome [13] or monogenic lupus [14].

There is mounting evidence that the epigenome, as a second dimension of genetic information superimposed onto the DNA helix, is also a key regulator of DDR/R. Indeed, since chromatin is the physiological template for DNA repair machinery, the structural context of chromatin environment may facilitate or alternatively make it more challenging for repair factors to maintain and restore genome integrity [15]. Thus, the dynamic nature of chromatin compaction is crucial to genome integrity through structurally restricting unstable repetitive elements on the one hand, while allowing access to repair factors upon damage on the other hand [16].

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Rheumatoid arthritis (RA) is a chronic, inflammatory rheumatic disease with relatively unknown genetic and epigenetic pathogenic components [17,18]. RA is characterized by aberrant adaptive and innate immune responses, including activation of monocytes, lymphocytes, synovial fibroblasts and production of inflammatory cytokines, reactive oxygen species, matrix degradation enzymes and auto-antibodies [19]. A few studies have proposed the involvement of deregulated DDR/R network in the pathogenesis of RA [20–22]. For example, human data have shown increased DNA damage load and apoptosis in $CD4^+/CD45RA^+$ T cells from RA patients, partly due to failure to produce sufficient mRNA transcripts/protein of the DNA repair kinase ataxia telangiectasia (AT) mutated (ATM) [21]. Moreover, epigenetic alterations affecting chromatin organization have been implicated in the pathogenesis of RA [23,24].

Herein, we sought to investigate factors and processes that can be associated to intracellular formation of DNA damage and deficient DNA repair, including chromatin structure, in patients with active RA. Furthermore, we tested the hypothesis that treatment influences the deregulated DDR/R network in these patients.

2. Materials and methods

2.1. Patients

Peripheral blood mononuclear cells (PBMCs) were isolated from 18 consecutive patients with active RA according to ACR/EULAR classification criteria [25], (untreated, n = 9, treated with conventional synthetic DMARDs ± corticosteroids, n = 5, treated with low dose (< 7.5 prednisolone daily) corticosteroids only, n = 4). Demographics and disease characteristics of patients including age, sex, disease duration, tender/swollen joint count(28), ESR (mm/1st h), CRP(mg/dl) and the activity index DAS28-ESR are shown in Table 1. Eight representative RA patients were re-examined 12 weeks after conventional synthetic disease-modifying antirheumatic drugs (DMARDs) ± corticosteroids and/or biological DMARDs treatment (Table 2). Sixty-five (n = 65) apparently healthy individuals served as controls. All individuals involved in the study gave their informed consent according to the declaration of Helsinki. The study was approved by Laikon Hospital Ethics Committee (Protocol Nr.:1348).

2.2. Alkaline single-cell gel electrophoresis (alkaline comet assay)

The single-cell gel electrophoresis assay was performed under alkaline conditions as previously described [26]. PBMCs were isolated from freshly drawn peripheral blood by standard Ficoll gradient centrifugation (Ficoll-Paque Plus, Sigma Aldrich) and stored in freezing medium [90% fetal bovine serum (FBS) + 10% DMSO] at −80 °C. Cells were suspended in low melting point agarose (1%) in PBS at 37 °C, and spread onto specifically designed slides purchased from Trevigen (CometAssay® HT Slide, Trevigen, USA). After lysis of cellular membranes at 4 °C for 2 h, slides were placed in a horizontal gel electrophoresis chamber and incubated in prechilled electrophoresis buffer for 40 min. Electrophoresis was performed for 30 min at 1 V/cm. Afterwards, slides were washed with neutralisation buffer and distilled H_2O and left to dry overnight. Gels were stained with SYBR Gold Nucleic Acid Gel Stain (Thermo Fischer Scientific) and analyzed with a fluorescence microscope (Zeiss Axiophot). Olive Tail Moments [OTM = (Tail Mean-Head Mean) x (% of DNA)/100] of at least 200 cells/treatment condition were evaluated. Comet parameters were analyzed by the ImageJ Analysis/Open Comet software. Experiments were performed in duplicate.

Table 1
Demographics and disease characteristics of RA patients at baseline.

Demographics and clinical characteristics	RA patients (n = 18)
Men/women, n	5/13
Age range (mean ± SD, years)	35–85 (64.9 ± 12.2)
Disease duration (mean ± SD, years)	6.5 ± 11.7
Tender joint count (28) (mean ± SD)	7.8 ± 3.7
Swollen joint count (28) (mean ± SD)	5.5 ± 3.5
ESR (mean ± SD, mm/1st h)	31.6 ± 14.7
CRP (mean ± SD, mg/dl)	1.3 ± 1.0
DAS28-ESR (mean ± SD)	5.1 ± 0.8
RF- and/or anti-CCP-positivity n (%)	14 (77.8)

ESR: erythrocyte sedimentation rate; *CRP*: C-reactive protein; *DAS*: Disease Activity Score; *RF*: rheumatoid factor; *anti-CCP*: anti-cyclic citrullinated peptide.

Table 2
Disease activity at baseline and after 12-week treatment.

RA patients (Gender, age)	DAS28-ESR baseline	Treatment baseline/add-on treatment	DAS28-ESR after treatment
Woman, 55	5.1	-/anti-TNF	4.2
Woman, 48	6.1	-/MTX + CS	2.6
Woman, 58	6.1	CS/anti-TNF	4.8
Man, 85	5.1	-/CS	3.6
Woman, 61	4.6	MTX + CS/anti-TNF	3.8
Woman, 75	5.2	CS/anti-TNF	4.2
Man, 62	4.3	-/MTX + CS	1.8
Man, 60	3.7	-/MTX	3.5

DAS: Disease Activity Score; *ESR*: erythrocyte sedimentation rate; *MTX*: methotrexate; *CS*: corticosteroids; *TNF*: tumor necrosis factor.

2.3. Western blot analysis

Western-blot analysis was performed in untreated PBMCs [27]. Briefly, PBMCs were harvested, rinsed in ice-cold PBS, and lysed in buffer containing 50 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 5 μg/ml benzamidine, 0.5 mM phenylmethylsulfonylfluoride, and 1% NP-40, followed by SDS-polyacrylamide gel electrophoresis. Subsequently, the proteins were transferred to nitrocellulose membranes, which were incubated for 40 min at room temperature with non-fat dry milk (5%) in 1xTris buffered saline (TBS) followed by incubation with specific primary antibodies to γH2AX (Ser139; Cell Signaling Technology), phosphorylated p53 binding protein-1 (p-53BP1; Cell Signaling Technology), and phosphorylated replication protein-32 (p-RPA32; Cell Signaling Technology) overnight at 4 °C. Then, the membranes were washed with 0.3% bovine serum albumin in 1xTBS and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. After washing with 1xTBS, the bound antibody complexes were visualized using the Western Blotting Detection Kit ECL Plus (Amersham) and XOMAT-AR film (Kodak).

2.4. Measurement of glutathione (GSH) and oxidized glutathione (GSSG)

Basal oxidative stress was measured using a luminescence-based system that detects and quantifies total glutathione, oxidized glutathione and the GSH/GSSG ratio according to manufacturer's experimental protocol (GSH/GSSG-Glo™ Assay, Promega). Briefly, 10^4 untreated PBMCs were plated in 96-well luminometer-compatible tissue culture plate (Corning Costar). Afterwards, 50 μl/well of Luciferin Generation Reagent was added to all wells, a brief shake followed and incubation at room temperature for 30 min. Subsequently, 100 μl/well of Luciferin Detection Reagent were added and after 15 min incubation the luminescence signal were read in Spectramax M3 microplate reader (Molecular Devices LLC, California).

2.5. Detection of abasic sites

The endogenous levels of abasic sites in PBMCs were evaluated using the OxiSelect Oxidative DNA Damage Quantitation Kit (AP sites)

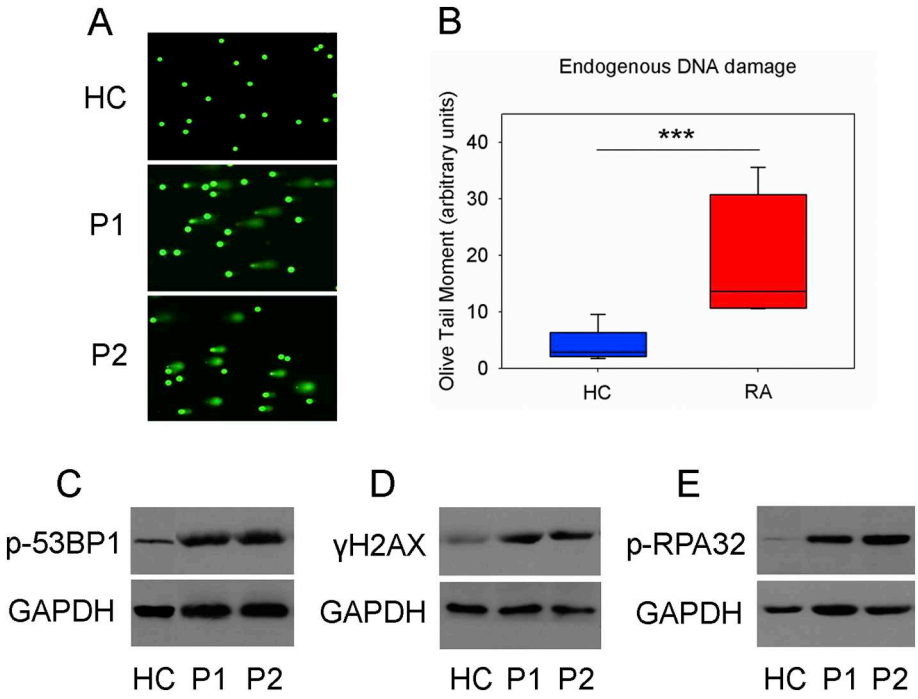

Fig. 1. High levels of endogenous DNA damage in patients with RA. (A) Representative alkaline comet assay images of untreated PBMCs from a control and two RA patients (P1 and P2). (B) Box plots showing statistical distribution of the endogenous DNA damage in untreated PBMCs from controls and RA patients. The horizontal lines within the boxes represent the median values of Olive Tail Moments (OTM) arbitrary units and the vertical lines extending above and below the box indicate maximum and minimum values, respectively. Two independent experiments were performed and 200 cells were scored per sample. Representative western-blots of 1 control and 2 RA patients (P1 and P2) using antibodies against critical factors of the DDR/R network, i.e. p-53BP1 (C), γH2AX (D), and p-RPA32 (E). GAPDH is used as a loading control for protein normalization. All error bars represent SD; ***$p < .001$ by Mann-Whitney *U* test.

according to manufacturer's experimental protocol (Cell Biolabs, Inc.). The assay kit uses an Aldehyde Reactive Probe (ARP) to react specifically with an aldehyde group on the open ring form of AP sites. This allows for the AP sites to be tagged with biotin, which is later detected with Streptavidin-Enzyme conjugate. The quantity of AP sites in unknown DNA sample is determined by comparing its absorbance with a standard curve generated from the provided DNA standard containing predetermined AP sites.

2.6. Measurement of DNA monoadducts along the N-ras gene

The total nucleotide excision repair (NER) capacity and the two subpathways of this fundamental mechanism, namely, global genome repair (GGR) and transcription-coupled repair (TCR), were assessed by measurements of DNA monoadducts along the transcribed N-ras gene [28]. Briefly, for the measurement of DNA monoadducts along the N-ras gene, freshly isolated PBMCs were treated with 100 μg/ml monohydroxymelphalan for 5 min at 37 °C in complete RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 2mM L-glutamine. Cells were subsequently incubated in drug-free medium for various times, harvested, and DNA monoadducts were measured along the Transcribed Strand (TS) and Non-Transcribed Strand (NTS) of the N-ras gene using Southern blot analysis. That is, genomic DNA was fully digested with *Eco*RI (NER capacity at the level of the whole N-ras gene) or *Bst*YI (NER capacity of different DNA fragments) and heated at 70 °C for 30 min for depurination of N-alkylated bases. Subsequently, the apurinic sites were converted to single-strand breaks by adding NaOH for 30 min at 37 °C, size fractionated using agarose gel electrophoresis and Southern blotted. Labeling of the oligonucleotides, hybridizations and calculation of the average frequency of monohydroxymelphalan-induced monoadducts in the restriction fragment of interest were performed as described previously [28]. The probe for detection of the entire gene was a 112-bp double-strand DNA fragment that was amplified using the following primer pair: forward, 5′-GTT-ATA-GATGGT-GAA-CC-TG-3′; reverse, 5′-ATA-CAC-AGA-GGAAGC-CTT-CG-3′. The probes employed for detecting the FN1, FN3 and FN6 fragments were single-stranded oligonucleotides (Supplementary Table 1). Particularly, fragment FN1 is upstream to the transcription start site of the gene, while FN3 and FN6 are located inside the gene. In all experiments an internal standard (part of the N-ras gene) was included.

2.7. Micrococcal nuclease (MN) digestion-based analysis of chromatin condensation

Isolation of nuclei from PBMCs, digestion with MN and analysis of chromatin condensation using Southern blot were performed as previously described [29]. Briefly, PBMCs were swollen in hypotonic buffer (10 mM Tris–HCl, pH 8.0, 10 mM NaCl, 5 mM $MgCl_2$) for 30 min on ice. After centrifugation (1400 *g*, 2 min) the cells were suspended in hypotonic buffer and homogenized in the presence of 0.3% Nonidet P-40. Nuclei were purified by centrifugation (1500 × *g*, 10 min) through hypotonic buffer containing 8.5% sucrose (w/v) and resuspended in 500 μl digestion buffer (100 mM Tris–HCl, pH 8.0, 50 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$). The isolated nuclei were immediately digested with micrococcal nuclease (1 U) for 1 min at 37°C and the digestion was stopped by adding an equal volume of stop solution (200 mM Tris–HCl, pH 8.0, 200 mM NaCl, 20 mM EDTA, 2% SDS, 200 μg/ml proteinase K). The control was an undigested, freshly lysed sample of nuclei. Genomic DNA was purified and then separated by electrophoresis in 1.5% agarose gels. After staining with ethidium bromide, the separated DNA was transferred to nitrocellulose and subsequently hybridized to the appropriate probe.

2.8. Statistical analysis

Continuous variables were compared among groups with Student's *t*-test, or Mann-Whitney *U* test when normal distribution did not apply, whereas paired comparisons were performed by paired t-test or Wilcoxon's test. Correlations were examined with Spearman's rank test. All statistical analyses were performed with SPSS v.24.0. Values are presented as mean ± SD. Results were considered significant when $p < .05$.

3. Results

3.1. Endogenous DNA damage accumulation in patients with active RA

The presence of endogenous DNA damage in PBMCs from patients with active RA was assessed using the alkaline comet assay measuring both SSBs and DSBs. As shown in Fig. 1A and B, the levels of endogenous DNA damage were almost 3-fold higher in patients compared to controls (mean ± SD 11.8 ± 7.3 versus 4.3 ± 2.2 olive tail moment (OTM) arbitrary units, respectively, $p < .001$) indicating that SSBs and/or DSBs accumulate in patients' cells in the absence of exogenous genotoxic insults. Although controls were not age–matched to patients, no evidence of any impact of age was found within those 65 healthy subjects (42 women/23 men), age range, mean ± SD (years): 19–95, 39.2 ± 14.9. To corroborate our results and examine for the presence of SSBs and DSBs separately, we also measured critical markers of the DDR/R network, including γH2AX (molecular marker of DSBs) [30], p-53BP1 (co-localizes with γH2AX at DSBs sites) [31] and p-RPA32 (indicator of SSBs) [32], using western-blot. We found induction of all three molecular markers in patients with active RA, suggesting the accumulation of both SSBs and DSBs in patients' cells (Fig. 1C-E).

3.2. Increased formation of DNA damage in patients with active RA

Accumulation of DNA damage in cells can be mediated either by augmented endogenous DNA damage formation and/or delayed/decreased efficiency of the DNA repair mechanisms, two possibilities that are not mutually exclusive. Therefore, we evaluated two critical endogenous factors/processes that lead to the intracellular formation of SSBs and DSBs, namely oxidative stress and AP sites, which are the most common type of lesions in DNA [33,34]. We found that PBMCs of 8 representative active RA patients exhibited significantly higher levels of oxidative stress [as indicated by the reduction of the glutathione (GSH) to oxidized glutathione (GSSG) ratio] (Fig. 2A) and increased number of AP sites compared with 10 healthy controls (Fig. 2B). Significant correlations were observed between individual endogenous DNA damage levels, as measured by alkaline comet assay, and oxidative stress ($r = -0.823$, $p < .001$) (Fig. 2C) or AP sites ($r = 0.859$, $p < .001$) (Fig. 2D), suggesting that the increased endogenous SSBs and DSBs may result, at least in part, from oxidative stress and/or the induction of the AP sites.

3.3. Intact transcription-coupled repair but deficient global genome repair capacity in patients with active RA

As for the likelihood of delayed/decreased efficiency of the DNA repair mechanisms, we evaluated the efficiency of the nucleotide excision repair, a highly versatile repair mechanism that can recognize and remove a wide variety of bulky, helix-distorting lesions from DNA [35]. Next, we examined the two sub-pathways of NER, i.e. TCR, which is responsible for the accelerated repair of lesions in the coding strand of active genes, and GGR that eliminates lesions from the non-transcribed DNA, including the non-coding strands of active genes [35]. For this purpose, we treated cells with the genotoxic agent monohydroxymelphalan, which induces exclusively NER-repaired damage [27] and the efficiency of nucleotide excision repair was measured at the active N-ras gene, the repair rate of which is representative of the total cellular nucleotide excision repair capacity [29].

Firstly, the efficiency of NER was measured at the level of the entire N-ras gene. We found that immediately after the end of the 5-min treatment with 100 μg/ml monohydroxymelphalan, DNA adduct levels were similar in PBMCs from active RA patients and healthy controls, indicating that the different repair kinetics observed between groups are rather due to the efficiency of DNA repair than the susceptibility of cells to damage formation (Supplementary Fig. 1A, B). Interestingly, we found significant differences in the efficiency of NER, with patients showing lower repair capacity (Supplementary Fig. 1A, B) and higher accumulation of DNA damage [expressed as the area under the curve (AUC) for DNA damage during the whole experiment] than healthy controls (Supplementary Fig. 1C), suggesting that PBMCs from active RA patients are characterized by defective NER.

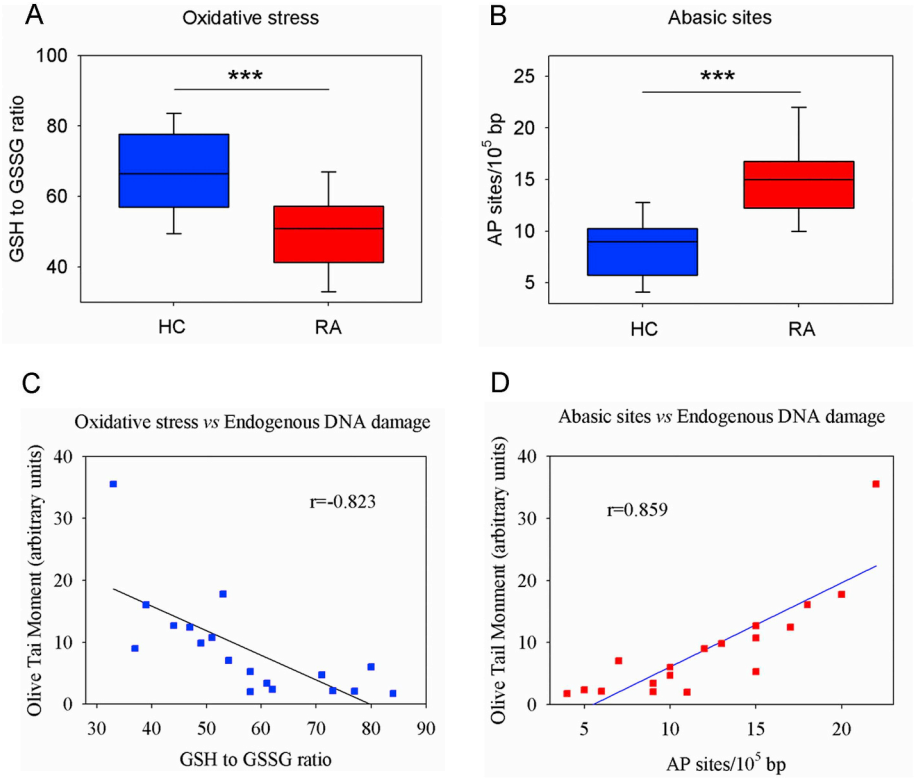

Fig. 2. Patients with RA show increased formation of DNA damage. Box plots showing statistical distribution of (A) the oxidative stress [expressed as the glutathione (GSH) to oxidized glutathione (GSSG) ratio] and (B) the abasic sites in PBMCs from controls and RA patients. The horizontal lines within the boxes represent the median value and the vertical lines extending above and below the box indicate maximum and minimum values, respectively. The correlation (C) between oxidative stress and the endogenous DNA damage (Spearman's $r = -0.823$) and (D) between abasic sites and the endogenous DNA damage (Spearman's $r = 0.859$) are shown. All error bars represent SD; ***$p < .001$ by Student's *t*-test.

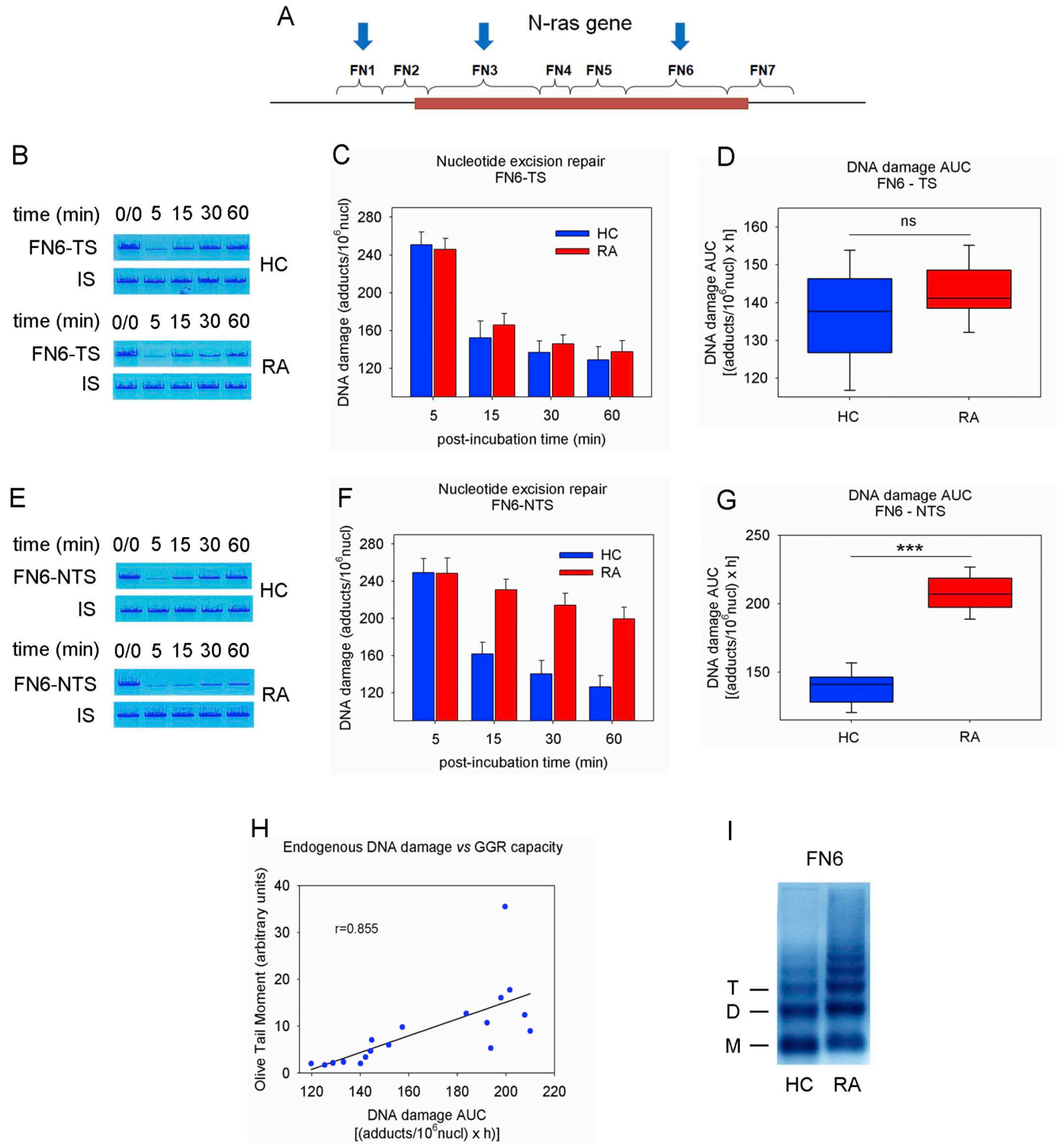

Fig. 3. The GGR subpathway of NER is defective in patients with RA – Focusing on the FN6 fragment. (A) PBMCs from controls and RA patients were treated ex vivo with the genotoxic agent monohydroxymelphalan that induces exclusively NER-repaired damage (monoadducts, i.e. monofunctional binding to a single site in the DNA molecule) and the efficiencies of the NER subpathways were measured at different DNA regions (FN1, FN3 and FN6). Representative autoradiograms for the Southern blot analysis at the transcribed strand (B) and at the non-transcribed strand (E) of the FN6 fragment (located inside the N-ras gene) from a representative control and a RA patient (P1). TS, transcribed strand; NTS, non-transcribed strand; IS, internal standard. The repair kinetics (mean ± SD) of monoadducts at the transcribed strand (C) and the non-transcribed strand (F) of the FN6 fragment is also shown. The accumulation of DNA damage (expressed as the AUC for DNA damage) at the transcribed (D) and non-transcribed strand (G) of the FN6 fragment. (H) Correlation between the endogenous DNA damage and the GGR capacity (expressed as the AUC for DNA damage at the non-transcribed strand of the FN6 fragment) from the same controls or RA patients (Spearman's r = 0.858). (I) Autoradiogram showing micrococcal nuclease sensitivity of the FN6 fragment in untreated PBMCs from a representative control and a RA patient. Symbols M, D, and T represent the positions of nucleosome monomer, dimer, and trimer, respectively. All error bars represent SD ***p < .001 by Mann-Whitney U test; ns, not significant.

To further investigate this important DNA repair mechanism, the efficiency of the two subpathways of NER was measured in a DNA region (FN6) located inside the N-ras gene (Fig. 3A). Focusing on the transcribed strand of the FN6 fragment, RA patients and healthy controls exhibited similar repair capacity (Fig. 3B, C) and very close accumulation of DNA damage (expressed as AUC) (Fig. 3D), indicating preserved TCR capacity in RA patients. On the other hand, the repair efficiency of the non-transcribed strand of the FN6 fragment differed, with active RA patients showing significantly lower adduct loss (Fig. 3E, F) and higher accumulation of DNA damage (expressed as AUC) than healthy controls (Fig. 3G; $p < .001$), indicating that these patients are characterized by defective GGR. Individual endogenous DNA damage levels inversely correlated with the GGR capacity, suggesting that the endogenous DNA damage observed in RA patients may be partly attributed to defects in the GGR subpathway of NER (Fig. 3H).

Similar results (preserved TCR and deficient GGR) were obtained in another DNA region (FN3 fragment) located inside the N-ras gene (Fig. 3A and Supplementary Fig. 2). In line with these results, in both strands of a silent DNA region (FN1 fragment) located outside the N-ras gene, RA patients showed reduced repair capacity and increased accumulation of DNA damage than healthy controls (Fig. 3A and Supplementary Fig. 3), again suggesting that RA patients show GGR-specific defects.

3.4. Condensed chromatin structure in active RA

Since the specific chromatin environment of a DNA lesion is considered an important epigenetic factor that may affect recognition of lesions and repair rates, and specifically GGR-mediated repair of DNA lesions [36,37] chromatin condensation was analyzed in the same DNA regions where the repair capacity was measured. In all respective DNA fragments, we found that PBMCs from active RA patients had more condensed chromatin structure than controls (FN6, Fig. 3I; FN3, Supplementary Fig. 2G; FN1, Supplementary Fig. 3G), suggesting that the reduced GGR capacity of these cells may result, at least in part, from changes in chromatin structure.

3.5. Decreased DNA damage accumulation and efficient global genome repair capacity following treatment of active RA

Endogenous DNA damage levels, DNA damage formation, TCR and GGR capacity and chromatin structure were re-examined 12 weeks after conventional synthetic disease-modifying antirheumatic drugs (DMARDs) ± corticosteroids and/or biological DMARDs treatment in 8 patients (Table 2). RA disease activity decreased in all 8 patients at 12 weeks compared to baseline (mean DAS28 from 5.0, range 3.7–6.1 to 3.6, range 1.8–4.8). We found indeed that treatment reduced the observed increase in endogenous DNA damage levels at baseline from 15.0 ± 9.2 (OTM, mean ± SD) to 5.9 ± 1.7, reaching control values ($p = .01$). Interestingly, those two patients who achieved disease remission (DAS28 of 2.6 and 1.8, respectively, Table 2) had the greatest reductions in OTM (from 16.1 to 6.1 and from 17.8 to 4.5, respectively). However, the small sample of patients does not allow for possible significant correlations between reductions in the levels of DNA damage and the magnitude of clinical response. Moreover, significant reductions were evident in oxidative stress [GSH/GSSG ratio (mean ± SD) baseline: 45.3 ± 8.6, after treatment: 64.1 ± 8.7; $p = .006$], and in the abasic sites, which decreased from 16.8 ± 3.2 to 8.8 ± 2.8 after treatment (AP sites, mean ± SD; $p < .001$) in all patients (Fig. 4A–C).

As for the NER capacity, focusing on the N-ras FN6 fragment, we found that only the repair efficiency of the non-transcribed strand (repaired by GGR) increased significantly after treatment (Fig. 4G, H), resulting in a significant decrease in the accumulation of the DNA damage in this strand (Fig. 4I). On the other hand, no changes were observed in the repair efficiency of the transcribed strand (repaired by TCR) (Fig. 4D, E) and in the accumulation of DNA damage in this strand (Fig. 4F). Moreover, treatment administration increased the looseness of the local chromatin structure in all RA patients examined, suggesting that the amelioration of the GGR defects correlates, at least in part, with changes in chromatin condensation (Fig. 4J).

Similar results were obtained in the FN3 fragment, which is also located inside the gene (Supplementary Fig. 4). As for the FN1 fragment (located outside the gene), treatment significantly increased the looseness of the local chromatin structure and the repair efficiency in both strands, while accumulation of DNA damage decreased in these DNA regions compared to baseline (Supplementary Fig. 5).

4. Discussion

Herein, we report that levels of endogenous DNA damage in peripheral blood mononuclear cells from patients with active RA are highly increased, as previously reported [20]. These findings are not RA-specific since patients with other systemic connective tissue diseases such as SLE and Systemic Sclerosis are also characterized by several-fold higher endogenous DSBs of DNA [27,38,39] which are associated with abnormalities of DNA repair machinery [[39], and our unpublished data]. Since RA disease duration ranged from one to many years and some of our patients had already been treated with DMARDs, it is unclear whether this result is the cause of disease onset or a result of long standing inflammatory arthritis. Such accumulation of endogenous DNA damage associated with deregulated DDR/R network, could arise from metabolic by-products, mostly ROS, prolonged stalling in DNA replication fork progression as this occurs during replication stress, V(D)J recombination and class switch recombination, chromosome mis-segregation events, telomere shortening, and chromothripsis, a phenomenon associated with the formation of micronuclei [40–44].

To understand the origin of DNA damage accumulation in active RA, we evaluated oxidative stress and levels of abasic sites, which are critical endogenous factors that lead to the intracellular formation of SSBs and DSBs [33,34]. We found significant correlation between endogenous DNA damage and oxidative stress or abasic sites in the same patients, suggesting that endogenous DNA damage accumulation may at least partly result from these factors/processes. These results are in line with previous studies showing that ROS produce multiple types of oxidative DNA damage in aerobic organisms, including oxidized DNA bases, oxidized sugar fragments, abasic sites and SSBs, which in turn triggers the induction of DSBs [45]. Increased production of ROS in association with an inflammatory response is a common feature of autoimmune diseases [46,47]. Indeed, there is mounting evidence that oxidative stress is a dynamic and complex phenomenon occurring in RA, as evidenced by increased intracellular ROS production, increased lipid peroxidation, protein oxidation, DNA damage and impaired enzymatic and non-enzymatic antioxidant defense system of the body [20,21,48–51]. On the other hand, DNA damage, as a primary insult, is also able to trigger increased ROS, which may in turn further increase oxidative damage [52] inducing a vicious circle and thus increasing DNA damage burden.

We further examined DNA repair mechanisms, such as the two subpathways of NER (TCR, GGR), as well as epigenetic factors, such as chromatin structure, that may affect DNA repair efficiency. NER is a fundamental mechanism repairing many different kinds of DNA adducts, including pyrimidine dimers, such as cyclobutane pyrimidine dimers and 6–4 photoproducts, which are produced by the UV component of sunlight, base lesions produced by reactive oxygen species or endogenous lipid peroxidation products, intrastrand cross-links and adducts formed by benzo[*a*]pyrene, various aromatic amines and cancer chemotherapeutic drugs such as cisplatin and melphalan [35]. Of note, NER efficiency is critical for cell survival [27] and has been associated with drug-response to chemotherapeutic agents in hematologic disorders [53]. While TCR acts only on lesions located at transcription sites blocking elongating RNA-polymerase complexes, GGR acts genome-wide [35]. Our results suggest that active RA patients are

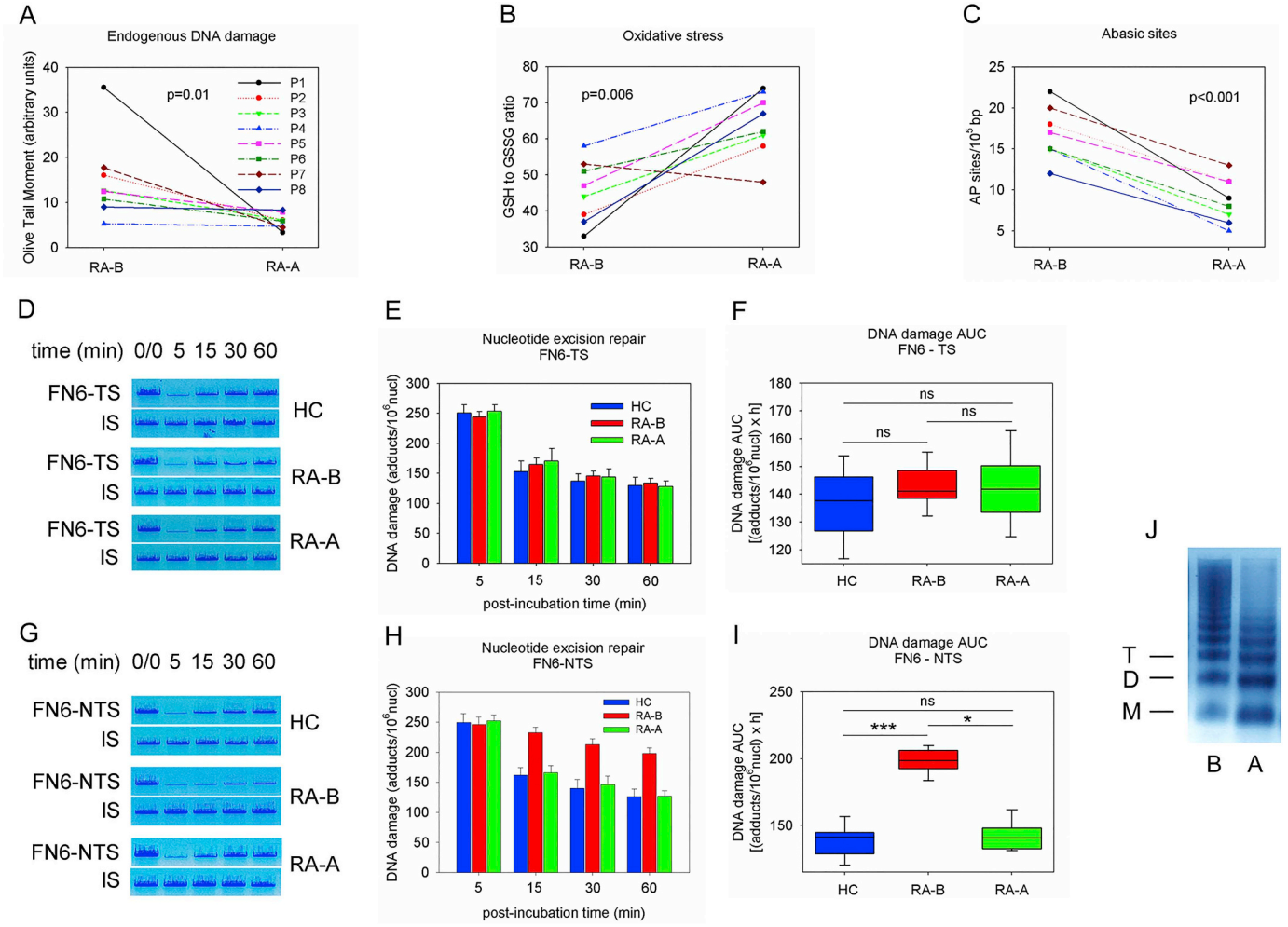

Fig. 4. The effect of antirheumatic treatment – Focusing on the FN6 fragment. Eight patients with RA were followed-up for 12 weeks after therapeutic treatment with conventional synthetic DMARDs and/or biological DMARDs. The individual endogenous DNA damage (A), the oxidative stress [expressed as the glutathione (GSH) to oxidized glutathione (GSSG) ratio] (B), and the AP sites(C) in PBMCs from RA patients at baseline (RA-B) and after (RA-A) therapy are shown. Representative autoradiograms for the Southern blot analysis at the transcribed strand (D) and at the non-transcribed strand (G) of the FN6 fragment (located inside the N-ras gene) from a representative control and a RA patient (P1) at baseline and after therapy. TS, transcribed strand; NTS, non-transcribed strand; IS, internal standard. The repair kinetics of monoadducts (mean ± SD) at the transcribed strand (E) and the non-transcribed strand (H) of the FN6 fragment at baseline and after treatment are presented. The accumulation of DNA damage (expressed as the AUC for DNA damage) at the transcribed strand (F) and the non-transcribed strand (I) of the FN6 fragment at baseline and after treatment are shown. The horizontal lines within the boxes represent the median value and the vertical lines extending above and below the box indicate maximum and minimum values, respectively. (J) Autoradiograms showing micrococcal nuclease sensitivity of the FN6 fragment in PBMCs from a representative RA patients at baseline (B) and after (A) therapy are presented. Symbols M, D, and T represent the positions of nucleosome monomer, dimer, and trimer, respectively. All error bars represent SD. *p < .05, ***p < .001 by Mann-Whitney U test; ns, not significant.

characterized by deficient GGR, while TCR is intact. More importantly, a significant inverse correlation was found between individual endogenous DNA damage load and GGR efficiency in the same subjects, suggesting that the increased endogenous DNA damage may be partly attributed to defects in the GGR subpathway of NER and the resulting accumulation of DNA damage. It is generally accepted that while TCR is dependent on transcription, GGR is greatly affected by chromatin structure, with more open chromatin structure being more accessible to GGR-related molecular components [36,37]. Therefore, the finding that all active RA patients exhibited more condensed chromatin structure than controls, suggests that the lower efficiency of GGR in these patients may at least partly result from their defective chromatin organization.

Finally, we provide evidence that, in parallel with treatment-induced reductions in RA disease activity, accumulation of DNA damage always decreased in the same patients. Shao et al. have also shown that RA patients under treatment have significantly lower endogenous DNA damage levels than untreated patients [21]. Decreased DNA endogenous damage in our treated patients was directly associated with a restored GGR capacity and a more open chromatin structure. Although no previous study has searched for reversible chromatin defects in RA, ex vivo anti-TNF treatment of T-cells from RA patients alters histone acetylation, thus, potentially affecting chromatin organization [54]. Accordingly, treatment of RA-fibroblast-like synoviocytes or PBMCs derived from RA patients by histone deacetylase inhibitors ex vivo led to significant downregulation of IL-6 production [55–57], while such drugs ameliorate collagen-induced arthritis [58]. More importantly, Givinostat, an oral histone deacetylase inhibitor was beneficial in children with systemic-onset juvenile idiopathic arthritis [59]. Taken together, these data may suggest an interaction between chromatin structure, DDR/R network, and disease activity in RA. Further studies are obviously needed to address how this aberrancy is induced and how might also contribute to other autoimmune diseases, as well as the exact specificity and cell types affected.

5. Conclusions

Our study demonstrates accumulation of endogenous DNA damage in patients with active RA, which is related with augmented formation of DNA damage, reduced DNA repair efficiency and changes in chromatin organization, all of which are reversible after treatment. Since the accumulation of endogenously generated DNA damage in patients with RA has a negative impact on the cellular health, including mutations and genomic instability, these results can be exploited for understanding pathogenesis and progression of this disease, as well as for new opportunities for treatment.

Conflict-of-interest disclosure

None of the authors has any potential financial conflict of interest related to this manuscript.

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Authors' contributions

VLS and PPS directed and coordinated the project, performed data acquisition and data analysis and drafted the manuscript. NIV and MP were responsible for the selection and clinical evaluation of patients, performed the experiments, analyzed the data, and wrote the manuscript. AA performed the experiments and reviewed the manuscript. All authors read and approved te final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.03.009.

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