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The NAD$^+$ precursor nicotinic acid improves genomic integrity in human peripheral blood mononuclear cells after X-irradiation

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A B S T R A C T

NAD$^+$ is an essential cofactor for enzymes catalyzing redox-reactions as well as an electron carrier in energy metabolism. Aside from this, NAD$^+$ consuming enzymes like poly(ADP-ribose) polymerases and sirtuins are important regulators involved in chromatin-restructuring processes during repair and epigenetics/transcriptional adaption. In order to replenish cellular NAD$^+$ levels after cleavage, synthesis starts from precursors such as nicotinamide, nicotinamide riboside or nicotinic acid to match the need for this essential molecule. In the present study, we investigated the impact of supplementation with nicotinic acid on resting and proliferating human mononuclear blood cells with a focus on DNA damage and repair processes.

We observed that nicotinic acid supplementation increased NAD$^+$ levels as well as DNA repair efficiency and enhanced genomic stability evaluated by micronucleus test after x-ray treatment. Interestingly, resting cells displayed lower basal levels of DNA breaks compared to proliferating cells, but break-induction rates were identical. Despite similar levels of p53 protein upregulation after irradiation, higher NAD$^+$ concentrations led to reduced acetylation of this protein, suggesting enhanced SIRT1 activity. Our data reveal that even in normal primary human cells cellular NAD$^+$ levels may be limiting under conditions of genotoxic stress and that boosting the NAD$^+$ system with nicotinic acid can improve genomic stability.

Keywords:
Human peripheral blood mononuclear cells (PBMC)
NAD$^+$
DNA repair
Genomic stability
Micronucleus formation
Nicotinic acid

1. Introduction

1.1. NAD$^+$ supplementation

Several studies have reported that administration of NAD$^+$ precursors such as nicotinamide, nicotinic acid (niacin, NA) or nicotinamide riboside can increase the intracellular NAD$^+$ concentration in various tissues or cellular compartments *in vitro* and *in vivo* [1–3]. In the past, nicotinic acid has been in clinical use, at millimolar concentrations in the tissue, as a cholesterol lowering drug displaying atheroprotective effects [4,5]. However, this treatment was accompanied by unwanted side effects (reviewed in [5]), most prominently prostaglandin-dependent vasodilation in the skin (flushing) [6] and hepatotoxicity [7] (for review, see [8]), with the latter becoming negligible by applying niacin in a different formulation [9] (reviewed in [10]). Nevertheless, high niacin dosing can result in discomforting flushes [11], but niacin is still in use [12,13] and also marketed as a food supplement. Several studies showed protective effects of high NAD$^+$ levels in model systems as well as in humans under certain pathological conditions or in the context of the aging process, possibly due to preserving energy production in mitochondria [14–20]. Apart from the important function of intracellular NAD$^+$ in mitochondrial respiration or as coenzyme in a vast range of redox-reactions, NAD$^+$ also participates in DNA repair and maintenance of genomic stability. J.B. Kirkland's group demonstrated the relevance of niacin status on genomic integrity, DNA repair and protection from carcinogenesis using animal models focusing on niacin deficiency [21–23]. The importance of NAD$^+$ in this context was highlighted in reports showing that NA deficiency results in increased chromosomal instability [24] and higher cancer incidence [25]. Likewise, high concentrations of nicotinamide or NA delayed carcinogenesis [26], improved repair capacity after γ-irradiation above 40 Gy in mouse melanoma cells

Abbreviations: IR, X-ray irradiation; MN, micronuclei; NA, nicotinic acid; PARP, poly(ADP-ribose) polymerase; PAR, poly(ADP-ribose); PBMC, human peripheral blood mononuclear cells; PHA-L, leucoagglutinin.

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[27] and facilitated recovery of neuronal functions after hypoxia [28]. There is also increasing evidence that micronutrients have an important impact on the maintenance of gross genomic stability. In a study by Fenech et al. it was shown that low intake of nicotinic acid was associated with an increased micronucleus frequency [29], a widely accepted measure of genomic stability [30].

1.2. Consumers of NAD^+: poly(ADP-ribose) polymerases and sirtuins

Immediately after a genotoxic insult cells activate various responses that contribute to cell survival or death. One of the first reactions is poly(ADP-ribosyl)ation (PARylation) of proteins mediated by nuclear poly(ADP-ribose) polymerases (PARPs) [31,32]. The most active enzyme in this regard is PARP1 (and to a lesser extent PARP2 [33]), which covalently attaches units of ADP-ribose in a stepwise fashion to target proteins including itself, synthesizing a negatively charged polymer by using NAD^+ as substrate. Depending on the level of DNA damage and intracellular NAD^+ status, PARP1 and its product poly(ADP-ribose) (PAR) mediate the recruitment of DNA repair factors to sites of lesions, facilitate DNA repair and help maintain genomic integrity under conditions of moderate stress [34,35]. In this scenario a tolerable proportion of total cellular NAD^+ is used for polymer synthesis. In contrast, drastic and irreversible NAD^+ depletion [36] as a result of hyperactivation of PARP1 under severe genotoxic stress conditions can lead to cell death [37,38]. This paradigm is also apparent in inflammatory diseases and neurodegenerative disorders [39,40] (reviewed in [41]). One parameter determining the cellular response to stress is the level of available NAD^+, which is crucial not only for PAR synthesis [42], but also for the enzymatic action of sirtuins (SIRTs) [43,44]. We could show that supplementation of human peripheral blood mononuclear cells (PBMC) with NA not only raises NAD^+ levels, but enhances PAR formation after genotoxic stress and protects from damaged-induced necrotic cell death [45]. Conversely, NA deficiency results in impaired PARP1 functions in rats [46,47].

Sirtuins are homologues of the yeast enzyme Sir2 [43,48] and have important functions in regulating cellular responses to particular types of signals and stressors [49,50]. They use NAD^+ in order to de-acetylate proteins, forming 2′O-acetyl-ADP-ribose as product [51]. Both PARPs and SIRTs have been implicated in genome stabilization [49,52–55] and crosstalk between members of the two enzyme families has been published [56–58], as was proposed already ten years ago [55]. For example, abrogation of cellular PAR formation by *Parp1* gene deletion, silencing or PARP inhibitors is known to sensitize cells to many genotoxic agents and to increase genomic instability [34]. In contrast, PARP1 overexpression leads to the suppression of DNA damage-induced genomic instability [59]. It was demonstrated that SIRT1 and PARP1 share some important tasks, *i.e.* both are regulating chromatin structure [52,60–63] and repair [64,65], and both dampen the activity of the transcription factor and stress response protein p53, SIRT1 by de-acetylation [66] and PARP1 by covalent and non-covalent modification by PARylation [67–69], although the impact of this modification is less well understood. Interestingly, it has been reported that also SIRT6 and PARP1 cooperate in DNA repair [70].

1.3. Mononuclear blood cells as a model system

Human PBMC are primary cells proficient in DNA damage response cascades and repair pathways and therefore an ideal model for DNA repair studies and most relevant to the understanding of biochemical and molecular mechanisms in human physiology [71–73]. Furthermore, they are widely used in epidemiological studies to investigate the correlation of various parameters including DNA repair capacity and cancer risk [74]. As DNA repair might vary throughout the cell cycle, analyzing resting and proliferating PBMC might yield different results. But quiescent PBMC in G_0 phase can be easily stimulated for proliferation by treatment with phytohemagglutinin (PHA-L) [75]. In this way, cells from the same donor can be monitored for their response to diverse treatments in the resting and the proliferating state, thus excluding inter-individual variations. PBMC have been reported to utilize supplemented NAD^+ precursors [76,77] and express nicotinate phosphoribosyltransferase as well as nicotinamide phosphoribosyltransferase, making this cell type a suitable model for our approach.

1.4. Aim

In the present work, we wanted to study whether increased cellular levels of NAD^+ can influence cellular responses after genotoxic treatment in PBMC from healthy, non-niacin-deficient subjects, as there is a lack of human data addressing this issue. We focused on the biological consequences of elevated NAD^+ levels in human PBMC regarding PARP1/SIRT activities or downstream effects related with genomic integrity. To assess how modulated NAD^+ levels may contribute to physiological or pathophysiological outcomes we set out to investigate various end points including (i) DNA damage, (ii) DNA repair, (iii) the influence on genomic stability and (iv) sirtuin-1 (SIRT1) activity after treatment with ionizing radiation (IR) using varying doses. It was of particular interest to find out whether supplementation of nicotinic acid at pharmacologically relevant concentrations is able to improve the cellular status in the context of DNA damage in normal human cells from healthy donors.

2. Materials and methods

2.1. Chemicals and reagents

Biocoll separating solution and fetal calf serum (FCS) were purchased from Biochrom (Berlin, Germany). Antibiotics and RPMI 1640 culture medium were from Invitrogen (Darmstadt, Germany), standard chemicals from Roth (Karlsruhe, Germany), Sigma-Aldrich (Munich, Germany) or Merck (Darmstadt, Germany) if not stated otherwise.

2.2. Isolation of peripheral blood mononuclear cells [45]

Blood sampling was carried out in accordance with the Declaration of Helsinki and with approval of the University of Konstanz Ethics Committee, from healthy donors aged 24–45 years giving informed consent. Venous blood was obtained using the S-Citrate-Monovette blood collection system from Sarstedt (Nümbrecht, Germany). Cells were separated *via* Biocoll gradient centrifugation. Briefly, the freshly drawn blood was mixed with an equal volume of PBS (137 mM sodium chloride; 10 mM disodium hydrogen phosphate; 3 mM potassium dihydrogen phosphate; pH 7.4) and layered on 15 ml of Biocoll separating solution, followed by centrifugation at 800 × *g* for 15 min at room temperature. The PBMC layer was collected and washed twice with PBS. Isolated cells were incubated in standard culture medium (RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C with 5% CO_2 in a humidified atmosphere. NA was added to the culture medium at a final concentration of 15 μM and cells were incubated 5 h before DNA damage induction.

2.3. Mitogen stimulation of cells

To investigate NAD^+ levels and DNA strand break repair at different cell cycle stages we challenged cells with X-irradiation, either

in G_0 phase or 44 h after mitogenic stimulation with leucoagglutinin (PHA-L; Roche, Mannheim, Germany) [78]. 5 μg/ml PHA-L was added to cell cultures in all assays as recommended by the manufacturer, except for micronucleus induction tests (20 μg/ml) to ensure maximum number of binucleated cells after cytochalasin block according to a well-established protocol by Fenech et al. [79].

2.4. Flow cytometric analysis of cell cycle

Methanol fixed cells (10^6/ml) were washed in PBS and incubated with 100 μg/ml RNase A for 1 h at room temperature. 10 μg/ml Propidium iodide was added. Samples were analyzed with LSR II (Beckton Dickinson, Heidelberg, Germany) and cell cycle distribution was quantified using FlowJo Software (Tree Star, Ashland, USA).

2.5. Detection of intracellular NAD^+ levels

Cellular NAD^+ concentration of proliferating cells was determined by an enzymatic cycling assay adapted from Jacobson et al. [80]. 5×10^5–1×10^6 cells per sample were irradiated with doses as indicated (2.5/5/10/25 Gy) in 500 μl PBS, incubated for 10 min at 37 °C to allow for PARP1 activity, and precipitated with ice-cold perchloric acid (0.5 M). After centrifugation at $1500 \times g$ for 10 min, the NAD^+ containing supernatant was processed for cycling assay. Briefly, supernatant was mixed with 350 μl of 0.33 M K_2HP0_4 (pH 7.5), centrifuged, and frozen at −20 °C. After thawing and centrifugation, 40 μl of each sample was transferred to a 96 well plate, mixed with 160 μl of buffer A (0.25 M H_3P0_4, 0.5 M NaOH) and 100 μl of buffer B (0.34 M bicine-NaOH pH 8.0, 2.9 mg/ml BSA, 14.3 mM EDTA, 1.4 mM MTT, 1.7 M EtOH, 5.7 mM phenazine ethosulfate, 0.14 mg/ml alcohol dehydrogenase) and incubated at 30 °C for 30 min. Absorption was measured at 550 nm (with 690 nm as a reference wavelength) in a 96-well-plate ELISA reader. Intracellular NAD^+ concentration was calculated based on defined NAD^+ standards analyzed in parallel.

2.6. Automated fluorimetric alkaline DNA unwinding (FADU) assay

For assessment of DNA strand breakage and repair, cell number was adjusted to 4×10^6 cells/ml suspended in culture medium. Aliquots of 100 μl were irradiated on ice (dose rate 8 Gy/min; energy 70 keV; 1.25 mm aluminum filter) using an X-ray source (RT 100; Müller, Hamburg, Germany). Subsequently, samples were incubated for DNA repair at 37 °C for the time periods indicated and then kept on ice until analysis by FADU. A modified and automated version of the FADU method was used for the assessment of induced DNA damage and repair as previously described [81]. Briefly, 900 μl of suspension buffer (0.25 M *meso*-inositol; 10 mM sodium phosphate, pH 7.4; 1 mM magnesium chloride) was mixed with each cell sample and 70 μl per well was transferred in triplicates onto a 96-well plate. The subsequent automated steps included cell lysis, alkaline DNA unwinding and neutralization of the samples, followed by addition of fluorescent dye SybrGreen (Invitrogen) to monitor the amount of DNA remaining double-stranded. T values represent SybrGreen fluorescence in samples without alkaline unwinding (total amount of double stranded DNA). P_0 values reflect the basal level of breaks in genomic DNA after alkaline unwinding in untreated controls. P_x values represent DNA strand breaks after irradiation of cells and indicated time of repair, expressed as% of P_0. Data are plotted on a logarithmic scale.

2.7. Western blot analysis of p53 and ac-p53 status

Cells were supplemented with NA for 5 h and with 1 μM trichostatin A (Enzo Life Science, Lörrach, Germany) for 1 h before irradiation. Trichostatin A inhibits class I/II histone deacetyases (HDACs), but not sirtuins (class III HDACs), thus ensuring the exclusive detection of SIRT related deacylase activity on p53. At distinct time points after DNA damage, cells were collected, washed in PBS and cell number was adjusted to 5×10^5 cells per sample. For cell lysis an appropriate volume of preheated 5× SDS sample buffer (100 mM Tris-HCl pH 8.0, 25% 2-mercaptoethanol, 5% glycerol, 12.5% SDS, 0.01% bromophenol blue) was added to the cell suspension and incubated for 5 min at 95 °C. Extracts were sonicated with 3–4 bursts of 15 s each. Proteins were separated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with the anti-p53 antibody (Calbiochem, Merck, Darmstadt, Germany) or anti-acp53 (Epitomics, Biomol, Hamburg, Germany). An ImageQuant LAS 4000 system was used for quantitative imaging of blots by Amersham ECL chemiluminescence (both from GE Healthcare, Munich, Germany).

2.8. In vitro cytokinesis-blocked micronucleus test

The induction of micronuclei was performed according to a protocol from Fenech with slight modifications [79]. Aliquots of 7.5×10^5 cells in 750 μl were irradiated in G_0 phase and then stimulated with 20 μg/ml PHA-L. X-irradiation of proliferating cells was 44 h after PHA-L stimulation. Cytochalasin B was added at a final concentration of 6 μg/ml at 44 h post-irradiation (Fig. 1A) or 48 h (1B). 72 h later, cells were transferred to glass slides by cytospin at $500 \times g$ for 3 min. After methanol fixation (10 min) cells were stained with Giemsa solution (Merck) diluted in Sörensen buffer (pH 6.8) (3:1) for 5 min. After drying, the cells were embedded with Mowiol. Micronuclei were scored in 500–1000 binucleated cells (BN) per sample. Micronucleus frequencies were calculated by dividing the total number of MN by the total number of BN cells scored.

2.9. Data presentation and statistical analysis

Data represent the mean ±SEM of the number of donors indicated, with each donor contributing one data point per condition. Every such data point is the mean of several replicates for each donor within one experiment. For statistical analysis, data were compared by two-way analysis of variances (ANOVA) and Bonferroni post-test using GraphPad Prism 7 (La Jolla, USA), if not stated otherwise. Where applicable, samples were analyzed by paired test to normalize for inter-individual variations. P-values <0.05 were considered significant (*), other P-values were labeled accordingly ** $P < 0.01$, *** $P < 0.001$.

3. Results and discussion

We have previously shown that NA supplementation reduces cell death 24 h after genotoxic stress in freshly isolated human PBMC and also causes a shift from necrosis to less harmful apoptosis, which was similar to PARP1 inhibitor treatment and correlated with the level of NAD^+ preservation [45]. In order to get deeper insight into the protective effect of NA supplementation, we determined its impact on DNA repair and genomic stability, *i.e.* micronucleus formation, after treatment with ionizing radiation, and we analyzed the impact of proliferation vs. quiescence on the respective parameters. For clarity, the general treatment schedule including the time points of NA supplementation, PHA-L stimulation to induce proliferation and irradiation is summarized in Fig. 1.

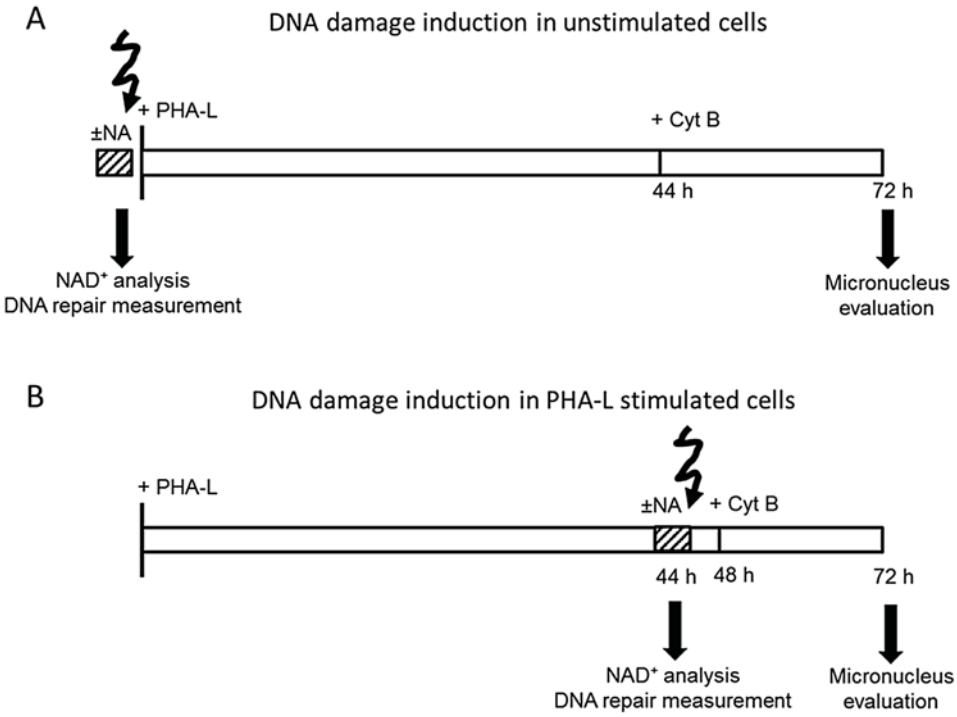

Fig. 1. Experimental treatment schedule.
Human PBMC were either exposed to ionizing radiation as unstimulated cells (A) or (B) 44 h after PHA-L addition and analyzed or cultured for the assays indicated. Cells were supplemented 5 h before irradiation with 15 μM nicotinic acid (NA) as indicated. For further details see Materials and Methods.

3.1. Cell cycle distribution and NAD⁺ content of unstimulated and PHA-L-stimulated PBMC

We used flow cytometry to monitor cell cycle distribution of PBMC. As depicted in Fig. 2, freshly isolated PBMC display a distinct peak representing the DNA content of cells in G_0/G_1 phase, as expected. After 44 h of stimulation with PHA-L, cells are found in all cell cycle phases, with 65–75% in G_0/G_1 and 20–30% in $S/G_2/M$, independent of supplementation. Thus, NA did not influence the cell cycle profile of PBMC during the time period of examination.

We tested PHA-L-stimulated samples for their physiological NAD^+ concentration (Table 1). The mean NAD^+ level in proliferating cells was approximately 280 μM, which could be raised by 15 μM NA supplementation to up to 390 μM, *i.e.* a nearly 1.4-fold increase within 5 h incubation ($p = 2.1 \times 10^{-5}$). The basal cellular NAD^+ status in unstimulated PBMC was increased 2-fold on average, as we have previously reported [45], reflecting a higher gain than in PHA-stimulated cells. This could be due to the prolonged time of maintaining the PBMCs in standard culture medium, which contains itself the NAD^+ precursor niacin, and may also be responsible for the generally higher NAD^+ concentration in stimulated PBMC compared to unstimulated PBMC [45].

3.2. Baseline DNA damage in unstimulated versus PHA-L-stimulated PBMC and the effect of NA supplementation

To estimate whether stimulation with PHA-L or NA supplementation *per se* influences baseline damage of the genome we analyzed our experimental FADU data by comparing the P_0 values (double-stranded DNA with physiological breaks) from PBMC of 6 different donors (Fig. 3).

In unstimulated cells (G_0 phase) the P_0 levels corresponded to approximately 85% of total double-stranded DNA. Proliferating cells exhibited higher amounts of endogenous strand breaks, with the mean P_0 values reaching only 67% of the respective T values. This is in line with other studies showing that the basal levels of strand breaks vary at different stages of the cell cycle, with highest levels observed during S phase [82] due to replication, which was also reported by Mayer and colleagues using the comet assay [83]. Our data reveal that NA supplementation changes the respective baseline P_0 values neither in unstimulated nor in stimulated PBMC. Therefore, whereas proliferation induced higher levels of physiological DNA-strand breaks, NA supplementation had no effect.

3.3. Effect of NA supplementation and proliferation on DNA damage induction and repair

To test if elevated NAD^+ levels can contribute to altered DNA repair in human PBMC of healthy subjects we damaged cells with different doses of IR after 5 h of NA pre-incubation and monitored DNA damage and DNA repair characteristics.

3.3.1. DNA damage induction is not affected by cell cycle

As expected, IR caused an identical dose-dependent induction of DNA strand breakage in both stimulated and unstimulated cells (Fig. 4), despite the fact that proliferating cells had a higher baseline level of strand breaks (Fig. 3). Thus, proliferation did not influence the ability of X-rays to inflict DNA breaks, leading to the same damage ratio.

3.3.2. Niacin supplementation maintains NAD⁺ concentration at high levels after IR

Of note, NAD^+ levels are strongly affected by massive DNA damage and subsequent PARP1 hyperactivation, as we have shown in freshly isolated PBMC [45], but NAD^+ levels and PARP1 activity both vary during the cell cycle with NAD^+ levels being lowest during S/G2 phase, possibly due to PARP1 activated by naturally occurring strand breaks during replication [84]. To test the effects of IR on NAD^+ levels in proliferating PBMC, we analyzed the intracellular NAD^+ concentration in PHA-L stimulated cells after

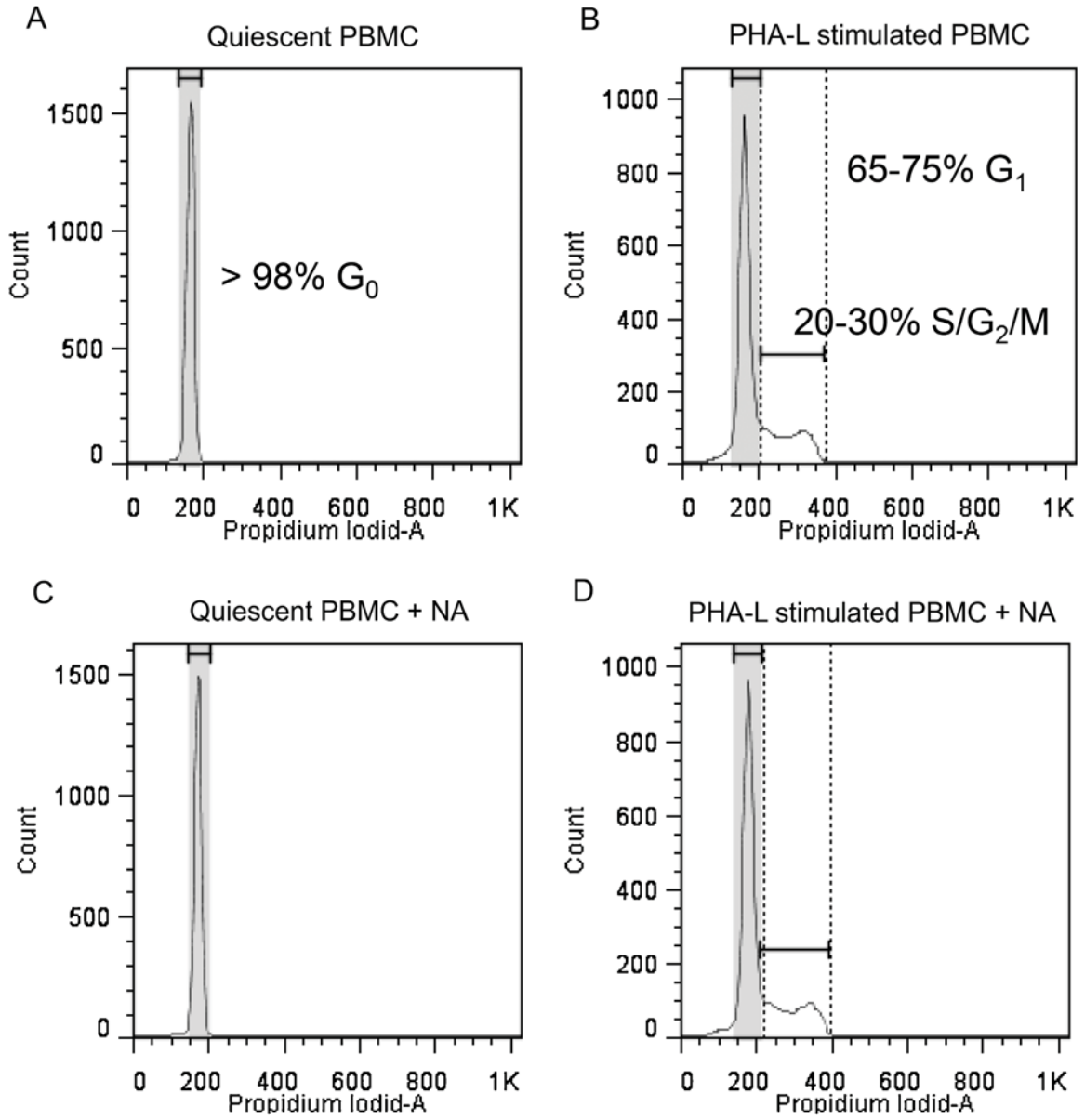

Fig. 2. Cell cycle distribution of PBMC.
Flow cytometric analysis of cell cycle distribution without (A/C) and after PHA-L (B/D) stimulation. Unstimulated PBMC rested in G_1/G_0 and PHA-L treated PBMC grew asynchronously 44 h after stimulation. NA supplementation has no influence on cell cycle distribution. DNA content is plotted against the cell count; histograms are representative of a single donor. Inserted percentages indicate mean values from three donors in independent experiments.

Table 1
Intracellular NAD^+ levels in [μM] of PBMC 44 h after PHA-L stimulation.

Donor #	NAD^+ concentration (without NA)	NAD^+ concentration + 15 μM NA	Fold increase
1	241.2	342.7	1.42
2	275.8	410.7	1.49
3	189.0	299.0	1.58
4	162.2	211.6	1.30
5	274.4	356.4	1.30
6	329.0	473.3	1.44
7	378.5	496.6	1.31
8	404.13	512.5	1.29
Mean ± SEM	281.8 ± 30.2	387.8 ± 37.1[a]	1.39 ± 0.039

[a] $P = 2.1 \times 10^5$ by paired *T*-test.

increasing doses of X-rays. Similar to our results in resting PBMC, the immediate and rapid cellular consumption of NAD^+ was dose-dependent and most pronounced after 25 Gy irradiation (Fig. 5), with loss of 53% of total NAD^+ in non-supplemented and 40% in NA-supplemented cells within the first 10 min after damage induction [45]. Independent of the irradiation dose applied, the supplemented cells always displayed significantly higher NAD^+ levels compared to non-supplemented PBMC. Thus, NA-supplementation

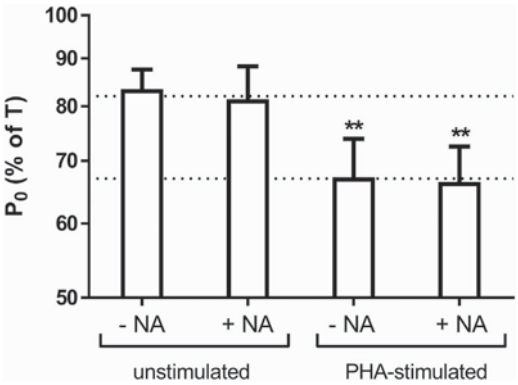

Fig. 3. Baseline levels of DNA damage in unstimulated and proliferating PBMC Endogenous levels of strand breaks in undamaged cells are expressed as percentage of fluorescent signal in control cells (P_0) in relation to the total amount of DNA (T). Each bar represents six independent experiments using PBMC *ex vivo* supplemented with nicotinic acid (+NA) or not (-NA). Statistical analysis by two-way ANOVA with Sidaks Multiple Comparison test.

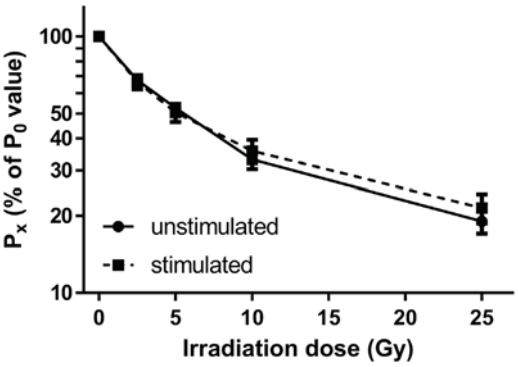

Fig. 4. Dose-dependent DNA damage induction by IR is independent of stimulation. DNA strand break induction (% of P_0) of unstimulated and PHA-L stimulated PBMC as a function of radiation dose. The data correspond to those shown in Figs. 7 and 8 and represent the DNA strand breakage induced by ionizing radiation as analyzed by the automated FADU assay.

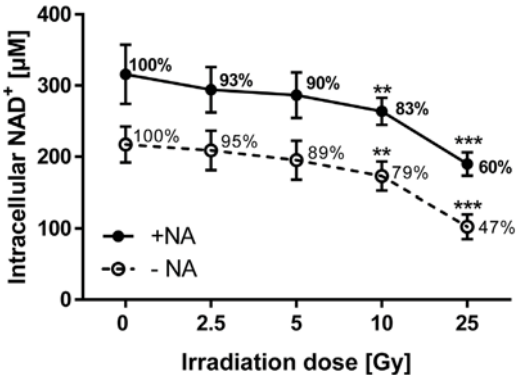

Fig. 5. Intracellular NAD^+ levels after IR with and without NA supplementation. Cells supplemented with NA (+NA) or not (-NA) were irradiated as indicated and incubated at 37 °C for 10 min to allow for poly(ADP-ribosyl)ation before NAD^+ concentration analysis. Data represent independent experiments using four different donors. Numbers indicate the remaining NAD^+ as percentage compared to non-irradiated controls. Asterisks indicate significant differences to controls.

was able to preserve intracellular NAD^+ at high concentrations also in proliferating cells, with absolute numbers at 25 Gy matching those of non-supplemented PBMC at 5 Gy. Supplementation with NAD^+ precursors rescued decreasing NAD^+ levels also in other settings like enhanced basic DNA damage in mice with mutated CSB (Cockayne Syndrome group B) protein [85] and human cells with mutated XPA (Xeroderma pigmentosum complementation group A) protein [86], and in human myocytes from patients with heart failure [87]. Likewise, inhibition of PARP1 during DNA damage in resting human PBMC [45] or fibroblasts [88] also preserved NAD^+ levels. We conclude that intracellular NAD^+ concentration can be maintained at higher levels by low-dose supplementation of NA even after massive DNA damage compared to non-supplemented PBMC.

3.3.3. NA supplementation diminishes acetylation of induced p53 after IR

As NAD^+ serves as substrate for both PARPs and sirtuins, we analyzed deacetylation activity of SIRT1 on its classical target p53 [66]. Upon cellular stressors such as DNA damage, p53 is stabilized and acts as transcriptional regulator [89]. Deacetylation of p53 by SIRT1 reduces its activity and renders cells less susceptible to apoptotic stimuli [90]. As displayed in Fig. 6, NA supplementation of PBMC did not influence p53 accumulation after low-dose irradiation, but reduced its acetylation. A similar finding on p53 has been reported for AROS, a stimulator of SIRT1 activity [91]. Thus maintained NAD^+ levels in our supplementation experiments also induced higher SIRT1 activity, at least as deduced from reduced levels of acetylated p53, a classical target of SIRT1. This observation has also been reported for application of PARP1 inhibitors, which abolish the consumption of NAD^+ by PARP1. Thus, maintained NAD^+ levels in our supplementation experiments probably also induce higher SIRT1 activity, a fact that has been reported for application of PARP1 inhibitors, which abolish the consumption of NAD^+ by PARP1 [55,58].

Although total cellular level of NAD^+ is still about 50% after high-dose irradiation (Fig. 5), which correlates to about 140 µM NAD^+ in non-supplemented cells (Table 1), concentrations in specific cellular compartments can be lower than that. Whereas mitochondria have a high NAD^+ content (about 400 µM) ([92]), which can sum up to 70% of total NAD^+ [93], cytosolic as well as nuclear NAD^+ concentrations reach only 100–150 µM. The latter two compartments display identical NAD^+ levels as there is free exchange of this molecule across the nuclear pore. In contrast, mitochondrial NAD^+ seems to be completely separated from the other pools, at least in humans [94]. The reported k_M value of human PARP1 is about 30–60 µM [95], whereas the k_M of SIRT1 is 150–200 µM (reviewed in [96]), which corresponds to NAD^+ concentrations just at limiting conditions for SIRT1 activity even in nuclei of undisturbed cells. In case of even rather low DNA damage, PARP1 may readily deplete the locally available nuclear pools of NAD^+, preventing SIRT1 from fulfilling its function as transcriptional regulator and chromatin organizer, and may also decrease SIRT1 expression by a so far unknown mechanism [85]. In line with this, it has been reported that cells challenged by DNA damaging agents [88], under pathophysiological conditions [85–87] or during aging [97] display high PAR levels, a lower NAD^+ content and decreased SIRT1 activity. Importantly, this can be rescued by PARP1 inhibition or replenishing the cellular NAD^+ pool by supplementation with NAD^+ precursors. Of note, acetylation of p53 in response to PARP1 inhibition or NAD^+ supplementation is reduced compared to situations with low NAD^+ content [87,88], suggesting that preserving sufficiently high NAD^+ concentration is a prerequisite for SIRT1-mediated de-acetylation of p53. Our results in human PBMC are in support of these data.

3.3.4. NA supplementation enhances DNA repair efficiency independent of proliferation

Having shown that NA supplementation preserves overall NAD^+ levels and enhances SIRT1 activity after irradiation, we set out to monitor the impact of NA on DNA repair after exposure of PBMC

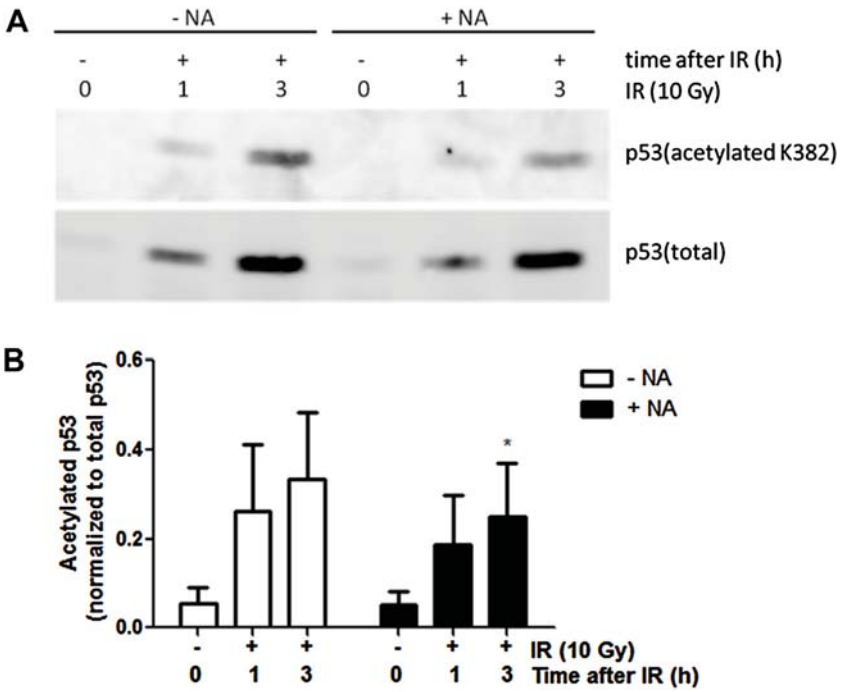

Fig. 6. Total p53 accumulation and K382-acetylation in response to IR.
PBMC were pre-treated −/+ NA, TSA and irradiated with 10 Gy. Levels of p53 and ac-p53 were analyzed in whole cell lysates *via* immunoblot analysis at indicated time points after irradiation. A representative blot is shown in (A) and quantitative analysis of band intensities for normalized acetylated p53 levels were shown for 6 independent experiments in (B). A significant decrease of p53-acetylation level is detectable at 3 h in NA-supplemented cells compared to control-irradiated cells.

to increasing doses of X-ray. As expected, in unstimulated cells 2.5 Gy induced moderate damage to DNA (Fig. 7A), whereas massive strand break induction was achieved with high-dose irradiation (25 Gy; Fig. 7D). Within 40 min repair time, rejoining of DNA breaks was evident from the partial recovery of fluorescent signals in PBMC from all donors tested. Strand break formation in supplemented PBMC was very similar compared to non-supplemented cells (Fig. 7A-D, compare values at 0 min), but interestingly, repair of strand breaks was slightly more efficient in supplemented cells compared to cells without additional NA treatment in a dose- and time-dependent manner already after application of 2.5 Gy.

In order to study the role of cell cycle progression in DNA damage formation and repair, we stimulated cells for 44 h with PHA-L and induced DNA damage by IR in these proliferating PBMC (Fig. 8), identical to the experimental setup in Fig. 7. The data obtained from PHA-stimulated cultures displayed a very similar picture as quiescent PBMC. Again, NA supplementation slightly, but significantly improved DNA repair in correlation to the applied dose and time of repair, although the difference was less pronounced compared to resting cells, *i.e.* reaching significance at 5 Gy or higher.

In summary, using the automated FADU assay [81] we were able to show that PHA-stimulation induced proliferation led to higher basal levels of strand breaks and NA-supplementation did not impact on this. NA-supplementation increased consistently the NAD^+ concentration in cells and maintained it at high levels even at the supralethal dose of 25 Gy irradiation compared to non-supplemented PBMC. These results are similar to data obtained from quiescent PBMC [45]. Most interestingly, NA treatment enhanced efficiency of DNA repair independent of proliferation status, but was slightly more effective in quiescent cells. This may be due to the fact that proliferating cells are more prone to cell death induction if challenged by IR [82,83] independent of NAD^+ pools. As a consequence, severely affected cells do not repair the damage anymore and die, whereas quiescent cells are less likely to induce apoptosis under these conditions. The overall mild stimulation of repair by NA-supplementation cannot be solely dependent on maintaining NAD^+ at high levels despite PARP1 activity, as low doses that do not hyperactivate PARP1, *i.e.* 2.5 and 5 Gy, display only a moderate reduction in NAD^+ as expected, but respective samples still show a significant increase in efficiency. This could be due to different mechanisms:

The structure of PAR synthesized by PARP1 can vary dependent on severity of DNA-damage [98,99], on the respective interaction partner [69,100], and also on the local NAD^+ concentration [100]. Whereas severity of damage (see Fig. 4) is the same, NAD^+ levels differ between NA-supplemented and non-supplemented cells by the factor of 1.4 (Table 1). Therefore subtle changes in the resulting PAR-structure (length, branching) may recruit repair factors with a different efficiency, leading to the observed differences in repair.

Sirtuins regulate a multitude of pathways, with SIRT1 involved in chromatin restructuring and regulation of repair [49], directly by targeting histones and indirectly by inactivating other regulator proteins such as p53 or p300, the acetylase targeting p53 [101,102]. P53 is the central transcription factor in stress-response [90,103–105]. In our experimental setup, p53 is induced after IR as expected. High levels of p53 not only stop the cell cycle to orchestrate repair, but can also induce apoptosis [106,107]. SIRT1 reduces the acetylated form of p53, thus dampen the adverse effects of high p53 activation [87,88].

Alternatively, restructured chromatin due to a different acetylation pattern may more susceptible to recognition and/or repair of damage. Although general chromatin acetylation is implicated in repair, there are also some reports about involvement of HDAC in early steps of DNA repair (reviewed in [108]). Whether this is relevant for our approach, and whether SIRT1 or other sirtuins are involved remains to be determined. In addition, chromatin is organized by PARylation (reviewed in [61,62]). DNA breakage is an immediate trigger of PARP1 activity, which leads to complex re-structuring of the chromatin at sites of damage, facilitating recruitment of repair proteins and activation of signaling cas-

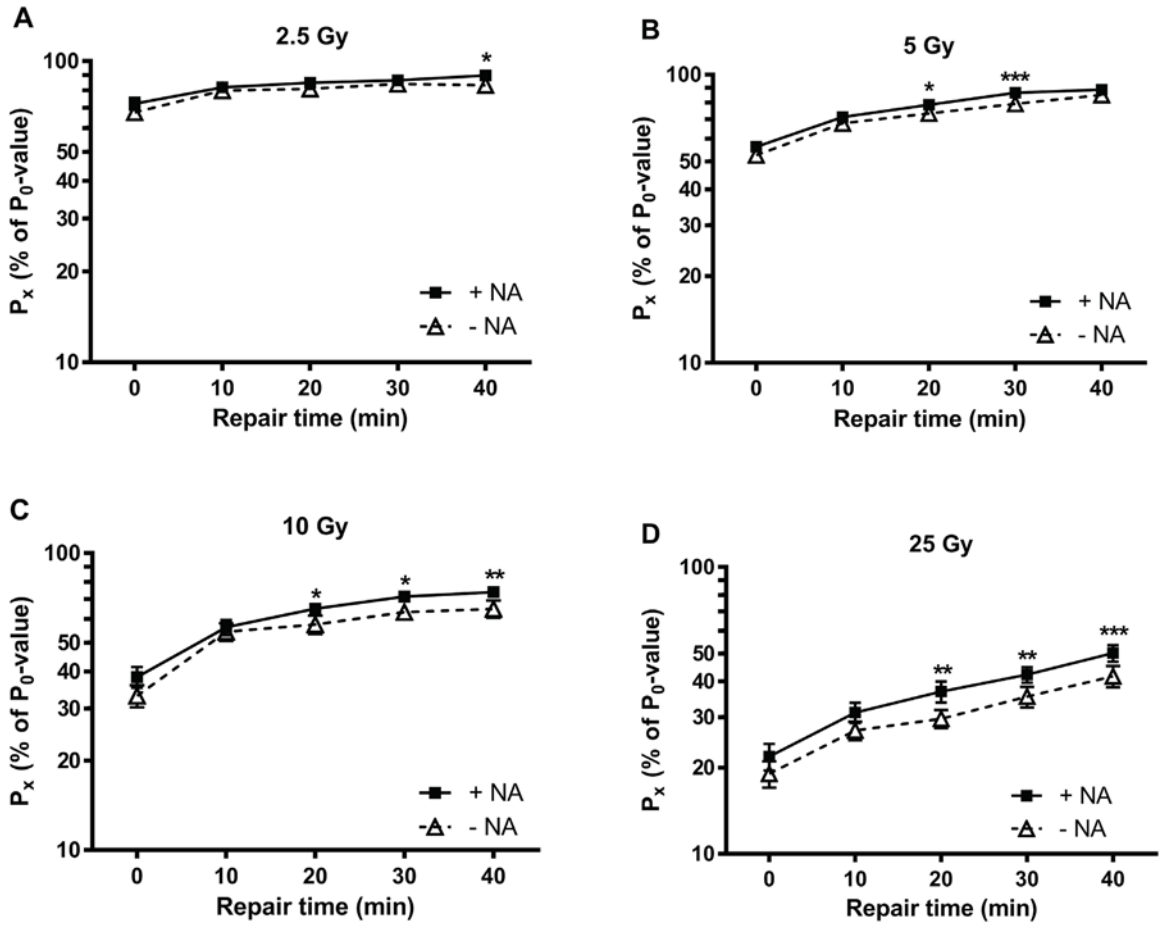

Fig. 7. DNA damage and repair in unstimulated PBMC.
Cells were irradiated in G_0. Strand break formation and subsequent DNA repair within the first 40 min after DNA damage was measured using the automated FADU assay. Values represent the mean fluorescence of double stranded DNA in relation to non-irradiated controls (P_0) as obtained in independent experiments covering (A) n = 12 donors (B) n = 13 donors (C) n = 11 donors (D) n = 10 donors.

cades (for review, see [109]). A faster opening of the chromatin with quicker recruitment of repair enzymes due to locally higher availability of NAD^+ and therefore differently structured PAR may account to the slight increase in DNA break resealing we observed.

3.4. *Effect of NA supplementation on genomic stability*

Studies have demonstrated a positive role of poly(ADP-ribosyl)ation in the maintenance of genomic integrity [59] and a negative influence by niacin deficiency [24]. Therefore we investigated the impact of NA supplementation on the formation of micronuclei in the low dose range of IR (1–5 Gy). The basal micronucleus frequency in undamaged cells was low, but MN frequency clearly increased in response to DNA damage (Fig. 9). Non-irradiated control cells displayed MN frequencies of 0.7% and 0.5% (Fig. 9A unstimulated/0 Gy, ±NA) and 1.5% and 1.1% (Fig. 9B stimulated/0 Gy, ±NA). Irradiation of cells in G_0 induced higher MN frequencies compared to cells damaged during proliferation. NA-treatment reduced MN frequency in proliferating as well as quiescent cells compared to non-supplemented PBMC, but this was more pronounced in proliferating cells. For example, MN frequency was not affected by NA supplementation in resting PBMC at 3 Gy, but was significantly reduced from 24% to 20% in PHA-L stimulated cells. At 5 Gy, NA supplementation reduced MN frequency for both conditions, from nearly 70% to 60% in cells in G_0, and from 36% to 30% in proliferating PBMC. These results are in line with the data obtained from our DNA break resealing measurements. As repair is more efficient in supplemented cells MN formation is slightly but significantly reduced.

Although there was no significant difference in basal MN formation or after 1 Gy irradiation, at doses ≥3 Gy lower MN frequencies were observed in cells damaged during proliferation compared to PBMC irradiated in G_0. This finding has been described for cells damaged during a distinct cell cycle phase [110] or with doses of IR leading to mitotic arrest [111]. Both conditions delay the appearance micronuclei, as these cells undergo a (temporary) division block and are therefore not included in the scoring of binucleated cells. The degree of IR induced division delay is dependent on the cell cycle position at the time of irradiation, with IR damage in S/G2 phase leading to a stronger delay [112,113] and generally resulting in lower MN frequencies, which is also apparent in our study. This effect was also reported for DNA damage induction in combination with PARP inhibitors, showing that resting cells were much less affected in comparison to proliferating cells [114]. The latter displayed reduced mutation rates but higher toxicity.

4. Conclusion

Niacin supplementation is generally thought to be beneficial in conditions of pathologies and aging with the ability to preserve mitochondrial energy production [16,17] with some caveats regarding side-effects of high-dose regimens. In addition, there are several reports on positive effects on genome stability or negative impact of NAD^+ deficiency [26–28]. In this study, we addressed the

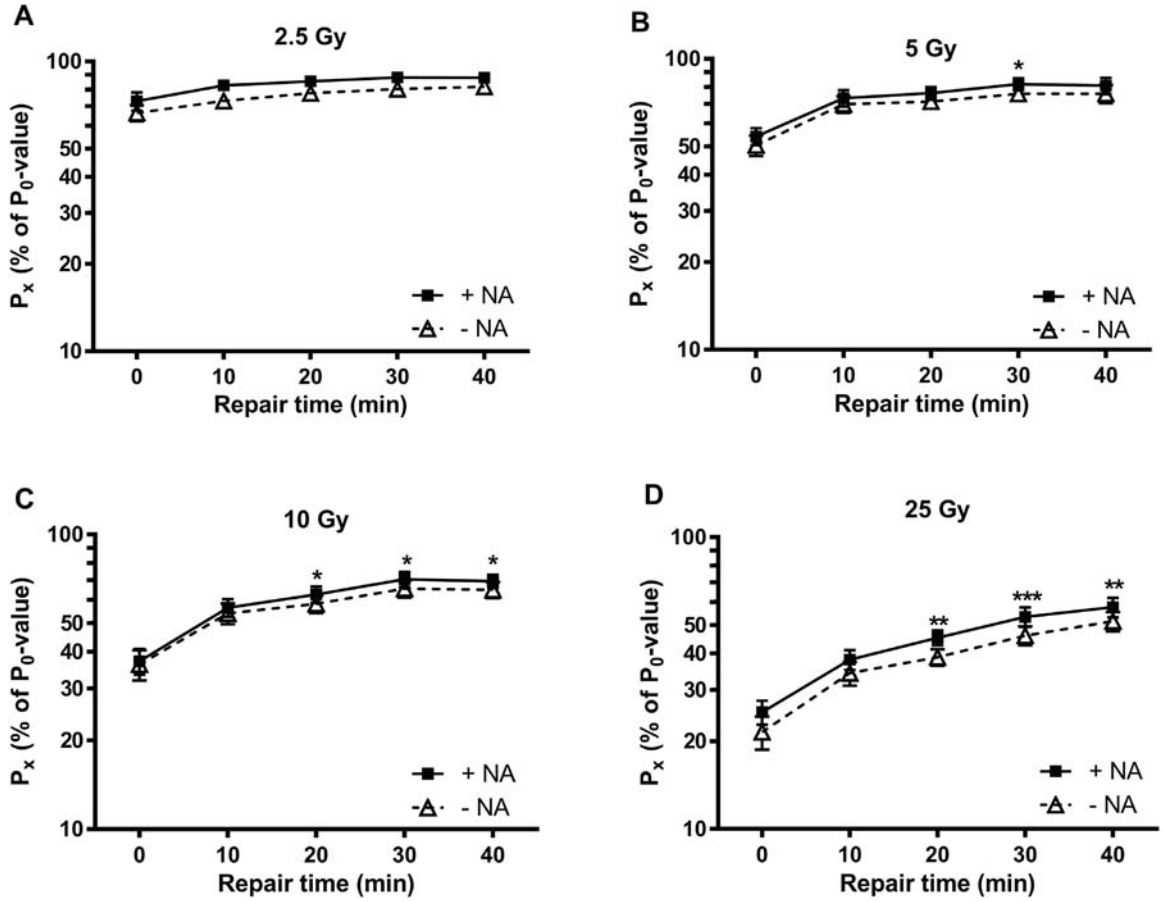

Fig. 8. DNA damage and repair in proliferating PBMC.
Cells were irradiated after PHA-L stimulation. Repair of strand breaks after genotoxic treatment was measured within the first 40 min by using the automated FADU assay. Values represent the mean fluorescence of double stranded DNA in relation to non-irradiated controls (P_0) as obtained in independent experiments covering (A) n = 8 donors (B) n = 10 donors (C) n = 8 donors (D) n = 9 donors.

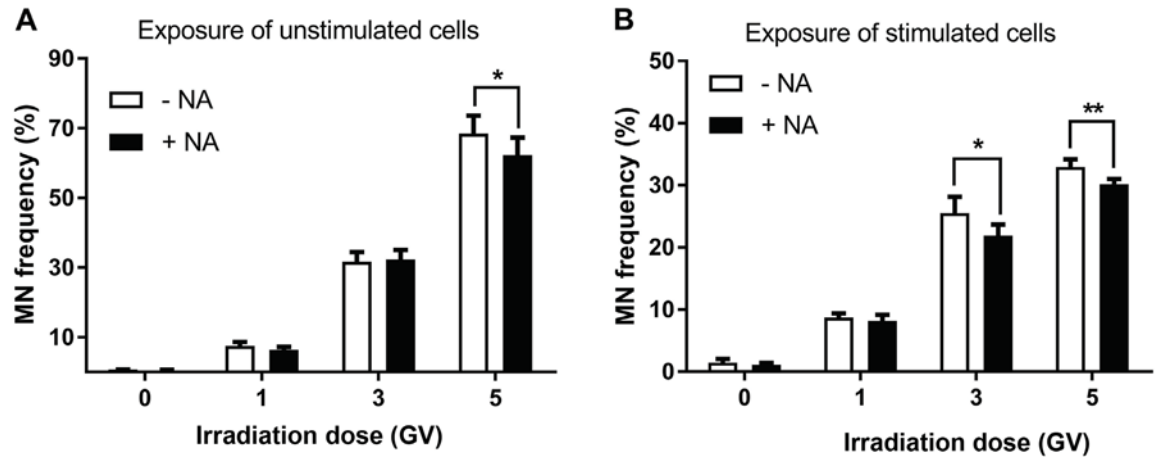

Fig. 9. Genomic stability in human PBMC exposed to DNA damage as assed by micronuclei formation.
(A) Unstimulated or (B) PHA-L stimulated cells were challenged with low doses of IR. Percentage of micronucleus frequency in binucleated cells was determined for (A) n = 7 and (B) n = 9 donors.

question of whether DNA repair is also affected by NA supplementation in normal human PBMC. Based on an automated version of the FADU technique [81] we monitored DNA strand break formation in PBMC after IR and the early phase of DNA repair after damage to discover immediate effects. Although the FADU assay cannot discriminate between single and double strand breaks, the former are known to be several fold more frequent than the latter after irradiation [115]. Our data indicate that even a short treatment with low doses of niacin (15 μM) is able to increase NAD^+ levels in proliferating PBMC similar to resting PBMC [45]. We could not observe any influence of NA-supplementation on the rate of DNA damage inflicted by irradiation. Interestingly, repair rates were slightly but significantly enhanced if NAD^+ levels were increased in resting as well as in proliferating PBMC. Accordingly, micronucleus

formation was marginally reduced after NA supplementation, suggesting a slightly better preservation of overall genomic stability. This slight difference could be based on the lower NAD^+ levels available in S-phase, which constrains the repair activity of proliferating cells. The observed results most likely depend on the availability of NAD^+ as substrate for two pathways, *i.e.* poly(ADP-ribosyl)ation, which is the main consumer of NAD^+ after genotoxic stress, and sirtuin-dependent protein de-acetylation. These enzymatic reactions regulate the DNA repair machinery and chromatin structure [63,64,85]. Especially PARPs and PAR have a pivotal role in preserving the genomic integrity as they regulate DNA repair processes and telomere length and can be detected at centrosomes and centromeres, thus ensuring correct distribution of the genetic material during cell division [116]. But there is ample evidence that also SIRT1 is involved in maintaining chromatin integrity by regulating different targets (reviewed in [117]). In line with this, knockout of SIRT1 and/or PARP1 led to changes in chromatin structure and alterations of histone marks [63]. In our study, the non-supplemented cells were not deficient in NAD^+, so it is unlikely that a general restructuring of chromatin took place in supplemented PBMC. It can be speculated that the higher availability of NAD^+ at the time of damage facilitates specific remodeling of the chromatin at the site of damage and thus leads to enhanced repair. In non-supplemented PBMC, PARP1 consumes substantial amounts of NAD^+ present in the nucleus after break induction, still working effectively even at concentrations of NAD^+ that disfavor SIRT1 activity [96]. PARP1 activity limits by this process the substrate availability for SIRT1, blocking de-acetylation of target proteins. In line with this, we provide evidence that NA-supplementation preserves SIRT1 enzymatic activity also in normal human PBMC as indicated by reduced levels of acetylated p53. So it can be speculated that maintaining activity of SIRT1 is improving repair efficiency, *i.e.* also by enhancing survival [87]. As it was shown in mice that inactivation of PARP1 leads to acceleration of aging, shortened life span and increased spontaneous carcinogenesis [118], it can be hypothesized that maintained PAR formation by NA supplementation is a potential strategy to counteract these effects. Notably, we monitored no adverse impact on the investigated parameters when NAD^+ levels were modulated. Based on our data, further usage of NA could be conceivable, as we showed that application of a low concentration (15 μM) NAD^+ precursor has a positive effect on genome integrity in our setting. NAD^+ dependent enzymes PARP and SIRT both influence genomic stability and metabolism, and adequate NAD^+ availability is likely to preserve their activities and functions. This could be favorable for human subjects with decreased PARP activity or low basal NAD^+ levels, which occurs during the aging process [119], in the pathogenesis of diseases [120] or cancer therapy [121,122]. High cellular NAD^+ levels as achieved by NA supplementation to maintain poly(ADP-ribosyl)ation and sirtuin activity thus have a plethora of positive effects, including enhanced repair and chromosomal stability.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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