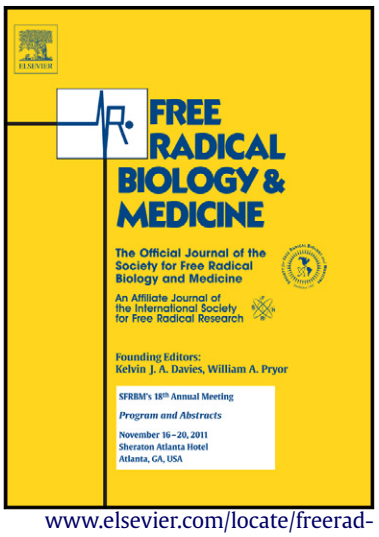

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Oxidative modification enhances the immunostimulatory effects of extracellular mitochondrial DNA on plasmacytoid dendritic cells

Kitti Pazmandi[a], Zsofia Agod[a], Brahma V. Kumar[a], Attila Szabo[a], Tunde Fekete[a], Viktoria Sogor[a], Agota Veres[a], Istvan Boldogh[b], Eva Rajnavolgyi[a], Arpad Lanyi[a] and Attila Bacsi[a,*]

[a]Department of Immunology, Faculty of Medicine, University of Debrecen, 98 Nagyerdei Blvd., Debrecen H-4012, Hungary;

[b]Department of Microbiology and Immunology, University of Texas Medical Branch at Galveston, 301 University Blvd., Galveston, TX 77555, USA.

*Corresponding author: Department of Immunology, Faculty of Medicine, University of Debrecen, 98 Nagyerdei Blvd., Debrecen H-4012, Hungary

Phone/Fax: +36 52 417 159, e-mail: bacsi.attila@gmail.com

Abbreviations: 7-AAD, 7-aminoactinomycin-D; 8-oxoG, 8-oxo-7,8-dihydroguanine; 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; DAMP, damage-associated molecular pattern; IFN, interferon; pDCs, plasmacytoid dendritic cells; mtDNA, mitochondrial DNA; ODN, oligodeoxynucleotide; OGG1, 8-oxoguanine DNA glycosylase 1; PBMC, peripheral blood mononuclear cells; ROS, reactive oxygen species, TFAM, mitochondrial transcription factor A; TLR, Toll-like receptor.

Abstract

Inflammation is associated with oxidative stress and characterized by elevated levels of damage-associated molecular pattern (DAMP) molecules released from injured or even living cells into the surrounding microenvironment. One of these endogenous danger signals is the extracellular mitochondrial DNA (mtDNA) containing evolutionary conserved unmethylated CpG repeats. Increased levels of reactive oxygen species (ROS) generated by recruited inflammatory cells modify mtDNA oxidatively resulting primarily in accumulation of 8-oxo-7,8-dihydroguanine (8-oxoG) lesions. In this study, we examined the impact of native and oxidatively modified mtDNAs on the phenotypic and functional properties of plasmacytoid dendritic cells (pDCs), which possess a fundamental role in the regulation of inflammation and T cell immunity. Treatment of human primary pDCs with native mtDNA up-regulated the expression of a co-stimulatory molecule (CD86), a specific maturation marker (CD83), and a main antigen-presenting molecule (HLA-DQ) on the cell surface, as well as increased TNF-α and IL-8 production from the cells. These effects were more apparent when pDCs were exposed to oxidatively modified mtDNA. Neither native nor oxidized mtDNA molecules were able to induce interferon (IFN)-α secretion from pDCs unless they formed a complex with human cathelicidin LL-37, an antimicrobial peptide. Interestingly, simultaneous administration of a Toll-like receptor (TLR)9 antagonist abrogated the effects of both native and oxidized mtDNAs on human pDCs. In a murine model, oxidized mtDNA also proved a more potent activator of pDCs compared to the native form, except for induction of IFN-α production. Collectively, we demonstrate here for the first time that elevated levels of 8-oxoG bases in the extracellular mtDNA induced by oxidative stress increase the immunostimulatory capacity of mtDNA on pDCs.

Highlights

We compared the immunostimulatory capacity of native and oxidized mtDNA on pDCs.8-Oxoguanin-containing mtDNA has a greater capacity to activate primary human pDCs.

In vivo, oxidized mtDNA also proved a more potent activator of pDC than native mtDNA.

Keywords

Plasmacytoid dendritic cells, Extracellular mitochondrial DNA, Oxidative stress, 8-oxoguanine base, Inflammation

Introduction

Mitochondria have crucial role in many cellular processes, including ATP, fatty acid and steroid hormone synthesis, the maintenance of Ca^{2+} homeostasis, thermogenesis and generation of reactive oxygen species (ROS). Furthermore, mitochondria are heavily integrated into pathways of cell death signaling, innate immunity and autophagy (reviewed in [1]). It is generally accepted that mitochondria are descended from a specialized, free-living, prokaryotic cell that survived endocytosis by another species of prokaryotes. In accordance with their prokaryotic origin, mitochondria still possess several morphological and biochemical characteristics of their bacterial ancestor, including a circular DNA genome containing unmethylated CpG oligodeoxynucleotide (ODN) sequences (reviewed in [2]). Therefore, mitochondrial DNA (mtDNA) molecules released by injured [3] or even living cells [4] are sensed as danger signals eliciting immunological responses (reviewed in [5]). Unmethylated CpG motifs in bacterial DNA [6], as well as in circulating mtDNA are recognized by Toll-like receptor 9 (TLR9) [7] expressed by various cells of the innate immune system [8, 9]. Among the leukocytes, human plasmacytoid dendritic cells (pDCs) are considered to be specialized for the recognition of nucleic acids of invading microbes, because in conjunction with TLR9 they also express single-stranded RNA-sensing TLR7 [10, 11]. Ligation of these endosomal TLRs initiates the secretion of various inflammatory cytokines and the production of large amounts of type I interferons (IFNs), which possess strong antiviral and immunomodulatory properties [12]. Because of their constitutively high level of TLR9 expression [13], human pDCs may serve as a primary sensor for mtDNA. Indeed, it has been previously reported that mtDNA fragments induce maturation, migration and type I IFN production of pDCs [14]. However, this former work has focused on the immunostimulatory properties of CpG islands and CpG-containing ODN derived from mtDNA [14]. In our study, we considered the pDC-activating potential of extracellular

mtDNA from another possible aspect, which has not been investigated yet.

Several lines of evidence indicate that pathogen-induced and even sterile inflammations are associated with oxidative stress generated by inflammatory cells recruited to the infected or injured tissues [15, 16]. This oxidative extracellular milieu and elevated levels of intracellular ROS are able to cause oxidative modification of mtDNA either before or after its release. The primary target of ROS in DNA is guanine due to its lowest redox potential among DNA bases, so 8-oxo-7,8-dihydroguanine (8-oxoG) is one of the most abundant base lesions [17, 18]. In mammals, the intra-helical 8-oxoG is recognized and excised by the *E. coli* Fpg homolog 8-oxoguanine DNA glycosylase 1 (OGG1) from nuclear and mitochondrial genome during base excision repair processes [19, 20]. The resulting free 8-oxoG base is capable of binding to OGG1 with high affinity, and the complex then functions as an activator of Ras and Rho family GTPases contributing to oxidative stress related cellular responses [21-23]. Here, our aim was to investigate the consequence if 8-oxoG is not removed from mtDNA and the oxidatively modified mtDNA is released from the cells. We hypothesized that accumulation of 8-oxoG during inflammation enhances the immunostimulatory capacity of mtDNA, and the 8-oxoG-enriched mtDNA has a greater potential to activate pDCs infiltrated into the inflamed tissues. To test this hypothesis we compared the phenotypic and functional properties of pDCs upon exposure to native or oxidatively modified mtDNA under *in vitro* as well as *in vivo* conditions.

Materials and methods

Cell cultures

Human promyelocytic leukemia HL-60 cells (American Type Culture Collection, CCL-240) and murine SP2/0-Ag14 cells (American Type Culture Collection, CRL-1581) were routinely grown in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine (Sigma-Aldrich), 100 U/ml penicillin, 100 ng/ml streptomycin and 10% heat-inactivated FBS (Life Technologies Corporation, Carlsbad, CA, USA). Both cell types were cultured at 37 ºC in 5% CO_2 humidified atmosphere.

Generation and detection of mitochondrial oxidative stress

Human HL-60 cells or murine SP2/0-Ag14 cells were loaded with 5 µM MitoSox™ Red probe (Life Technologies Corporation) and incubated for 10 min at 37 ºC protected from light. After that cells were washed gently three times with warm PBS buffer (Sigma-Aldrich) to remove the excess fluorescent dye and were cultured at a density of $5x10^6$ cells/ml in RPMI 1640 medium (Sigma-Aldrich) supplemented with the above mentioned components. The MitoSox™ Red loaded cells were exposed to increasing concentration of antimycin A (50 µg/ml, 100 µg/ml, 200 µg/ml; Sigma-Aldrich) for 2 h. The changes in MitoSox™ Red fluorescence intensity were measured at 580 nm using a BD FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and data were analyzed by FlowJo software (Treestar, Ashland, OR, USA). In parallel experiments the viability of the antimycin A-exposed cells were determined by 7-aminoactinomycin-D (7-AAD; 10 µg/ml; Sigma-Aldrich) staining using flow cytometric analysis.

Isolation of mitochondrial DNA

The mtDNA was extracted from untreated and antimycin A-treated human HL-60 cells and murine SP2/0-Ag14 cells using Biovision Mitochondrial DNA Isolation Kit (Gentaur Belgium BVBA, Kampenhout, Belgium), according to the manufacturer's instructions. Briefly, a total of $5x10^7$ cells were collected by centrifugation at 600 *g* for 5 min at 4 C° and washed with ice-cold PBS. After centrifugation (600 *g*, 5 min, 4 C°) cells were resuspended in cytosol extraction buffer and incubated on ice for 10 min. Then the cells were homogenized with ice-cold Dounce tissue grinder. The homogenate was centrifuged at 700 *g* for 10 min at 4 C° to remove nuclei and intact cells. Mitochondria were pelleted from the supernatant by centrifugation at 10000 *g* for 30 min at 4 C°. This centrifugation step was repeated after resuspending the pellet in fresh ice-cold cytosol extraction buffer. After that isolated mitochondria were lysed, and mtDNA was purified using standard ethanol precipitation. Namely, the enzyme-lysed mitochondria were mixed with absolute ethanol and kept at -20 C° for 10 min. The precipitated mtDNA was centrifuged at 16000 *g* for 5 min at room temperature and the pellet was washed 2 times with 70 % ethanol. After removing the trace amount ethanol mtDNA was air dried (5 min) and dissolved in TE buffer (Life Technologies Corporation). The concentration and purity of isolated mtDNA were determined by NanoDrop spectrophotometer (Thermo Scientific, Rockford, IL, USA). In case of every mtDNA sample the A260/280 ratio was between 1.8 and 1.9. To test the possibility of contamination of the mtDNA samples with nuclear DNA, the NovaQUANT Human Mitochondrial to Nuclear DNA Ratio Kit (MerkMillipore, Darmstadt, Germany) was used according to the manufacturer's recommendations. The mtDNA samples were probed for two mitochondrial genes (ND1, ND6) and for two nuclear DNA marker genes (BECN1, NEB)

using the ABI StepOne Real Time PCR System (Life Technologies Corporation). It was found that in contrast to the mitochondrial genes, none of the tested nuclear DNA genes could be detected in the mtDNA samples. The integrity of mtDNA molecules prepared from untreated and antimycin A-treated cells was analyzed by a standard agarose gel electrophoresis. Mitochondrial DNAs were run on 0.8 % agarose gel and then visualized by SYBR Green I fluorescent intercalating dye under ultraviolet light.

Detection of 8-oxoG in the isolated mtDNA samples using dot blot analysis

To determine the 8-oxoG content of mtDNA extracted from untreated and antimycin A-treated cells, 1 µg of mtDNA was spotted onto a nitrocellulose membrane. After drying the non-specific binding was blocked in TBS-T buffer (50 mM Tris, 0.5 M NaCl, 0.05% Tween-20, pH 7.4) containing 5% dry milk for 1 h at room temperature. Following the blocking step the membrane was incubated with mouse anti-8-oxoguanine monoclonal antibody (1:1000, clone 483.15, MerkMillipore) in TBS-T buffer containing 5% dry milk for 1 h at room temperature. After incubation the membrane was washed three times with TBS-T buffer and anti-mouse IgG conjugated with HRP (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used as a secondary antibody at a dilution of 1:10000. After 1 h incubation and repeated wash steps blot results were visualized by ECL system (SuperSignal West Femto Chemiluminescent Substrate; Thermo Scientific). Spot signals were quantified using the image analysis software Kodak 1D 3.6 (Eastman Kodak Company, Rochester, NY, USA). After detection of 8-oxoG in the mtDNA samples, the same membrane was washed thoroughly with distilled deionized water and stained with ethidium bromide (Sigma-Aldrich) dissolved in 1X TAE (Tris-acetate-EDTA) buffer for 30 min at room temperature. After rinsing the membrane with 1X TAE buffer, ethidium bromide binding was visualized and

photographed under UV light in Alpha Imager HP System (ProteinSimple, CA, USA).

Isolation of primary human pDCs

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized leukocyte-enriched buffy coats by standard Ficoll-Paque (GE Healthcare) density gradient centrifugation. Untouched human pDCs were purified from PBMC by negative selection using immunomagnetic cell separation kit (Miltenyi Biotec, Bergish Gladbach, Germany), according to the manufacturer's instruction. After separation on VarioMACS magnet, the purity of $BDCA2^+$ $BDCA4^+$ $CD123^+$ pDCs was >98% as confirmed by flow cytometry.

Human buffy coats were obtained from healthy blood donors drawn at the Regional Blood Center of Hungarian National Blood Transfusion Service (Debrecen, Hungary) in accordance with the written approval of the Director of the National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Centre (Debrecen, Hungary).

In vitro stimulation of primary human pDC

Freshly isolated primary human pDCs were seeded at $1x10^5$ cells/well in 96-well flat-bottom plates in RPMI 1640 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine (Sigma-Aldrich), 100 U/ml penicillin, 100 ng/ml streptomycin, 10% heat-inactivated FBS (Life Technologies Corporation) and 50 ng/ml recombinant human IL-3 (PeproTech, London, UK). For stimulation, pDCs were exposed for 24 h to 7 µg of mtDNA prepared from untreated and antimycin A-treated human HL-60 cells. An optimal concentration of mtDNA for cell activation was determined in preliminary experiments. In control experiments, pDCs

were pre-incubated for 1 h with TLR9 inhibitory ODN TTAGGG oligonucleotide (InvivoGen, San Diego, CA, USA) and then co-treated with 7 μg of mtDNAs for 24 h (mtDNA: inhibitory ODN ratio, 1:5). For the induction of human pDCs' type I IFN secretion, 35 μg of LL-37 protein (Sigma-Aldrich) were premixed with 7 μg of mtDNA (peptide: mtDNA mass ratio of 5:1) in sterile PBS (PAA Laboratories GmbH) for 30 min at room temperature. After incubation the mix was added to the primary pDC cultures (final concentration was 175 μg/ml of LL-37 and 35 μg/ ml of mtDNA). The treated cells were incubated at 37 ºC in 5% CO_2 humidified atmosphere for 24 h and then supernatants were collected and stored at -70 ºC until cytokine measurements.

Phenotypic analysis of primary human pDCs by flow cytometry

Cell surface protein expression was analyzed by staining the cells with PE-labeled human monoclonal antibodies against CD86 (R&D System, Minneapolis, MN, USA), HLA-DQ (BioLegend, San Diego, CA, USA) and PE-Cy5-labeled anti-CD83 (BD Pharmingen, San Diego, CA, USA) monoclonal antibodies. Isotype-matched control antibodies were obtained from BD Pharmingen. Fluorescence intensities were measured by FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems) and data analysis was performed by FlowJo software (TreeStar).

Measurement of cytokine and chemokine secretion of primary human pDCs

ELISA kits (BD OptEIA; BD Biosciences, San Diego, CA, USA) were used to quantify IL-8 chemokine, as well as TNF-α cytokine. Human IFN-α ELISA kit (with a detection limit of 12.5 pg/ml) was purchased from PBL InterferonSource (Piscataway, NJ,

USA). Assays were performed according to the manufacturer's instructions. Absorbance measurements were performed with a Synergy HT micro plate reader (Bio-Tek Instruments, Winooski, VT, USA) at 450 nm.

In vivo mice experiments

For *in vivo* treatments, 6- to 8-week-old 129Sv female mice (The Jackson Laboratory, Bar Harbor, ME, USA) were injected with 200 µl of sterile PBS (Sigma-Aldrich) as a control or 200 µl of mtDNA preparations i.v. into the lateral tail vein. The preparations contained 10 µg of mtDNA isolated from untreated or from antimycin A-treated murine SP2/0-Ag14 cells mixed with 30 µl of cationic liposomal transfection reagent (DOTAP; Sigma-Aldrich) dissolved in sterile PBS (Sigma-Aldrich). Mice were sacrificed and blood was collected by cardiac puncture at 6 h after treatments. For analysis of *in vivo* cytokine and chemokine production, sera were collected from whole blood by centrifugation at 3500 *g* for 5 min at room temperature and tested for IFN-α, TNF-α, IL-6 and CXCL1/KC proteins using mouse specific ELISA kits (mouse IFN-α ELISA kit [with a detection limit of 50 pg/ml] from TSZ Scientific LLC., and mouse TNF-α, IL-6, CXCL1/KC ELISA kits from R&D Systems). Blood cells were stained with FITC-conjugated anti-CD86, PE-conjugated I-A/I-E (both from BioLegend), APC-conjugated mPDCA-1 (Miltenyi Biotec) mouse antigen specific monoclonal antibodies and the respective isotype controls from the same sources for 20 min in the dark and resuspended in 1x BD FACS™ Lysing Solution (BD Bioscience, San Jose, CA, USA) for 30 min at room temperature to remove red blood cells. Following repeated wash steps with PBS (Sigma-Aldrich), the phenotypic changes of the mouse cells were determined by flow cytometric analysis. The mouse pDC population was identified by the mouse specific pDC marker, mPDCA-1, and the phenotypic analysis was performed on the

gated mPDCA-1$^+$ pDC population.

Care and handling of animals followed the Helsinki Declaration, European Union regulations and adhered to the guidelines of the Committee for Research and Ethical Issues of IASP. All animal study protocols were approved by the Animal Care and Protection Committee at the University of Debrecen (#7/2011/DE MAB). Animals were maintained in the pathogen-free animal facility of the University of Debrecen.

Statistical analysis

Data from the different treatment groups were analyzed by Student's paired *t* test or ANOVA, followed by Bonferroni *post hoc* analyses for least-significant differences. Data analysis was performed with GraphPad Prism v.6. software (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered to be statistically significant at $P < 0.05$.

Results

Inhibition of mitochondrial electron transport chain at complex III results in elevated 8-oxoG levels in mtDNA

Antimycin A, a well-known specific inhibitor of mitochondrial electron transport chain at complex III [24], was applied to induce oxidative modification of mtDNA in both human HL-60 and murine SP2/0-Ag14 cells. To determine the optimal amount for generation of mtDNA damage, cells were treated with increasing concentrations of antimycin A for 2 h. Mitochondrial ROS production was assessed using MitoSox™ Red, a mitochondrial superoxide indicator, while cellular viability was determined by 7-AAD staining. Exposure of either human or murine cells to 100 µg/ml of antimycin A resulted in a significant increase in the MitoSox™ Red fluorescence without significant cellular toxicity (Suppl. Fig. 1). Higher concentration of antimycin A (200 µg/ml) did not cause further increase in MitoSox™ Red fluorescence intensity but triggered a 30% drop in cell viability (Suppl. Fig. 1). Based on these observations, for *in vitro* and *in vivo* pDC stimulations, native mtDNA was extracted from untreated human and murine cells, whereas oxidatively modified mtDNA was isolated from cells exposed to 100 µg/ml of antimycin A.

To test whether antimycin A treatment of the cells led to oxidative modification of their mtDNA, 8-oxoG levels correlating with the oxidized state of the nucleic acid [17, 18] were investigated in the purified mtDNA samples by dot blot method. We found that the amount of 8-oxoG in the mtDNA from antimycin A-treated human HL-60 cells were ~5 times higher than in mtDNA from untreated cells (Fig. 1A). A similar increase in 8-oxoG levels was observed in the mtDNA samples extracted from the antimycin A-treated murine SP2/0-Ag14 cells (Fig. 1B).

In addition to induction of base lesions, oxidative stress may also cause strand breaks resulting in DNA fragmentation. To compare the integrity of native and oxidatively modified mtDNA molecules after extraction, mtDNA samples were electrophoresed on a standard agarose gel and stained with SYBR Green I intercalating fluorescent dye. The electrophoretic mobility of the native and oxidized mtDNA isolated from either the HL-60 or the SP2/0-Ag14 cells was identical, suggesting that antimycin A-treatment does not cause detectable mtDNA fragmentation (Suppl. Fig. 2).

Mitochondrial DNA with higher 8-oxoG content has a greater capacity to induce phenotypic changes on primary human pDCs

To investigate the possible differences in the immunostimulatory properties of the two forms of mtDNAs, freshly isolated primary human pDCs were exposed to native or oxidatively modified mtDNA extracted from human HL-60 cells. Expression of a co-stimulatory molecule (CD86), a specific maturation marker (CD83), and a main antigen-presenting molecule (HLA-DQ) on pDCs was evaluated by means of flow cytometry. Treatment of pDCs with native mtDNA resulted in a significant increase in the expression of CD86 (Fig. 2A) and CD83 (Fig. 2C), while it markedly, but not significantly enhanced the HLA-DQ levels (Fig. 2B) on the cell surface. When cells were exposed to high 8-oxoG-containing mtDNA, a further increase in the expression of all tested molecules was detected indicating that the oxidatively modified mtDNA has a greater potential to induce phenotypic changes on pDCs (Fig. 2A-C).

Previous studies have provided evidence that TLR9 is a specific receptor for microbial DNA [9], therefore we supposed that the effects of mtDNA containing CpG motifs on human pDCs are mediated via TLR9 selectively expressed in these cells. To test this hypothesis,

mtDNA treatments were repeated in the presence of TTAGGG ODN, a specific competitive inhibitor of TLR9. The results showed that pre-treatment of the cells with the TLR9 antagonist, prevented the above described phenotypic changes on pDCs, independently from the state of oxidation of mtDNAs (Fig. 2A-C). These observations strongly suggest that the phenotypic maturation of pDCs induced by native as well as oxidized mtDNA treatments was mainly mediated through TLR9.

Cytokine and chemokine production of primary human pDCs is potentiated by the presence of high level of 8-oxoG in mtDNA

To further examine the effects of high 8-oxoG content of mtDNA on pDC functions, freshly isolated pDCs were treated with native and oxidized mtDNA purified from non-treated or oxidative stress-exposed human HL-60 cells, respectively. Levels of TNF-α and IL-8 were determined in the supernatants of the cell cultures by means of ELISA. The administration of native mtDNA augmented the secretion of both TNF-α (Fig. 3A) and IL-8 (Fig. 3B); however, it induced statistically significant increase only in the case of the former one (Fig. 3A). Compared to the treatment with native mtDNA, the stimulation of primary pDCs with the oxidized form of mtDNA resulted in an enhanced production of both mediators (Fig. 3A and B). The observed increase in the release of IL-8 was more conspicuous than that of TNF-α (Fig. 3A and B). Similarly to the phenomenon observed in the phenotypic analyses, the presence of a TLR9 antagonist prevented the enhanced TNF-α and IL-8 production from pDCs induced by either native or oxidatively modified mtDNA (Fig. 3A and B) suggesting that the activation of pDCs was initiated by TLR9-dependent recognition of mtDNAs.

Neither native nor oxidatively modified mtDNA alone is able to induce IFN-α secretion of

primary human pDCs

As pDCs are considered to be professional type I IFN-producing cells [25], the effect of the mtDNA treatments on IFN-α secretion was also investigated. The concentration of IFN-α was measured in the supernatants of the native and oxidized mtDNA-exposed primary human pDCs by means of ELISA. We found that administration of neither native nor oxidatively modified mtDNA induced IFN-α secretion of the cells (Fig. 4). Previous studies have reported that nuclear DNA alone is not able to trigger the production of IFN-α by human pDCs, while nuclear DNA pre-incubated with human cathelicidin (LL-37), an antimicrobial peptide, is capable of doing so [26]. Based on this previous finding, mtDNA samples were pre-incubated with LL-37 before addition to the cell cultures. In complex with LL-37, both native and oxidized forms of mtDNA were able to provoke IFN-α production by pDCs (Fig. 4). It is worth noting that the native form resulted in significantly more IFN-α release than the oxidatively modified form. Furthermore, simultaneous administration of a TLR9 antagonist totally inhibited the secretion of this mediator induced by both kinds of mtDNA (Fig. 4).

Oxidized mtDNA is a more potent in vivo activator of pDCs than its native form

To test the hypothesis that oxidatively modified mtDNA is a more potent activator of pDCs *in vivo* than the native form, mtDNA was extracted from either untreated or antimycin A-treated murine SP2/0-Ag14 cells and intravenously injected into 129Sv mice. It has been previously reported that estrogens enhance pDCs' immune responses after TLR-7/TLR-9 stimulation in mice [27]. In order to get a more powerful and easily detectable immune activation after administration of mtDNA, only female animals were included in our study. Peripheral blood samples were collected after 6 h incubation and multi-color flow cytometric

analysis was performed to identify mouse pDCs and assess their phenotypic changes. *In vivo* treatment with native mtDNA induced an increase in the expression levels of both CD86 co-stimulatory (Fig. 5A) and I-A/I-E antigen-presenting molecules (Fig. 5B) on mouse pDCs; however, the changes were found to be statistically significant in the latter case only. Administration of oxidized mtDNA triggered a significantly stronger increase in the expression of both investigated cell surface molecules than that of the native form (Fig. 5A and B).

To compare the *in vivo* effects of the two forms of mtDNA on cytokine and chemokine levels, serum samples of 129Sv mice injected with native and 8-oxoG-enriched mtDNAs isolated from untreated and antimycin A-treated murine SP2/0-Ag14 cells were analyzed for IL-6, TNF-α and CXCL1/KC by means of ELISA. In sera of mice injected with oxidized mtDNA, statistically significant increases in the levels of IL-6 (Fig. 6A), TNF-α (Fig. 6B) and CXCL1/KC (Fig. 6C) were detected. On the contrary, in the sera of native mtDNA-injected mice, insignificant elevations could be observed in the levels of all these inflammatory mediators (Fig. 6A-C).

To evaluate the ability of the two distinct forms of mtDNA to induce type I IFN secretion *in vivo,* the amount of IFN-α in the serum samples was also measured. Both forms of mtDNA significantly enhanced its production; however, exposure of mice to oxidatively modified mtDNA led to a less induction of IFN-α (Fig. 6D), similarly to our *in vitro* findings with human pDCs (Fig. 4).

Discussion

Plasma level of mtDNA is elevated during severe sepsis and traumatic injury; moreover, it is associated with injury severity and predicts the risk of post-traumatic systemic inflammatory response syndrome [28, 29]. Mitochondrial DNA has been also detected in synovial fluids of patients with rheumatoid arthritis [30]. In addition, a recent study showed that regardless of disease activity, circulating mtDNA levels distinguish patients with systemic lupus erythematosus from non-inflammatory controls with high sensitivity [31]. Furthermore, circulating mtDNA can activate human neutrophils through TLR9, leading to their migration and degranulation [3]. These data indicate that extracellular mtDNA acting as a DAMP can contribute to innate immune responses. As mtDNA can be released from various cell types during infections or under inflammatory conditions closely associated with oxidative stress [32, 33], in this work we examined the immunostimulatory properties of oxidatively damaged mtDNA on pDCs, which exert a crucial role in the regulation of inflammation and T cell immunity [34].

Mitochondrial DNA is more vulnerable to oxidative damage than nuclear DNA for several reasons. Mitochondria are the primary sites of ROS production within the cells, and mtDNA is partially associated with the inner mitochondrial membrane in close proximity to the electron transport chain [35, 36]. Furthermore, mtDNA is not protected by histones and mitochondrial DNA repair is thought to be less efficient than nuclear DNA repair [35]. As a result of these facts, the steady state levels of oxidized bases in mtDNA are several times higher than in nuclear DNA [37]. Among the oxidative-damage DNA lesions, 8-oxoG is the most prevalent product of the oxidative attack on DNA. It accumulates in mtDNA with age and in patients suffering from certain types of cancer or neurodegenerative diseases associated with chronic inflammation (reviewed in [38]). This base damage has particular biological relevance because, unless repaired, it induces G:C to T:A transversion mutations with high

frequencies [39]. Mitochondria contain enzymes whose function is to remove these lesions, thus reversing the effects of oxidative insult and preventing mutations [40, 41]. However, when oxidative damage to DNA is excessive and mtDNA is released before repair, e.g., under inflammatory processes, it may contain high level of 8-oxoG [42]. In this study we investigated the immunomodulating potential of this 8-oxoG-enriched mtDNA on both human and murine pDCs.

To mimic oxidative stress conditions we treated the cells with antimycin A, which binds to cytochrome *b* and inhibits electron flow from semiquinone to ubiquinone, consequently increasing the steady state concentration of semiquinone and resulting in electron escape from complex III [43, 44]. This impairment of the electron transport system can increase mitochondrial ROS generation leading to oxidative damage of mtDNA [45]. In good agreement with these previous findings, we detected several times higher levels of 8-oxoG in mtDNA isolated from antimycin A-exposed cells than those from untreated cells. Repair of mitochondrial oxidative DNA lesions occurs predominantly through the base excision repair pathway [19]. Excision of 8-oxoG is initiated by OGG1, which recognizes and removes the base in a free form by cleavage of the glycosylic bond between the damaged base and the deoxyribose residue. The resulting abasic site is cleaved by AP endonuclease-1 to generate a gap that is filled by DNA polymerase followed by action of DNA ligase to complete the repair process [19, 46]. Agarose gel analysis of the mtDNA samples indicated that the isolation procedure resulted in partial fragmentation of mtDNA, whereas antimycin A-treatment did not result in further detectable changes in mtDNA integrity, despite the fact that during repair of 8-oxoG in DNA transient single strand breaks are generated [47].

In a recent study it was found that full-length mtDNA possessed immunostimulatory activity only if it was transfected into the pDCs using the cationic reagent DOTAP [14]. In that study, however, the authors investigated exclusively the IFN-α production of mtDNA-

exposed pDCs. Our study confirms this previous observation and extends it in several ways. First, we have also shown here that neither native nor oxidatively modified mtDNA alone was able to induce IFN-α secretion of human pDCs. However, when mtDNA molecules were pre-incubated with human cathelicidin (LL-37), an antimibrobial peptide, prior administration to the cells, both the native and the oxidized forms induced IFN-α production by pDCs. Besides its antimicrobial properties, LL-37 contributes to the uptake of extracellular DNA by various cell types binding the DNA by virtue of its opposite charges. As previously reported, LL-37 can convert inert self nuclear DNA into a potent inducer of IFN-α production from pDCs by protecting extracellular DNA from degradation and forming aggregated and condensed complexes with DNA that are delivered to and retained within early endocytic compartments to trigger TLR9 [26]. Furthermore, binding of LL-37 to CpG-rich bacterial DNA significantly reduces the time required for pDCs and B cells to sense the presence of bacterial DNA via TLR [48]. In addition, LL-37 also has the ability to efficiently transport self-DNA into cytosolic compartments of monocytes, leading to activation of the cells in a TLR-independent manner [49]. Our results demonstrating that a specific competitive inhibitor of TLR9 almost completely abolished the ability of mtDNA to induce phenotypic and functional changes on pDCs confirm previous observations that mtDNA containing multiple CpG motifs binds and activates TLR9 in human pDCs [14]. Previous findings indicate that single-stranded mitochondrial ODN molecules are immunostimulatory on pDCs only after DOTAP transfection, whereas double-stranded mitochondrial ODN is able to induce pDCs' maturation [14]. Our observations that exposure to mtDNA samples containing full-length mtDNA and mtDNA fragments with various lengths can elicit activation of pDCs without transfection are in line with these earlier results, and support that human pDCs are able to engulf DNA molecules specifically those with higher-order structures. It has recently been reported that mtDNA remained in association with the mitochondrial transcription factor A (TFAM) upon

release from necrotic cells [50]. TFAM and mtDNA interact with receptors for advanced glycation end-products and TLR9, respectively, to promote activation of pDCs [50]. In our experiments, we isolated mtDNA from cultured human and murine cells for further investigation and in every mtDNA sample the A260/280 ratio was between 1.8 and 1.9, excluding a significant protein contamination. Therefore, contribution of TFAM proteins to the observed immunostimulatory effects of mtDNA on pDCs in our experimental system seems very unlikely.

Our results from both *in vitro* and *in vivo* experiments indicate that the oxidatively modified extracellular mtDNA possesses a greater potential to activate pDCs than native mtDNA. In line with our observations, in a prior study, purified mtDNA and even an ODN that lacked CpG motifs but contained a single 8-oxoG residue induced arthritis when injected intra-articularly in mice [30]. In contrast, an ODN with exactly the same sequence, except that it lacked the oxidized base, was totally inert *in vivo* [30]. In another study, CpG ODN molecules, in which a guanine base was substituted with 8-oxoG, induced a significantly higher amount of TNF-α from RAW264.7 macrophage-like cells than control unsubstituted ones [51]. The 8-oxoG-containing DNA-induced increase in TNF-α production was also observed in primary cultured macrophages isolated from wild-type mice, but not observed in those from TLR9 knockout mice [51]. In addition, subcutaneous injection of 8-oxoG-containing CpG ODN led to an increase in footpad swelling compared to that of regular CpG ODN [51].

In our *in vivo* experiments the phenotypic properties of murine pDCs in peripheral blood were investigated by means of flow cytometry, whereas cytokine and chemokine levels in the serum samples were measured by ELISA. It is important to note that while flow cytometric analysis provides unambiguous data about the pDCs' phenotypic changes, the detected cytokines and chemokine could be released by diverse cell types responding to

mtDNA treatment. In humans, TLR9 expression in mononuclear blood cells and lymphoid tissues is restricted to B cells and pDCs, whereas in mice, TLR9 expression has also been demonstrated in macrophages, myeloid DCs, and activated T cells in addition to pDCs and B cells [52]. Despite this fact, the results of our *in vivo* experiments correlate very well with those of cell culture assays. Both in human pDC cultures and mice, treatment with oxidized mtDNA resulted in much higher production of all tested mediators, except IFN-α, than stimulation with the native form. The native form of mtDNA proved to be a better inductor of IFN-α release. Based on these observations, it seems that effects of oxidized mtDNA on TLR9-expressing cells are similar to those of type B CpG ODNs. It has been previously shown that type B CpG ODNs predominantly activate pDCs in a manner that results in phenotypic changes and pro-inflammatory cytokine production, as well as weak induction of type I IFN responses [53]. The functional activity of type B CpG ODNs is attributed to their localization to lysosome-associated membrane protein 1-positive endosomes, while activation of TLR9 by type A CpG ODNs occurs in transferrin receptor-positive endosomes [54]. Although, prior X-ray crystal and NMR structures had showed that DNA with 8-oxoG lesions appears virtually identical to the corresponding unmodified duplex, recent thermodynamic studies have indicated that 8-oxoG has a destabilizing influence [55]. The presence of 8-oxoG has a profound effect on the level and nature of DNA hydration indicating that the environment around an 8-oxoG:C is fundamentally different than that found at G:C [55]. The instability of the 8-oxoG modification is attributed to changes in the hydrophilicity of the base and may influence the recognition of oxidized mtDNA by TLR9.

While increased levels of extracellular mtDNA were detected during several pathological conditions such as chronic inflammations [30], trauma [3], neurodevelopmental disorders [56], or different infections [57], the clinical relevance of elevated 8-oxoG levels in released mtDNA has not yet been investigated. In a prior study extracellular mtDNA and

oxidized DNA (8-oxo-7,8-dihydro-2'-deoxyguanosine, 8-oxodG) were detected in the synovial fluid samples of the great majority of patients with rheumatoid arthritis, but were absent or present at low levels in the samples of healthy controls [42]. Furthermore, a significant correlation was observed between the levels of 8-oxodG and rheumatoid factor positivity. Higher concentrations of 8-oxodG (>1.5 ng/ml) in the synovial fluid were also associated with more severe clinical manifestations, but the correlations were found not to be statistically significant [42]. It is worth mentioning that source of the elevated oxidatively damaged DNA adducts (mitochondrial DNA or nuclear DNA) was not identified in that study. Although further studies are needed to reveal the clinical significance of our observations, our data indicate that a positive correlation between the 8-oxoG level in extracellular mtDNA and the severity of clinical symptoms may exist in those diseases in which activation of pDCs and/or other TLR-9-expressing cells has pivotal role in the pathogenesis.

In conclusion, we have demonstrated that the oxidatively modified, extracellular mtDNA has a greater potential to activate human pDCs and to initiate innate immune responses in experimental animals. Our results suggest that oxidized mtDNA originated from stressed, damaged or even dying cells during inflammation exacerbates acute and chronic immune processes by eliciting the production of chemokines and pro-inflammatory cytokines from TLR9-expressing cells.

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Disclosure

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1. Detection of 8-oxoG contents of the isolated mtDNAs. Oxidatively modified mtDNA was purified from antimycin A-exposed human HL-60 (A) or murine Sp2/0-14Ag (B) cells. Native mtDNA was extracted from untreated cells and used as controls. Levels of 8-oxoG in the isolated mtDNA samples were determined by means of dot blot method. Diagrams display the intensities of 12 dots from three independent experiments and results are presented as means ± SE. The lower panels show representative dot blots of four parallel samples of oxidized and native mtDNA isolated from the indicated cell lines and same blots stained with ethidium bromide as DNA loading controls. **** $p<0.0001$ vs. mtDNA from untreated cells. Ox-mtDNA: oxidatively modified mtDNA.

Figure 2. Phenotypic analysis of primary human pDCs treated with native or oxidatively modified mtDNA. Freshly isolated primary human pDCs were exposed to native or oxidatively modified mtDNA extracted from untreated or antimycin A-exposed HL-60 cells. In parallel experiments pDCs were pre-incubated for 1 h with specific TLR9 antagonist (TTAGGG) and then treated with native or oxidized mtDNA. Following a 24-h activation, expression of CD86 (A), HLA-DQ (B) and CD83 (C) was determined by flow cytometry. Relative fluorescence was calculated using the respective isotype-matched control for each monoclonal antibody. Data are presented as means ± SE of three independent experiments. ** $p<0.01$, **** $p<0.0001$ vs. untreated cells; ## $p<0.01$, #### $p<0.0001$ vs. mtDNA- or ox-mtDNA-treated cells. Ox-mtDNA: oxidatively modified mtDNA.

Figure 3. Cytokine and chemokine secretion of primary human pDCs in response to native or oxidized mtDNA. ELISA was used to assess the concentration of released TNF-α (A) and IL-

8 (B) from primary human pDCs in response to a 24-h exposure to native or oxidatively modified mtDNA purified from untreated or antimycin A-treated HL-60 human cells. In parallel experiments, mtDNA was administered in combination with a specific TLR9 inhibitor (TTAGGG). Data are presented as means ± SE of three independent experiments. * $p<0.05$ vs. untreated cells; # $p<0.05$ vs. mtDNA- or ox-mtDNA-treated cells. Ox-mtDNA: oxidatively modified mtDNA.

Figure 4. Type I IFN production from primary human pDCs in response to native or oxidized mtDNA. Freshly isolated primary human pDCs were treated with native or oxidatively modified mtDNA purified from untreated and oxidative stress-exposed HL-60 human cells alone or in complex with LL-37 protein in the absence or presence of TLR9 antagonist (TTAGGG). After a 24-h incubation the levels of IFN-α in the supernatant of cells were determined by ELISA. Data are presented as means± SE of three individual experiments. *** $p<0.001$, **** $p<0.0001$ vs. untreated cells. Ox-mtDNA: oxidatively modified mtDNA, N/D: not determined.

Figure 5. Phenotypic analysis of blood pDCs isolated from mice injected with native or oxidatively modified mtDNA. For *in vivo* stimulation of pDCs, 129Sv female mice were injected with sterile PBS as a control or native and oxidatively modified mtDNA preparations isolated from untreated cells or antimycin A-exposed murine SP2/0-Ag14 cells in complex with DOTAP cationic liposomal transfection reagent. Blood cells from mice were collected 6 h after PBS or mtDNAs injection and stained as described in Materials and Methods. Mouse pDCs were gated as mPDCA-1^+ cells and the expression of mouse CD86 (A) and I-A/I-E (B) was determined on the gated mPDCA-1^+ pDC population. Relative fluorescence was calculated using the respective isotype-matched control for each monoclonal antibody. Data

are presented as means ± SE of 13 animals in four independent experiments. ** p<0.01, **** p<0.0001 vs. PBS-treated control mice. Ox-mtDNA: oxidatively modified mtDNA.

Figure 6. Cytokine and chemokine levels in the sera of mice injected with native or oxidized mtDNA. Sera from 129Sv mice were collected at 6 h after injection with PBS (control), as well as native or oxidatively modified mtDNA extracted from untreated or antimycin A-exposed murine SP2/0-Ag14 cells and then tested for IL-6 (A), TNF-α (B), CXCL1/KC (C), and IFN-α (D) proteins using specific ELISA kits. Data are presented as means ± SE of 17 animals in four independent experiments. *** p<0.001, **** p<0.0001 vs. PBS-treated control mice. Ox-mtDNA: oxidatively modified mtDNA, N/D: not determined.

Figure 1

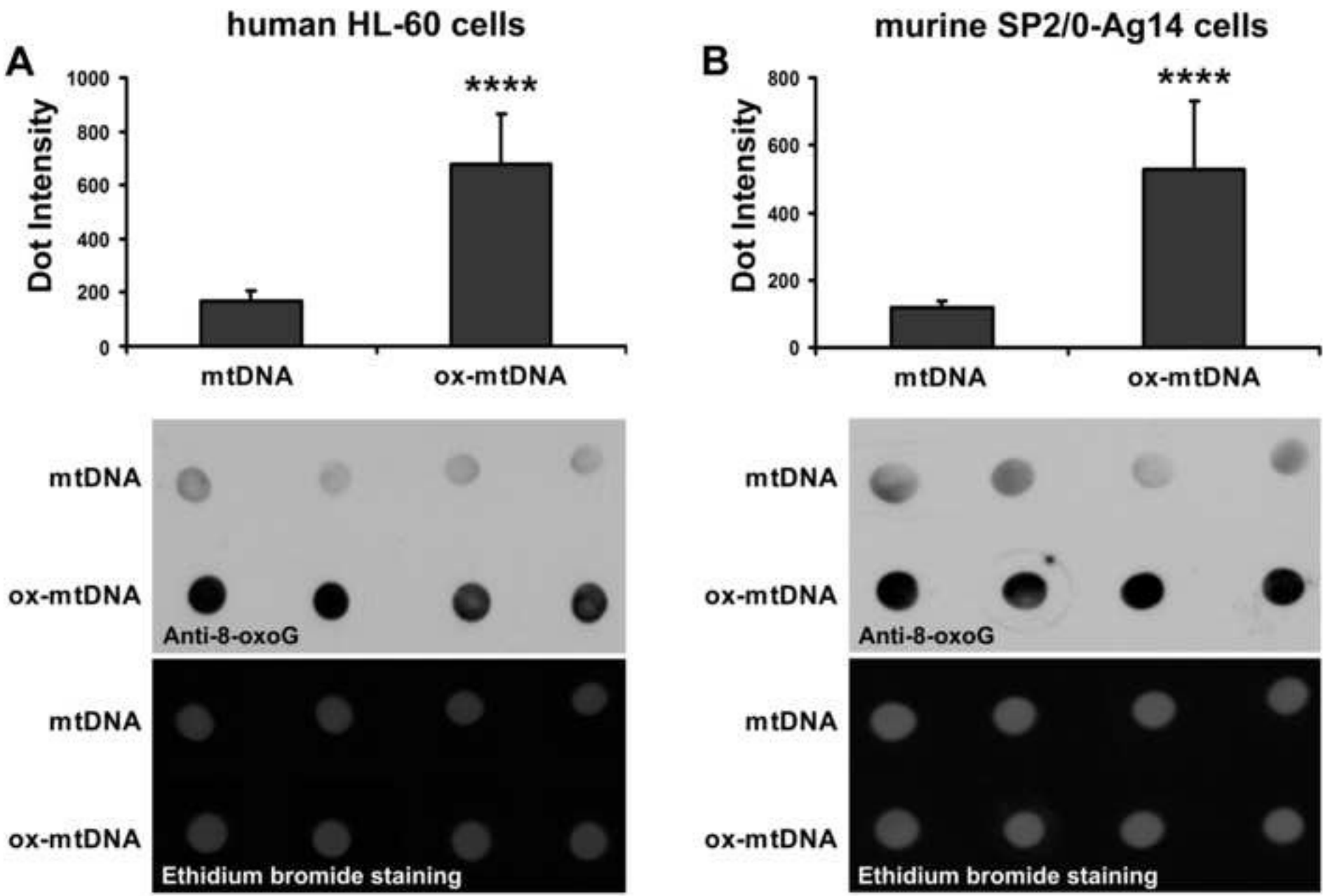


Figure 2

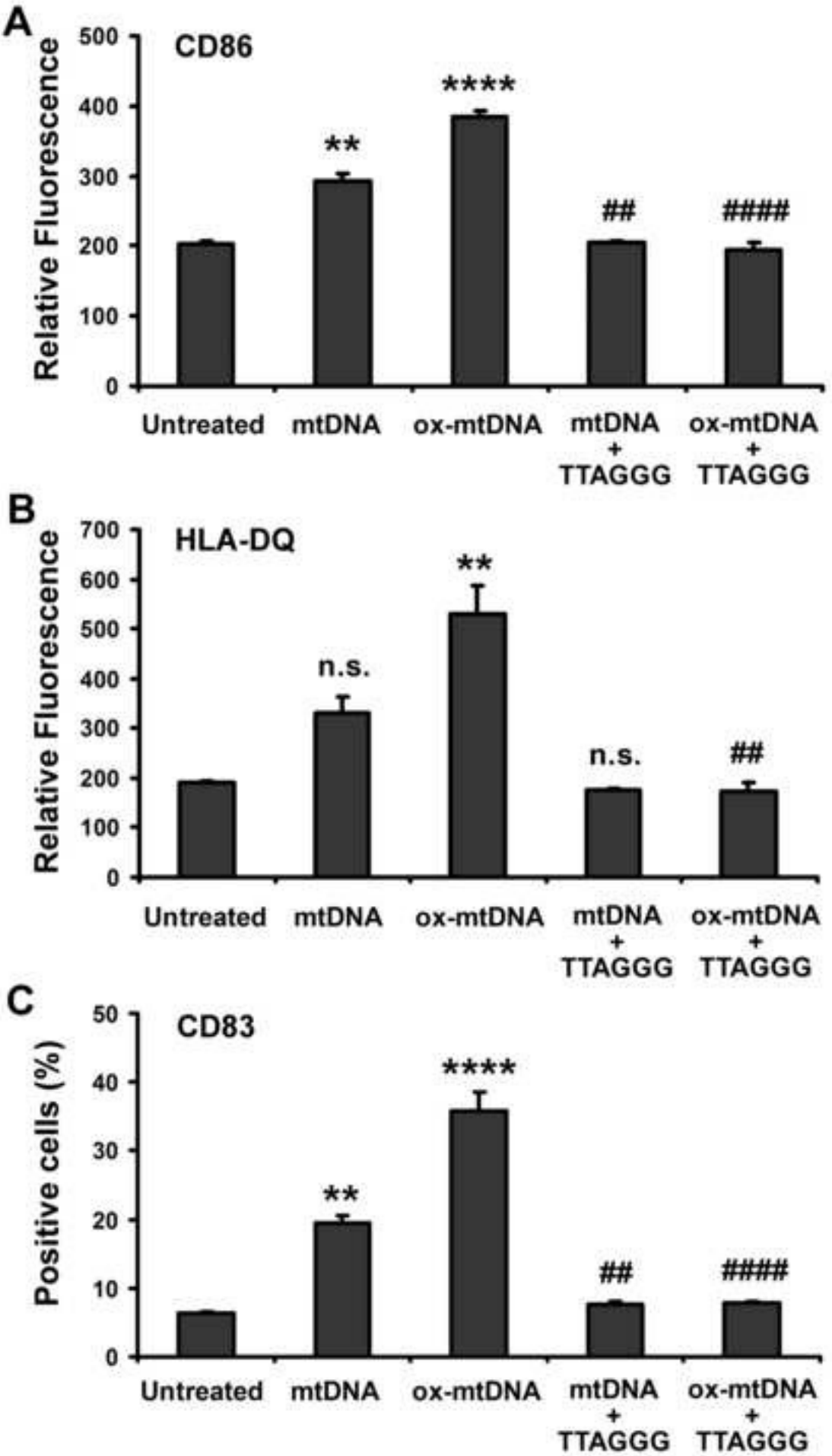
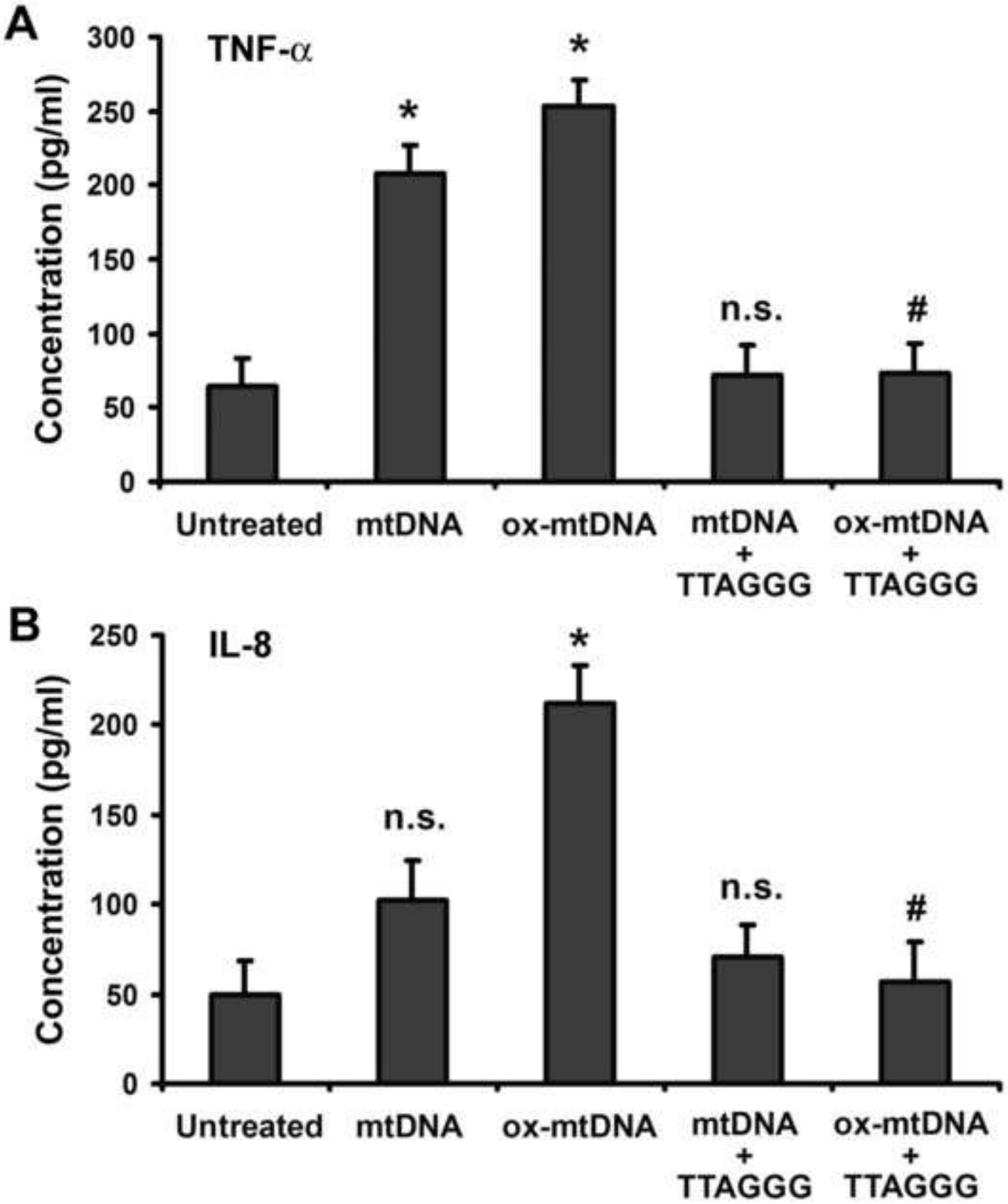


Figure 3



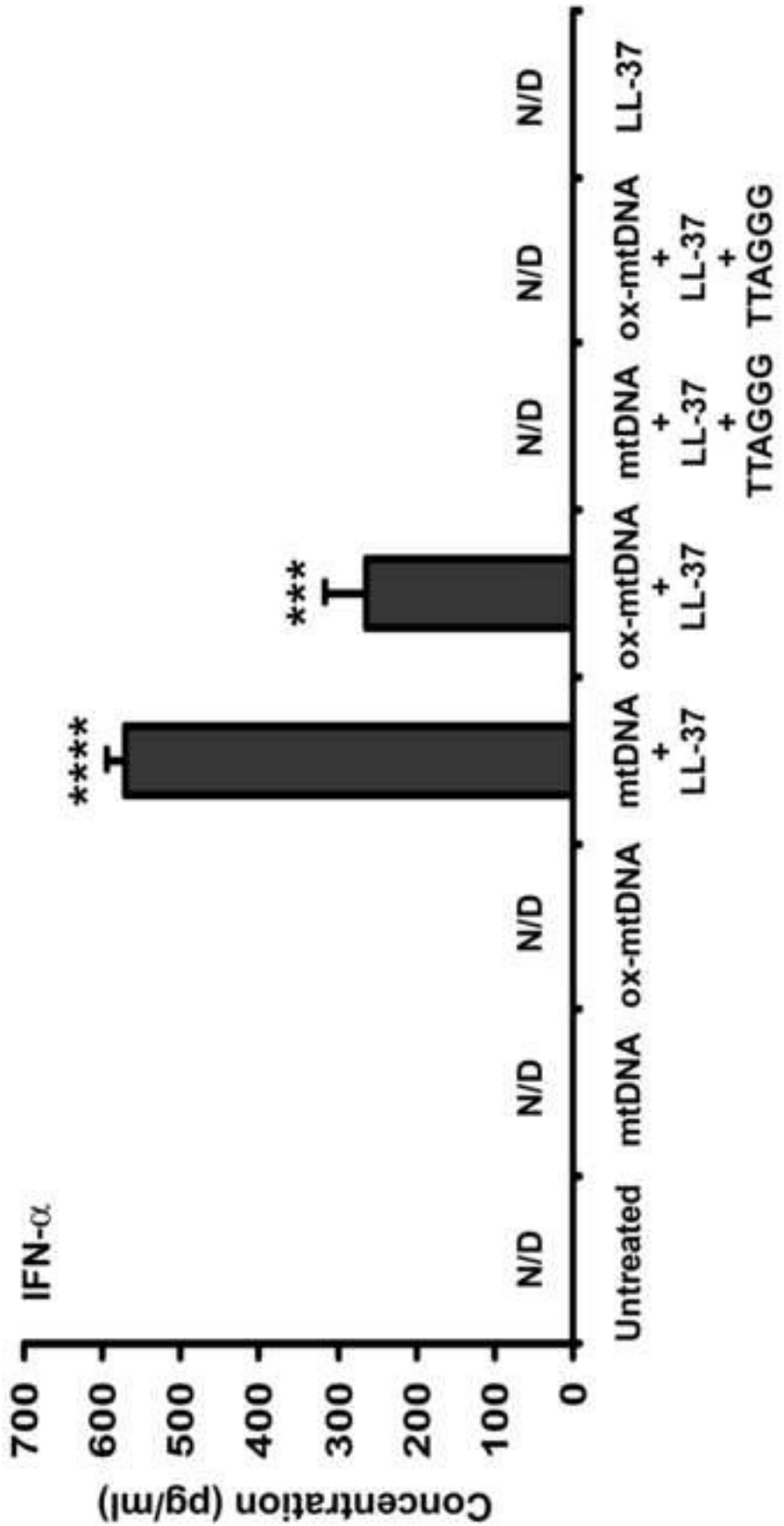
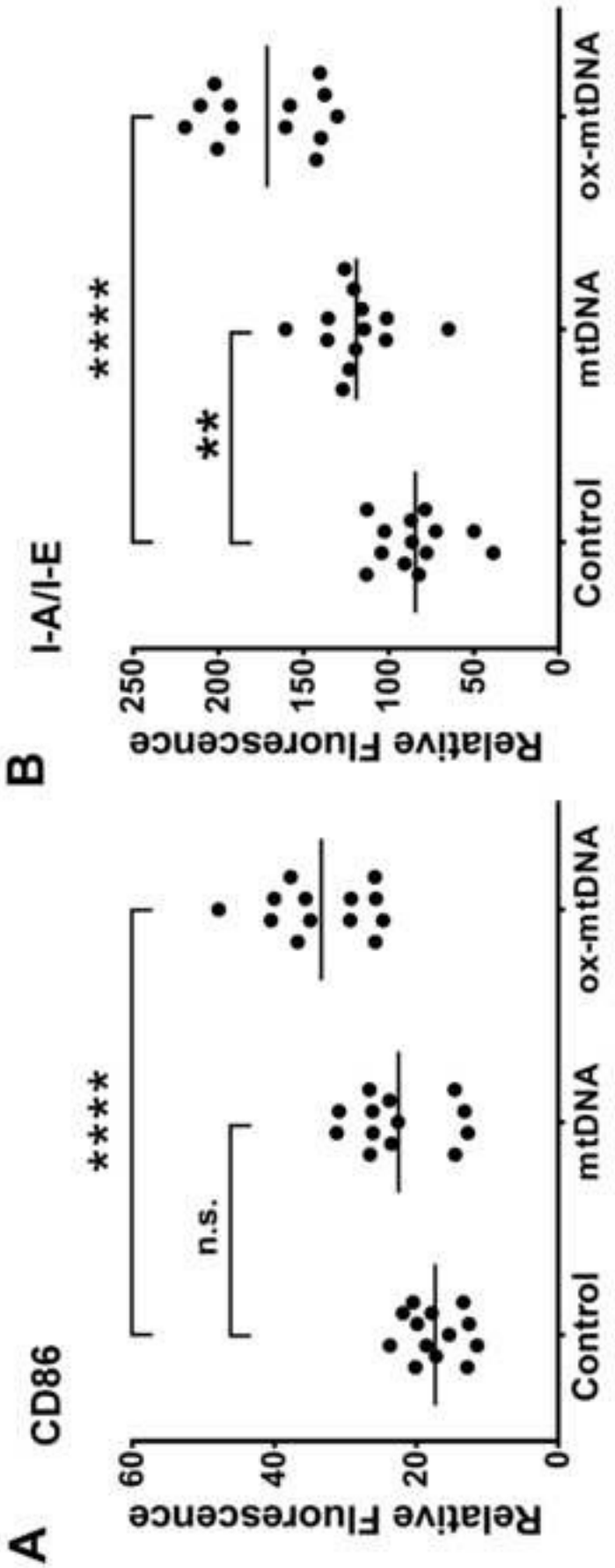

IFN-α
Concentration (pg/ml)
700
600
500
400
300
200
100
0
****
***
N/D
N/D
N/D
N/D
N/D
Untreated
mtDNA
ox-mtDNA
mtDNA + LL-37
ox-mtDNA + LL-37
mtDNA + LL-37 + TTAGGG
ox-mtDNA + LL-37 + TTAGGG
LL-37

Figure 4


A
CD86
Relative Fluorescence
60
40
20
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Control
mtDNA
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B
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250
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150
100
50
0
**
****
Control
mtDNA
ox-mtDNA

Figure 5

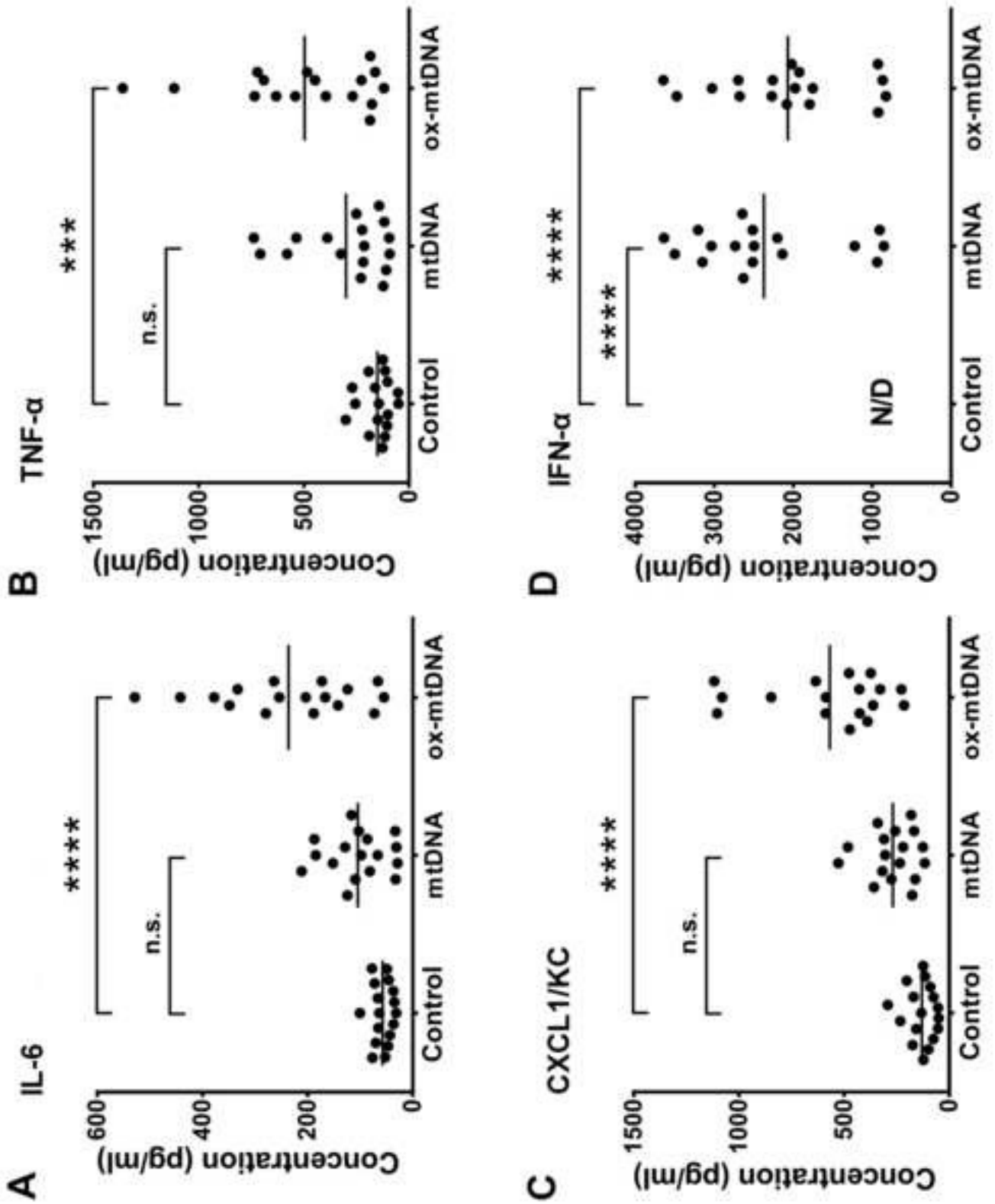

Figure 6