



Original article

Oxidized base 8-oxoguanine, a product of DNA repair processes, contributes to dendritic cell activation

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ABSTRACT

A growing body of evidence suggests that elevated levels of reactive oxygen species (ROS) in the airways caused by exposure to gas phase pollutants or particulate matter are able to activate dendritic cells (DCs); however, the exact mechanisms are still unclear. When present in excess, ROS can modify macromolecules including DNA. One of the most abundant DNA base lesions is 7,8-dihydro-8-oxoguanine (8-oxoG), which is repaired by the 8-oxoguanine DNA glycosylase 1 (OGG1)-initiated base excision repair (BER) (OGG1-BER) pathway. Studies have also demonstrated that in addition to its role in repairing oxidized purines, OGG1 has guanine nucleotide exchange factor activity when bound to 8-oxoG. In the present study, we tested the hypothesis that exposure to 8-oxoG, the specific product of OGG1-BER, induces functional changes of DCs. Supporting our hypothesis, transcriptome analysis revealed that in mouse lungs, out of 95 genes associated with DCs' function, 22 or 42 were significantly upregulated after a single or multiple intranasal 8-oxoG challenges, respectively. In a murine model of allergic airway inflammation, significantly increased serum levels of ovalbumin (OVA)-specific IgE antibodies were detected in mice sensitized via nasal challenges with OVA + 8-oxoG compared to those challenged with OVA alone. Furthermore, exposure of primary human monocyte-derived DCs (moDC) to 8-oxoG base resulted in significantly enhanced expression of cell surface molecules (CD40, CD86, CD83, HLA-DQ) and augmented the secretion of pro-inflammatory mediators IL-6, TNF and IL-8, whereas it did not considerably influence the production of the anti-inflammatory cytokine IL-10. The stimulatory effects of 8-oxoG on human moDCs were abolished upon siRNA-mediated OGG1 depletion. Collectively, these data suggest that OGG1-BER-generated 8-oxoG base-driven cell signaling activates DCs, which may contribute to initiation of both the innate and adaptive immune responses under conditions of oxidative stress.

1. Introduction

The lungs are constantly exposed to a large number of environmental stimuli including various antigens and compounds with oxidative potential. Dendritic cells (DCs), forming a network beneath the airway epithelium, continuously monitor the inhaled materials to sense potentially harmful microbes and substances. When DCs detect danger and/or damage signals during internalization of foreign or self-antigens, they become activated, release pro-inflammatory cytokines and chemokines, migrate to the draining lymph nodes, where they present

antigen to naïve T lymphocytes and thus initiate adaptive immune responses [1]. An increasing body of evidence suggests that reactive oxygen species (ROS) in the airways induced by exposure to gas phase pollutants such as ozone [2] and nitrogen oxides [3], or to pollens [4,5] and particulate matters [6] are all able to activate DCs; however, the exact mechanisms still need to be defined.

Increased levels of ROS can damage all types of biological macromolecules, i.e., proteins, lipids, and even DNA [7]. The primary target of free-radicals in the DNA is guanine, due to its lowest oxidation potential among the DNA bases, and several oxidation products can be

Abbreviations: 7-AAD, 7-aminoactinomycin-D; 8-oxoG, 7,8-dihydro-8-oxoguanine; BER, base excision repair; DCs, dendritic cells; moDCs, monocyte-derived DCs; MyD88, myeloid differentiation primary response 88; OGG1, 8-oxoguanine DNA glycosylase 1; OGG1-BER, OGG1-initiated BER; OVA, ovalbumin; ROS, reactive oxygen species; Tfh, follicular helper T cells; TLR, Toll-like receptor

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formed from it [8,9]. Among these oxidation products 7,8-dihydro-8-oxoguanine (8-oxoG) is the most abundant and its accumulation in DNA is considered to be one of the best biomarkers of oxidative stress [10]. If 8-oxoG is present in the DNA and encountered by the DNA replication machinery, adenine can be misincorporated into the newly synthesized DNA strand; therefore, 8-oxoG is considered as a mutagenic base lesion [11]. To prevent its accumulation and mutagenic effects, the intrahelical 8-oxoG is recognized and excised primarily by 8-oxoguanine DNA glycosylase 1 (OGG1) during base excision repair (BER) processes [12,13].

In a recent series of publications we have shown that the release of the 8-oxoG base from DNA, and its binding to cytosolic OGG1 mediates the activation of small GTPases, including K-Ras [14,15], Rac1 [16] and RhoA [17], and subsequent downstream signaling molecules [15,18,19]. Furthermore, OGG1 has also been shown to bind 8-oxoG lesions in promoter sites of NF- κ B-activated genes, thus facilitating rapid transcriptional responses following oxidative stress [19]. In addition, it has recently been demonstrated that OGG1-initiated BER (OGG1-BER) mimicked by a single 8-oxoG challenge increases gene expression involved in homeostatic, immune system-related, and macrophage activation processes mediated by chemokines, cytokines, integrin and interleukin signaling pathways [20]. On the other hand, ongoing OGG1-BER during intermittent exposure to environmental oxidative agents and/or during chronic inflammation induces gene expression resulting in epithelial alterations, increased smooth muscle mass and collagen deposits in the airways [21].

In this particular study, we tested the hypothesis that OGG1-BER processes producing 8-oxoG can induce DCs activation. To this end, we utilized murine models and the *in vivo* obtained data were supported by *in vitro* studies using human monocyte-derived DCs (moDC).

2. Materials and methods

2.1. Treatment of animals for transcriptome analysis

Animal experiments were performed according to the NIH Guide for Care and Use of Experimental Animals and approved by the UTMB Animal Care and Use Committee (approval no. 0807044A). Eight-week-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME, USA) were used for these studies. Mice ($n = 5$ per group) were challenged intranasally (i.n.) on day 0 (single challenge) or days 0, 2 and 4 (multiple challenge) with 60 μ L of pH-balanced 8-oxoG (Cayman Chemicals, Ann Arbor, MI, USA) solution (pH: 7.4; 1 μ M), or saline under mild anesthesia [14]. The lipopolysaccharide concentration was below detectable levels in all reagents. Animals were sacrificed at various time points (0, 30, 60 and 120 min) after a single or multiple challenges to isolate lung RNA as previously described [20,21].

2.2. RNA isolation

After intranasal challenge, mouse lungs were excised and homogenized in lysis buffer (Qiagen, Valencia, CA, USA) with a TissueMiser® (Fisher, Pittsburgh, PA, USA). RNA was extracted using an RNeasy kit (Qiagen) as previously described [20,21]. The RNA concentration was determined spectrophotometrically on an Epoch Take-3™ system (BioTek, Winooski, VT, USA) using Gen5 version 2.01 software. Equal amounts of RNA from each mouse lung within an experimental group ($n = 5$) were pooled and analyzed in triplicate.

2.3. Next-generation RNA sequencing

Library construction and deep sequencing analysis were performed in UTMB's Next-Generation Sequencing (NGS) Core Facility (Director: Dr. Thomas G. Wood) on an Illumina HiSeq 1000 sequencing system (Illumina Inc., San Diego, CA, USA) as previously described [20,21]. Briefly, poly(A)⁺ RNA was selected from total RNA (1 μ g) with poly(T)

oligo-attached magnetic beads. Bound RNA was fragmented by incubation at 94 °C for 8 min in 19.5 μ L of fragmentation buffer (Illumina, Part # 15016648). First- and second-strand synthesis, adapter ligation, and amplification of the library were performed using the Illumina TruSeq RNA Sample Preparation kit per the manufacturer's instructions. Samples were tracked through the "index tags" incorporated into the adapters. Library quality was evaluated using an Agilent DNA-1000 chip on an Agilent 2100 Bioanalyzer. Library DNA templates were quantitated by qPCR and a known-size reference standard.

Cluster formation of the library DNA templates was performed using the TruSeq PE Cluster Kit version 3 (Illumina) and the Illumina cBot workstation under the conditions recommended by the manufacturer. Template input was adjusted to obtain a cluster density of 700–1000 K/mm². Paired-end, 50-base sequencing-by-synthesis was performed with a TruSeq SBS kit version 3 (Illumina) on an Illumina HiSeq 1000 per the manufacturer's protocols. Base calls were converted to sequence reads using CASAVA-1.8.2. Sequence data were analyzed with the Bowtie2, Tophat and Cufflinks programs using the National Center for Biotechnology Information's (NCBI's) mouse (*Mus musculus*) genome build reference mm10. RNA-Seq data have been deposited in the NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession numbers [GSE61095](#) and [GSE65031](#). Reads per kilobase of transcript per million (RPKM) were normalized to the corresponding control for each experimental group [22].

To confirm transcript levels for selected genes, an SAB biosciences RT Profiler PCR Array assay (PAMM-090A, Qiagen) was used per the manufacturer's instructions, using SYBR®Green. qRT-PCR was performed on an ABI7000 Sequence Detector (Life Technologies, Grand Island, NY). Quantification of changes in gene expression was calculated by using the $\Delta\Delta C_t$ method and unstimulated cells as the calibrator and then normalized to GAPDH [23].

2.4. Gene ontology analysis

Heat maps and hierarchical clusters from whole transcriptomes were constructed with Morpheus online software from Broad Institute (<https://software.broadinstitute.org/morpheus>). Venn diagrams were constructed using online Venny 2.1 software (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>). To create a list of genes that are documented to be linked to activation of murine DCs a gene ontology browser at the Mouse Genome Informatics website (MGI, The Jackson Laboratory, Bar Harbor, Maine, http://www.informatics.jax.org/vocab/gene_ontology/) and the gene list of the Mouse Dendritic and Antigen Presenting Cell RT² Profiler PCR Array (Qiagen) were utilized.

2.5. Treatment of animals for the measurement of IgM and IgE production

Eight-week-old female BALB/c mice ($n = 8$ per group) were challenged intranasally with 8 μ g/mouse ovalbumin (OVA, Grade V, A5503, Sigma-Aldrich, St. Louis, MO, USA) alone or in combination with 60 μ L of 1 μ M pH-balanced 8-oxoG solution on days 0–4, 18 and 28 under mild anesthesia. In controls, intranasal challenges were performed with 60 μ L of PBS. On day 30, animals were sacrificed and sera were collected for the measurement of IgM or IgE production. The levels of OVA-specific IgM and IgE in the mice' sera were measured by ELISA kit (Southern Biotech, Birmingham, AL, USA), according to the manufacturer's instructions. Absorbance was detected by a Synergy HT microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 450 nm.

2.6. Generation and treatment of moDCs

Leukocyte-enriched buffy coats were obtained from healthy blood donors drawn at the Regional Blood Center of the 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 (Hungary). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient, and monocytes were separated from PBMCs by positive selection using magnetic anti-CD14 microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany), according to the manufacturer's instructions. For gene silencing experiments freshly isolated monocytes were electroporated as described below. For DC differentiation electroporated and non-electroporated monocytes were cultured in 24-well tissue culture plates at a density of 1×10^6 cells/ml in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO, USA), supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Biosera Ltd, Ringmer, UK), 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), 80 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech EC, London), and 100 ng/ml IL-4 (Peprotech EC, London, UK). On day 2, the same amounts of GM-CSF and IL-4 were added to the cell cultures. The moDCs were challenged at day 5 of culturing for 24 h with increasing concentration of 8-oxoG (1, 10 or 100 µM), Toll-like receptor (TLR) 7/8 ligand CL075 (0.5 µg/ml, InvivoGen, San Diego, CA, USA) or TLR9 agonist CpG-B (5 µM, ODN2006; Hycult Biotech, Uden, The Netherlands). To inhibit myeloid differentiation primary response 88 (MyD88)-dependent pathways, cells were pre-incubated with the MyD88 inhibitory peptide (40 µM, Pepinh-MYD, InvivoGen) or its negative control peptide (40 µM, Pepinh-Control, InvivoGen) for 6 h and then exposed to 8-oxoG base or TLR agonists for an additional 24 h. During incubation periods cells were maintained at 37 °C in a humidified CO₂ incubator.

2.7. Depletion of OGG1 expression

Freshly isolated monocytes were electroporated with 3 µM siGENOME Smartpool OGG1-specific or siGENOME non-targeting small interfering RNA (siRNA; Dharmacon, Pittsburgh, PA, USA) in Opti-MEM medium (Life Technologies) in 4-mm cuvettes (Bio-Rad Laboratories GmbH, Munich, Germany) using GenePulser Xcell instrument (Bio-Rad). Following transfection the cells were seeded as described above. During DC differentiation IL-4 and GM-CSF were replenished on day 2. On day 5, depletion of OGG1 was determined by western blotting.

2.8. Western blot analysis

Cells were lysed in Laemmli buffer and the protein samples were separated on SDS–PAGE gels, and transferred onto nitrocellulose membranes using wet electro-blotting. Nonspecific binding was blocked by TBS-Tween containing 5% non-fat dry milk for 1 h at room temperature and probed with antibody recognizing OGG1 (rabbit monoclonal; Abcam, Cambridge, UK) overnight at 4 °C. Primary antibody was detected using horseradish peroxidase-conjugated secondary antibody (anti-rabbit; Bio-Rad Laboratories, Munich, Germany) for 1 h at room temperature. Anti-β-actin was used as a loading control (mouse monoclonal; Santa-Cruz Biotechnology, Santa Cruz, CA, USA). Protein samples were visualized by ECL system (SuperSignal West Pico/Femto chemiluminescent substrate; Thermo Scientific, Rockford, IL, USA). The protein bands were scanned, and band density was determined using Kodak 1D Image Analysis software, version 3.6 (Kodak Digital Science Imaging, Eastman Kodak, New Haven, CT, USA). Relative density was calculated by the ratio of OGG1 versus β-actin band intensities.

2.9. Flow cytometric analysis

Cell surface protein expression was analyzed by staining the cells with FITC-labeled monoclonal antibodies against CD40 and CD209, PE-labeled anti-CD14, anti-CD86 and anti-HLA-DQ, PE-Cy5-labeled anti-CD83 and APC-conjugated anti-CD1a and isotype-matched control antibodies. All of the monoclonal antibodies and the isotype-matched control antibodies were obtained from BioLegend (San Diego, CA,

USA). Fluorescence intensities were measured by FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, Franklin Lakes, NJ, USA). In all flow cytometric measurements isotype-matched control antibodies were used to detect non-specific background signals according to the manufacturer's recommendation. Relative fluorescence intensity values were calculated as a ratio of median fluorescence intensity of specific to non-specific isotype-matched antibodies. Data analysis was performed by FlowJo software (TreeStar, Ashland, OR, USA). Cell viability was determined by 7-aminoactinomycin-D (7-AAD; 10 µg/ml; Sigma-Aldrich) staining for 15 min immediately before flow cytometric analysis.

2.10. Measurement of cytokine and chemokine secretion of moDCs

Human ELISA kits (BD OptEIA; BD Biosciences, San Diego, CA, USA) were used to quantify IL-6, TNF, IL-10 cytokines, as well as IL-8 chemokine in moDC supernatants. Assays were performed according to the manufacturer's instructions. Absorbance measurements were performed using a Synergy HT microplate reader (Bio-Tek Instruments) at 450 nm.

2.11. Detection of intracellular ROS levels in moDCs

Five-day human moDCs were collected and washed with warm PBS and loaded with 50 µM 2',7'-dihydrochlorofluorescein diacetate (H₂DCF-DA; Invitrogen, Carlsbad, CA, USA) at 37 °C for 20 min. After removing the excess of fluorescent dye by repeated washing steps, cells were plated in 96-well black polystyrene plate at a density of 2×10^5 cells/200 µl in complete RPMI 1640 medium (Sigma-Aldrich). Cells were then exposed to increasing concentrations of 8-oxoG (1, 10 or 100 µM) or 100 µM H₂O₂ (as a positive control) and changes in DCF fluorescence intensity were detected in every 15 min at 530 nm by a Synergy HT microplate reader (Bio-Tek Instruments).

2.12. DNA extraction from moDCs for the detection of its 8-oxoG content

DNA was extracted from human moDCs treated with 8-oxoG (10 and 100 µM) or H₂O₂ (5 mM) for 1 h by salting out method as previously described [24] with some modifications. Briefly, a total of 2×10^6 cells were collected and washed with warm PBS. After washing step cells were resuspended in 250 µl of TNES buffer (10 mM Tris pH: 7.5, 400 mM NaCl, 100 mM EDTA pH: 8, 0.6% SDS) and mixed with 4 µl of proteinase K (Sigma-Aldrich). The mixture was kept in water bath at 56 °C for 1 h. After incubation 85 µl of 6 M NaCl was added and mixed vigorously. Samples were centrifuged at 15000 g for 15 min at 4 °C and then supernatants were collected in new Eppendorf tubes. Equal volume of cold absolute ethanol was added, mixed, and the tubes were kept at 4 °C for 10 min. The precipitated DNA was centrifuged at 15000 g for 10 min at 4 °C and the pellet was washed 2 times with 70% ethanol and allowed to air dry. The DNA was dissolved in 20 µl of TE buffer (Life Technologies Corporation) and used for dot blot experiments. The concentration and purity of isolated DNA were determined by NanoDrop spectrophotometer (Thermo Scientific, Rockford, IL, USA).

2.13. Dot blot analysis

To determine the 8-oxoG content in DNA extracted from 8-oxoG- and H₂O₂-treated moDCs, 2 µg of DNA was spotted onto a nitrocellulose membrane. After 1 h drying non-specific binding sites of the membrane were blocked for 1 h at room temperature with TBS-T buffer (50 mM Tris, 0.5 M NaCl, 0.05% Tween-20, pH 7.4) containing 5% dry milk. Following the blocking step the membrane was incubated with mouse anti-8-oxoG monoclonal antibody (1:1000, clone 483.15, MerckMillipore) 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. As a secondary antibody anti-mouse IgG conjugated

with horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used at a dilution of 1:10000. After 1 h incubation and repeated washing steps blot signals were visualized by ECL system (SuperSignal West Femto Chemiluminescent Substrate; Thermo Fisher Scientific). Spot intensities were quantified using the Image Studio Lite Software version 5.2 (LI-COR Biosciences, Lincoln, Nebraska USA). After detection of 8-oxoG in DNA, the same membrane was washed thoroughly with distilled deionized water and stained with ethidium bromide (1:1000, Sigma-Aldrich) dissolved in 1X TAE (Tris-acetate-EDTA) buffer for 1 h at room temperature. After rinsing the membrane with 1X TAE buffer, ethidium bromide binding was visualized and photographed under UV light in Azure c600 Imaging System (Azure Biosystems, Dublin CA, USA).

2.14. Statistical analysis

Data analyses were performed using Student's *t*-test or ANOVA, followed by Bonferroni post hoc tests with GraphPad Prism v.6. software (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Intranasal exposure of mice to the OGG1-BER product 8-oxoG induces changes in the expression of genes related to DC functions

We have recently described the effects of OGG1-BER process on global transcriptome of murine lungs [20,21]. To mimic OGG1-mediated generation of 8-oxoG in the airways, mice were challenged intranasally with 8-oxoG base, the specific product of OGG1-BER [25]. Mice were either exposed to a single dose of 8-oxoG or repeatedly challenged on days 0, 2 and 4 [20,21]. Total RNA was isolated from the lungs harvested at 0, 30, 60 and 120 min after single or third repeated challenge on day 4, pooled and subjected to RNA sequencing (Fig. 1A). After single challenge a total of 23,337 transcripts, while after repeated challenges a total of 18,678 transcripts were identified (GEO Series accession number [GSE61095](#) as well as [GSE65031](#)) [20,21]. In this particular study, we aimed to examine whether OGG1-BER processes can alter the expression of genes related to the functions of lung DCs. In order to do so, an online database (MGI) and the gene list from previous publications were utilized to create a list of genes that are documented to be associated with activation of murine DCs (Supplementary Table I). This gene list was “overlaid” onto the data set from RNA sequencing of samples collected after single and multiple 8-oxoG challenges. Clustering of the selected genes (cytokines, chemokines and their receptors; antigen uptake; antigen presentation; cell surface receptors; signal transduction) and fold changes of gene expression levels are displayed on a heat map (Fig. 1B) and shown in Supplementary Table II. The number of significantly upregulated (≥ 2 -fold) and downregulated (≤ -2 -fold) genes at each time point is depicted in Fig. 1C. After a single 8-oxoG challenge altogether 22 out of 95 genes related to DC activation and function were found to be significantly upregulated (Fig. 1B and Supplementary Table II). Multiple challenges with 8-oxoG induced more remarkable changes compared to a single challenge as they triggered upregulation of altogether 42 out of 95 genes associated with DC functions (Fig. 1B and Supplementary Table II). After exposure to a single 8-oxoG dose the total number of significantly upregulated genes did not show changes over the time, while in response to multiple exposures gene expression peaked at 60 min and did not exhibit further increase at 120 min (Fig. 1C). Venn diagrams display the numbers of unique and differentially expressed overlapping genes at each time point after 8-oxoG challenges (Fig. 1D) corresponding to the transcripts shown in Supplementary Table II. The highest number of differentially expressed genes was observed at 60 min after multiple challenges when significant upregulation of 22 unique genes as well as 12 overlapping genes with the single challenge were detected (Fig. 1D). Despite the fact

that none of the selected 95 genes are exclusively expressed by mouse lung DCs, coordinated upregulation of *Il12b* and several genes associated with antigen presentation suggests that OGG1-BER-mediated release of 8-oxoG may have an impact on DC activation.

3.2. Intranasal coadministration of OVA and the OGG1-BER product 8-oxoG intensifies OVA-specific IgE production in mice

It has previously been demonstrated that intranasal sensitization and challenge with OVA even in the absence of an adjuvant elicit all of the immunologic features of IgE-mediated allergic airway inflammation in murine lungs [26]. Thus, we used this model to investigate the immunomodulatory role of OGG1-driven DNA BER processes. As expected, repeated intranasal challenges of mice (Fig. 2A) with adjuvant-free OVA induced a significant increase in the serum levels of both OVA-specific IgM and IgE antibodies (Fig. 2B and C). Combined administration of OVA and 8-oxoG base resulted in a slight increase in the amount of OVA-specific IgM antibodies (Fig. 2B), whereas it triggered a significant rise in OVA-specific IgE production (Fig. 2C). Recent advances in follicular helper T cell (Tfh) biology have revealed that antigen presentation by DCs is necessary and sufficient to initiate Tfh differentiation program (reviewed in Ref. [27]). Since Tfh cells play a critical role in the regulation of IgE antibody production in allergic immune responses to airborne allergens [28], our observations strongly support the contribution of the OGG1-BER system to the activation of DCs in murine lungs.

3.3. Exogenous 8-oxoG alters the phenotype and changes cytokine and chemokine production of human moDCs

Results obtained from murine experiments prompted us to further investigate the direct effects of exogenous 8-oxoG on human moDCs. To this end, immature moDCs were treated with increasing concentrations (1, 10, and 100 μ M) of 8-oxoG, then expression of CD40 and CD86 costimulatory molecules, CD83 maturation marker, as well as HLA-DQ antigen-presenting molecule on moDCs was analyzed by flow cytometry (Fig. 3A–D). As we anticipated, exposure to exogenous 8-oxoG enhanced the expression of activation/maturation-associated cell surface molecules; however, only the highest applied concentration of 8-oxoG (100 μ M) induced statistically significant changes in the expression of all investigated markers (Fig. 3A–D).

To further examine the effects of exogenous 8-oxoG on the activation of human moDCs, cells were exposed to 1, 10, or 100 μ M 8-oxoG for 24 h, then levels of secreted IL-6, TNF- α and IL-10 cytokines and the IL-8 chemokine present in cell culture supernatants were measured by ELISA (Fig. 4A–D). Treatment of moDCs with 8-oxoG increased secretion of the pro-inflammatory cytokines IL-6, TNF and the chemokine IL-8 (Fig. 4A–C) in a concentration-dependent manner, whereas it had no effect on production of the anti-inflammatory cytokine IL-10 (Fig. 4D). Altogether, these data suggest that exogenous 8-oxoG alone is able to induce the activation and maturation of human moDCs via increasing the expression of antigen presenting and costimulatory molecules on their surface, and enhancing their pro-inflammatory and chemotactic factor production.

Several guanosine derivatives, which are structurally related to 8-oxoG, as well as oligodeoxynucleotides containing 8-oxoG have been reported to be potent immunostimulatory agents and induce activation of immune cells in a TLR7-, TLR7/8- or TLR9-dependent manner [29–32]. Therefore, we tested the involvement of these endosomal TLRs in the exogenous 8-oxoG-triggered activation of moDCs. Inhibition of MyD88 adaptor protein-mediated signaling cascades with Pepinh-MYD, a blocker of MyD88 homodimerization [33], did not influence either the 8-oxoG-induced expression of CD86 and CD83 (Supplementary Figs. 1A and B) or the production of IL-6 and IL-8 by moDCs (Supplementary Figs. 1C and D). In control experiments, inhibition of MyD88 significantly blocked the CL075- (a TLR7/8 ligand) or CpG-B- (a

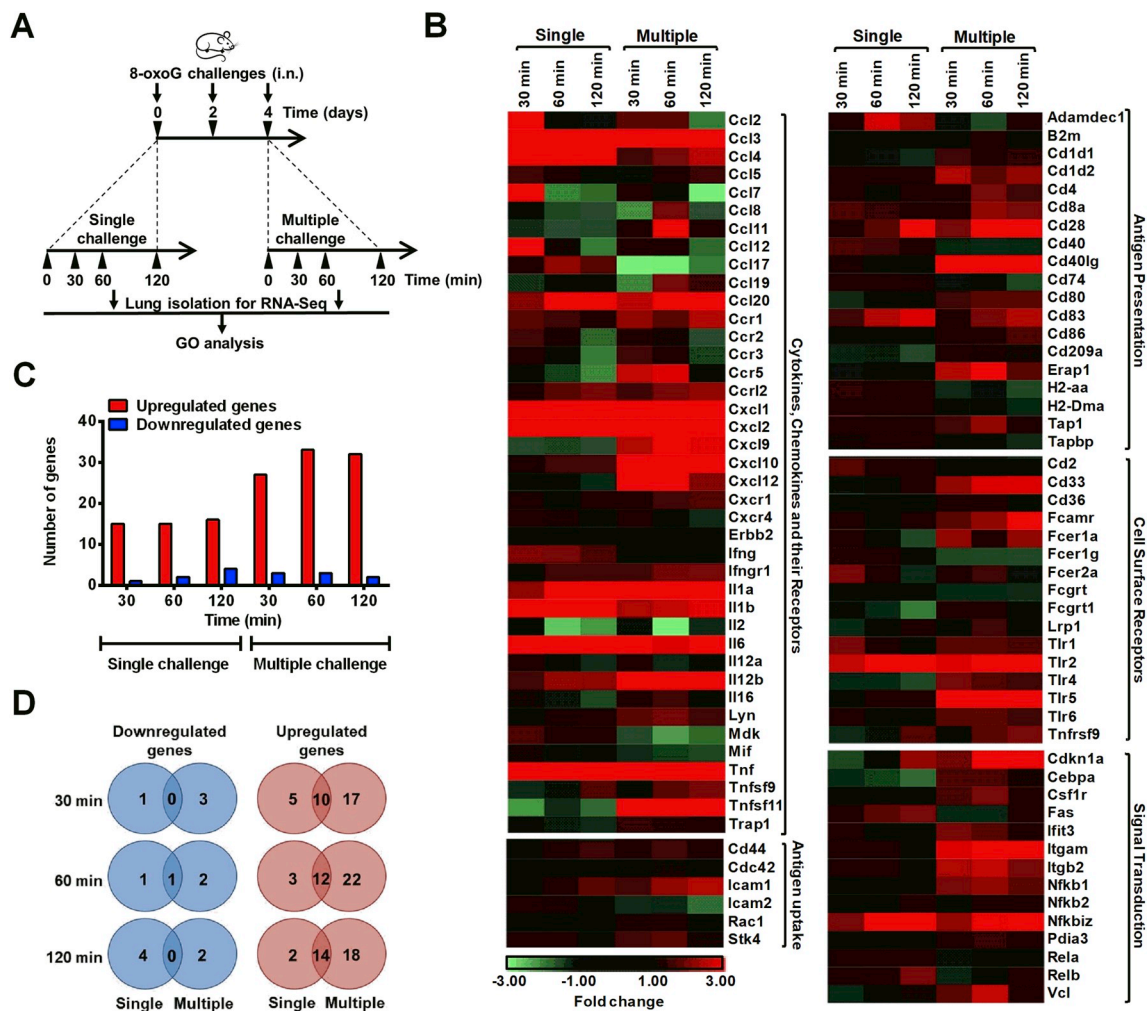


Fig. 1. Gene expression changes induced by the OGG1-BER product 8-oxoG are associated with DC activation in mice. Eight-week-old female BALB/c mice ($n = 5$ per group) were exposed to a single intranasal dose of OGG1-BER product 8-oxoG base (on day 0) or repeatedly challenged with it on days 0, 2 and 4. Lungs were harvested 0, 30, 60 and 120 min after the single challenge or the last challenge on day 4. Total RNA was subjected to RNA-Seq analysis as previously described [20,21]. Schematic representation of the experimental design is shown in panel (A). (B) The clustering of the selected genes associated with "DC activation" is demonstrated on heat map. (C) The diagram indicates the number of significantly upregulated (≥ 2 -fold) or downregulated (≤ -2 -fold) genes after single or repeated (multiple) 8-oxoG challenges. (D) Venn diagrams show the numbers of unique and overlapping transcripts altered by single or multiple 8-oxoG challenges linked to DC activation.

TLR9 agonist) initiated activation of the cells (Supplementary Figs. 1A–D). These results indicate that endosomal TLRs are not involved in the recognition of exogenous 8-oxoG base and thus in the induction of moDC activation.

3.4. Expression of OGG1 is required for 8-oxoG-induced activation of human moDCs

To confirm the involvement of OGG1 in the 8-oxoG-induced phenotypic and functional changes of human moDCs, the expression of OGG1 was downregulated by specific siRNA-mediated silencing. The efficacy of OGG1 depletion was verified by western blot analysis (Fig. 5A and B). We found that OGG1-specific siRNA downregulated the protein level of OGG1 by $\sim 80\%$ compared to the untreated or negative control siRNA-treated moDCs (Fig. 5B). In addition, the percentage of 7-AAD negative, viable cells were nearly 100% in all samples treated with negative control or OGG1-specific siRNAs, indicating that siRNA treatments did not influence the viability of the cells (Fig. 5C). Furthermore, on day 5 of differentiation, immature OGG1-silenced moDCs displayed normal DC phenotype showing low CD14 and high CD209 and CD1a levels (Fig. 5D and E) similar to non-silenced cells. In

addition, neither the baseline expression of the main activation markers such as CD86 and CD83 (Fig. 6A and B) nor the baseline production of IL-6 and IL-8 proinflammatory mediators (Fig. 6C) were influenced by OGG1 depletion. These data demonstrate that OGG1 silencing does not interfere with the differentiation and maturation processes in moDCs.

Next, we analyzed whether the 8-oxoG triggered signaling was OGG1-dependent. To this end, OGG1-depleted moDCs were exposed to 8-oxoG at various concentrations (10 μM and 100 μM) which consistently upregulated the expression of the tested cell surface markers and increased the production of cytokines in the previous set of experiments (Figs. 3 and 4). Strikingly, as shown in Fig. 7, OGG1 silencing abrogated the 8-oxoG-induced increase in the expression of CD86 (Fig. 7A) and CD83 (Fig. 7B) as well as in the secretion of IL-6 cytokine (Fig. 7C) and IL-8 chemokine (Fig. 7D) as compared to the negative control siRNA-transfected cells. These findings underpin our hypothesis that free 8-oxoG bases can activate human DCs in an OGG1-dependent manner.

Under oxidative stress conditions nonproductive binding of OGG1 to 8-oxoG in promoter sequences could be an epigenetic mechanism to augment the expression of proinflammatory genes [19]. Based on this previous observation we tested whether OGG1 depletion renders

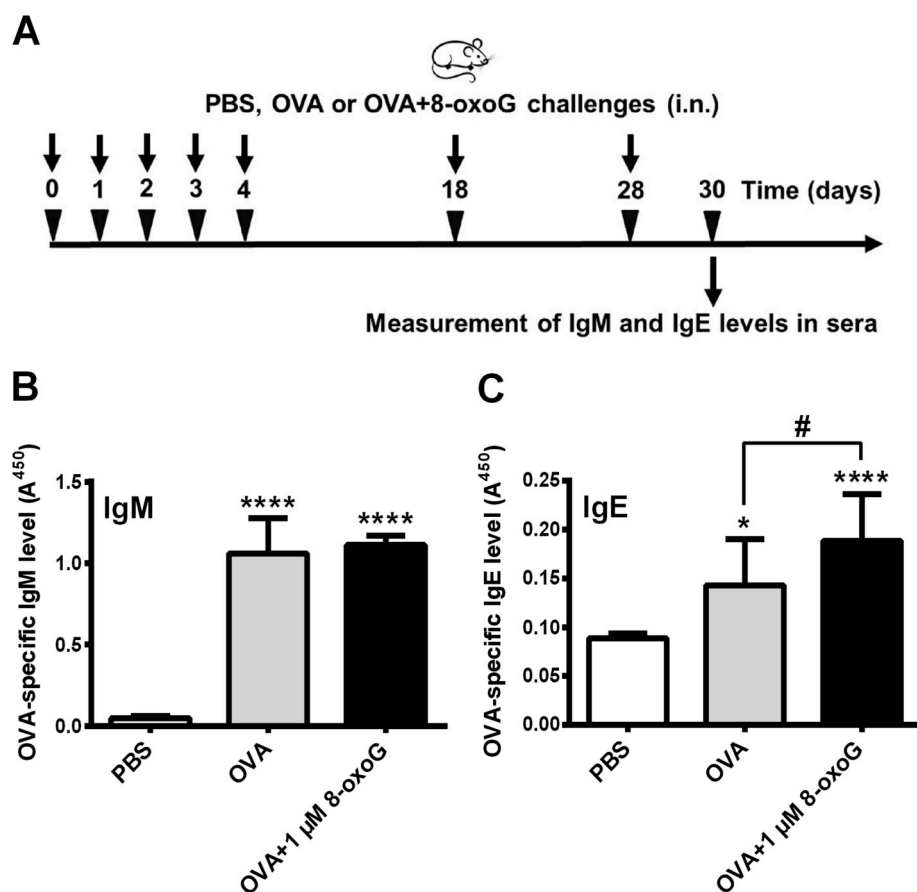


Fig. 2. Intranasal coadministration of OVA and 8-oxoG intensifies OVA-specific IgE production in mice. Eight-week-old female BALB/c mice ($n = 8$ per group) were challenged intranasally with OVA alone or in combination with 1 μ M 8-oxoG solution on days 0–4, 18 and 28 under mild anesthesia as indicated on the timeline of the experiments (A). On day 30, animals were sacrificed and sera were collected for measuring OVA-specific IgM (B) or IgE (C) levels by ELISA. Data are presented as means $\pm$ SD of three independent experiments and analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. * $p < 0.05$, **** $p < 0.0001$ vs PBS and # $p < 0.05$ vs. OVA.

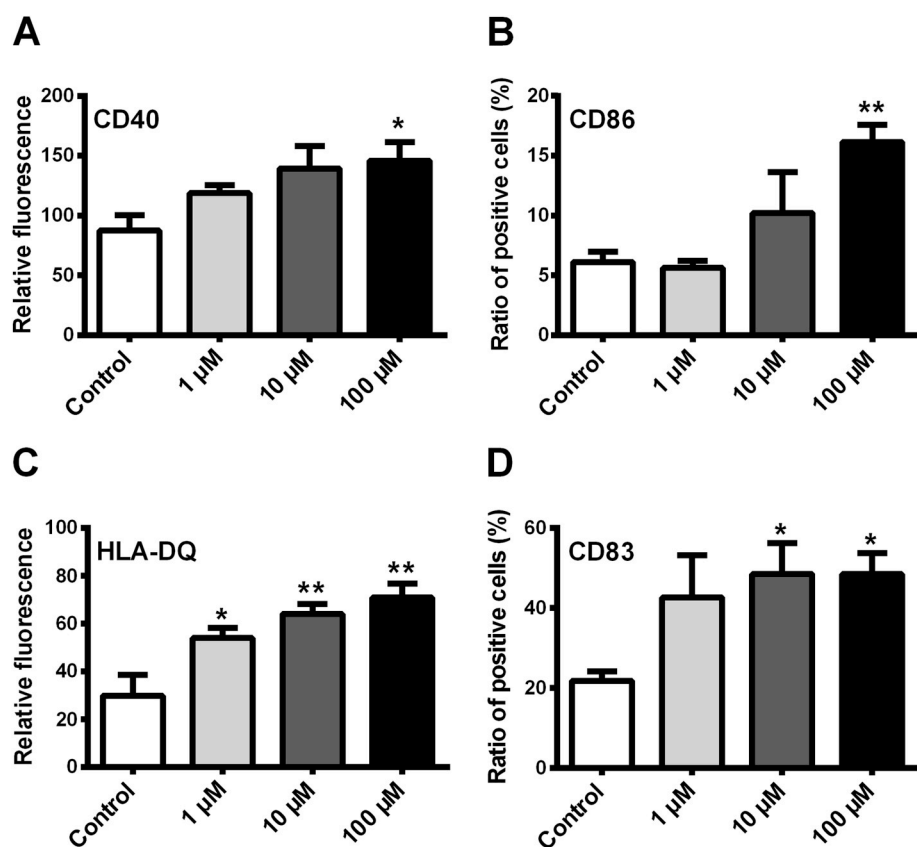


Fig. 3. 8-OxoG treatment induces phenotypic changes of moDCs. Human moDCs were exposed to increasing concentrations (1, 10 and 100 μ M) of 8-oxoG for 24 h and the expression of CD40 (A), CD86 (B), HLA-DQ (C), and CD83 (D) was determined by flow cytometry. Relative fluorescence was calculated using the respective isotype-matched control for each monoclonal antibody. Data are presented as means $\pm$ SD of four independent experiments and analyzed by one-way ANOVA followed by Bonferroni's test as post-hoc. * $P < 0.05$, ** $P < 0.01$ vs control.

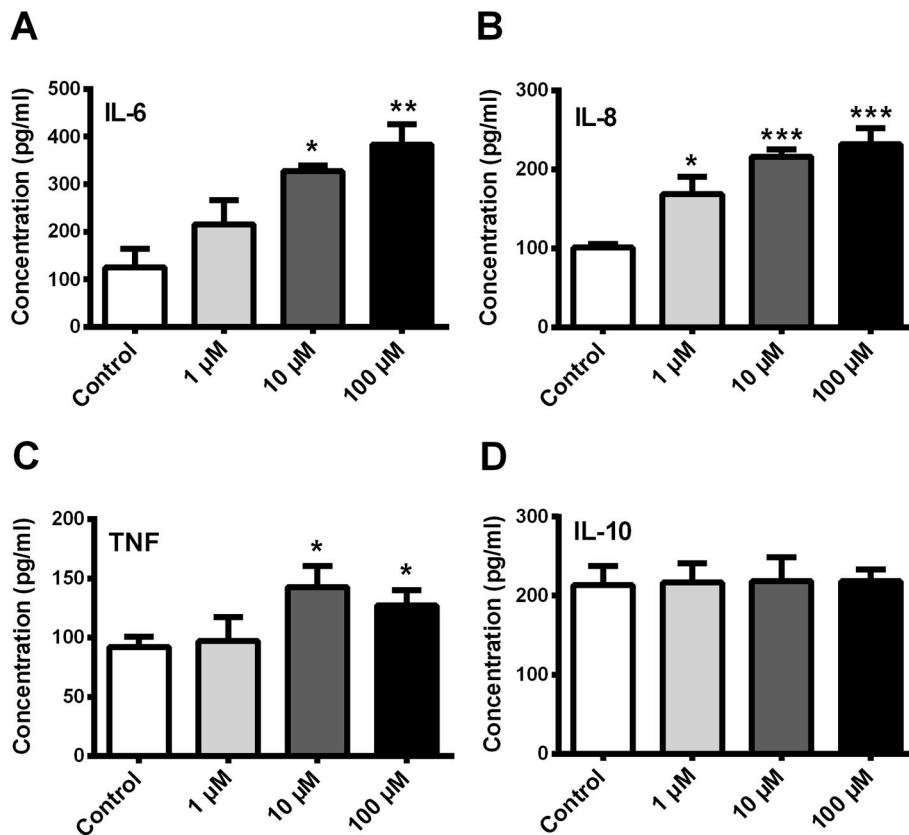


Fig. 4. Exogenous 8-oxoG augments the cytokine and chemokine secretion of moDCs. Human moDCs were treated with an increasing amount (1, 10 and 100 μM) of 8-oxoG for 24 h, then the concentration of released IL-6 (A), IL-8 (B), TNF (C) and IL-10 (D) was assessed by ELISA from cell culture supernatants. Data are presented as means $\pm$ SD of four independent experiments and analyzed by one-way ANOVA followed by Bonferroni's test as post-hoc. *P < 0.05, **P < 0.01, ***P < 0.001 vs control.

moDCs unresponsive to 8-oxoG treatment due to the lack of OGG1-driven transcriptional initiation. To this end, we measured the effects of exogenous 8-oxoG on intracellular ROS levels in moDCs loaded with redox-sensitive H₂DCF-DA. No changes in DCF fluorescence intensities were observed in 8-oxoG-exposed moDCs (Supplementary Fig. 2A). Furthermore, we also evaluated the extent of oxidative DNA damage in moDCs exposed to exogenous 8-oxoG using dot blot analysis. It was found that treatment of cells with exogenous 8-oxoG did not elevate 8-oxoG formation in DNA (Supplementary Fig. 2B). In control experiments, a significant increase both in intracellular DCF signals and in 8-oxoG content in DNA was detected in H₂O₂-treated moDCs as compared to untreated ones (Supplementary Figs. 2A and B). These data together indicate that exposure to exogenous 8-oxoG does not induce oxidative stress in human moDCs or formation of 8-oxoG in DNA; therefore, it does not trigger OGG1 binding to promoter sequences to facilitate the recruitment of site-specific transcription factors.

4. Discussion

As sentinels of the immune system, DCs constantly sample their microenvironment providing a crucial first line of cellular immune defense in the peripheral tissues. They capture, process and present antigens, and then migrate to secondary lymphoid organs where they interact with cognate naïve T cell clones to initiate their activation. Therefore, DCs play an essential role in linking innate and adaptive immune responses. Immature DCs respond to a broad spectrum of environmental danger or endogenous damage signals by extensive differentiation or maturation [34]. In reply to external or internal stimuli, DCs, along with other cells, orchestrate an inflammatory response. A plethora of molecules released from different cellular compartments or organelles by either living cells under stress conditions or from dying cells act as endogenous damage signals (reviewed in Ref. [35]). Several lines of evidence indicate that exposure of the lung to environmental oxidants (e.g., various gases, smoke, fine particles, chemicals) increase

8-oxoG levels in the DNA of lung resident and even in peripheral blood cells, as well as the exogenous 8-oxoG levels in body fluids (e.g., serum, urine, sputum, bronchoalveolar lavage fluid) (reviewed in Ref. [36]). Recently, we have demonstrated that extracellular mitochondrial DNA with higher 8-oxoG content has a potent immunostimulatory capacity on a special subset of DCs [31]. In this work we examined whether free 8-oxoG base, the product of OGG1-BER, exerts an immunomodulatory effect on DCs.

In our previous studies, effects of OGG1-BER were examined by genome-wide analysis of the mouse lung transcriptome. Mouse lungs were challenged with free 8-oxoG base and gene expression changes were assessed by next generation RNA-sequencing [20,21]. Transcriptome analysis revealed that both single and multiple challenges with free 8-oxoG base upregulate mRNAs associated with various biological processes including homeostasis, immune system, macrophage activation, responses to stimulus and metabolism [20,21]. Among the immune system processes inflammation was represented by increased expression levels of chemokines/cytokines, interleukins and various signaling molecules upon 8-oxoG challenge [20]. These previous findings suggested that among the genes altered by OGG1 signaling, several ones may affect function of DCs whose activation might contribute to oxidative stress-associated inflammatory responses. Therefore, based on online database and literature data we selected 95 genes that are previously proven to be related to DC activation and function. Our previous transcriptome analysis data were used to reveal how the expression of these 95 genes changed after a single and multiple 8-oxoG challenges. We found that a relatively small fraction of these selected genes associated with DC function (22 out of 95) was upregulated after a single challenge. However, this is not surprising given the fact that pulmonary DCs are sparsely distributed in the airways, representing around 1% of all cells in the epithelium [37]. Despite this limitation, our analysis revealed that 8-oxoG base increased expression of many genes linked to DC functions in innate and adaptive immune responses. For example, both single and multiple challenges with 8-oxoG

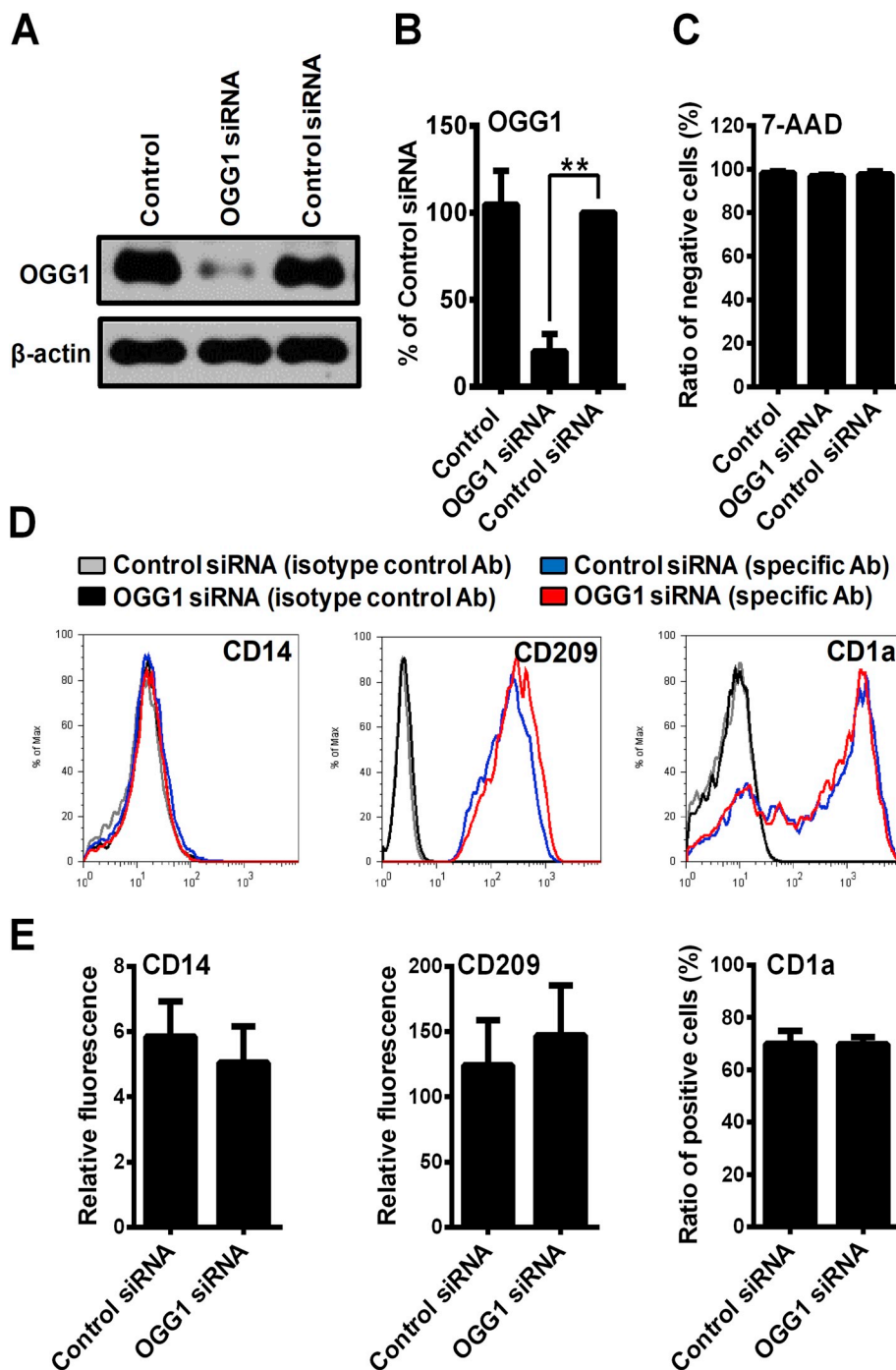


Fig. 5. Downregulation of OGG1 expression does not alter the viability and the differentiation processes of moDCs. Freshly isolated human monocytes (day 0) were electroporated with OGG1-specific or non-targeting (control) siRNA. (A) At day 5, depletion of OGG1 in moDCs was verified by western blotting. Representative blots from four independent experiments are shown. (B) The bar graph indicates silencing efficiency of OGG1 as compared with negative control siRNA, which is taken as 100%. The means $\pm$ SD of four independent experiments are shown. The cell viability (C) and the expression level of CD14, CD209 and CD1a (D and E) cell surface proteins were analyzed by flow cytometry. Representative histograms from 4 independent experiments are shown in panel (D), whereas bar graphs represent the means $\pm$ SD of four individual experiments in (C) and (E). Data were analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. $^{**}p < 0.01$ vs control siRNA.

upregulated genes associated with pro-inflammatory mediators including chemokines (Ccl3, Ccl20, Cxcl1, Cxcl2) and cytokines (Il1a, Il1b, Il6, Tnf). The chemokines CCL3 and CCL20 regulate migration of DCs, which upon activation mature into effective antigen presenting cells [38].

Antigen presentation by DCs is required for the activation of naive T lymphocytes, such as Tfh cells which then initiate the differentiation of B cells into antibody-producing plasma cells and promote class switching [39]. In our experiments, due to the limiting number of lung DCs, we assessed the adaptive immune system activating capacity of 8-oxoG-exposed DCs in an indirect manner by measuring serum levels of allergen-induced specific immunoglobulins, the production of which are associated with DC activation. To this end, we utilized an adjuvant-free OVA model of experimental allergic airway inflammation. A large

body of evidence suggests that non-adjuvant-aided respiratory exposure of naive rodents to inert antigen is likely to result in inhalation tolerance. However, several reports of successful sensitization to inhaled OVA are published in the literature (reviewed in Ref. [40]). The endotoxin contamination of OVA applied in these studies may be involved in the abrogation of inhalation tolerance. Indeed, mice lacking the LPS receptor appeared unable to develop Th2 responses in the absence of alum adjuvant [41]. In good agreement with these observations, crude OVA (A5503, Sigma-Aldrich), which induced marked OVA-specific antibody responses in our experiments, was also shown to contain a trace amount of endotoxin (52 EU/mg, based on the *Limulus* amoebocyte lysate assay [42]). We have found that co-administration of 8-oxoG base with OVA significantly increased the OVA-specific IgE production in mice. In a recent study, the authors have elegantly demonstrated that

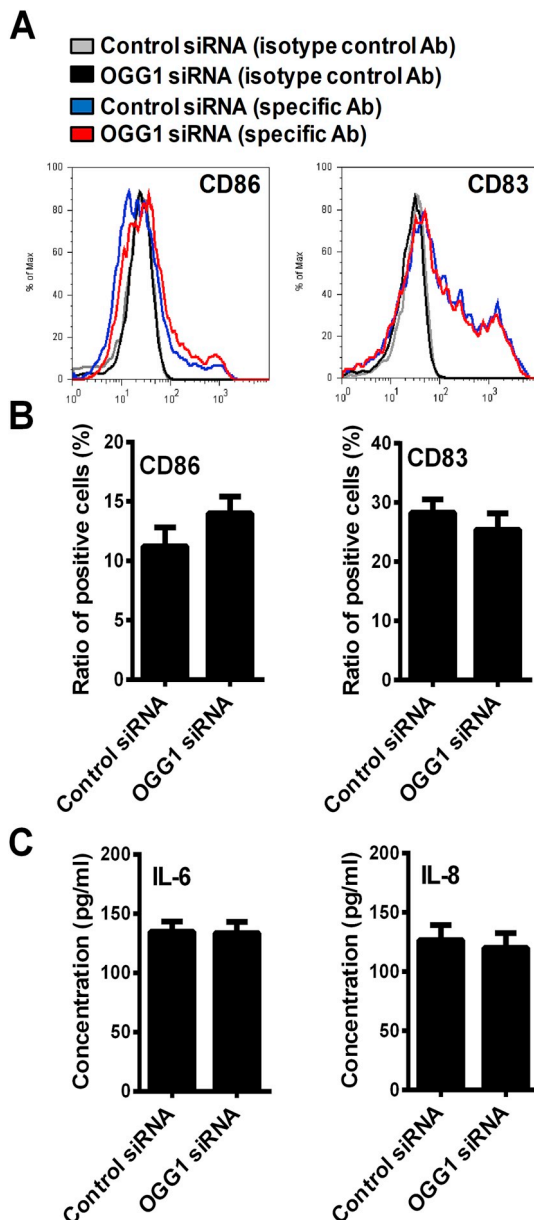


Fig. 6. OGG1 silencing does not influence the maturation state of moDCs. Freshly isolated human monocytes (day 0) were electroporated with OGG1-specific or non-targeting (control) siRNA. At day 5 of OGG1 depletion the expression levels of CD86 and CD83 (A and B) maturation markers were assessed by flow cytometry and the concentrations of the IL-6 pro-inflammatory cytokine and IL-8 chemokine (C) were determined from cell culture supernatants by ELISA. Representative histograms from 4 independent experiments are shown in panel (A), whereas bar graphs represent the means $\pm$ SD of four individual experiments in (B) and (C).

Tfh cells are essential for the production of IgE antibodies in immune responses to inhaled allergens [28]. In adoptive transfer models, Th2 and Tfh cells were isolated from mice previously exposed to OVA plus IL-33 and transferred intravenously to naive T cell-deficient (*Tcrb*^{-/-}) or IL-7 receptor α chain-deficient (*Il7r*^{-/-}) mice. Furthermore, OVA plus IL-33-exposed IL-4-IRES-eGFP (*Aget*) reporter mice were challenged intranasally with OVA to identify the anatomic location of Th2 and Tfh cells during type 2 immune responses. Results from these experiments indicated that Th2 cells were mobile and mediated type 2 cytokine production and inflammation in the airways; whereas, Tfh cells remained in the draining lymph nodes and contributed to the sustained IgE production by plasma cells [28]. In another study using

mice in which MHC class II expression was restricted to conventional DCs and absent from B cells, the initial steps of Tfh differentiation, including turning on the ability of CD4⁺ T cells to express Bcl6 and CXCR5 and homing to B-cell follicles were found to require cognate-interactions with DCs [43]. From another point of view, it has been shown that inhalation of diesel exhaust particles (DEPs) leads to formation of oxidatively modified guanine bases in DNA both in mouse [44] and human lungs [45]. However, exposure to DEPs can not only trigger OGG1-BER mechanism but also promote IgE responses to neoantigens [46]. Indeed, after challenge with the neoantigen (keyhole limpet haemocyanin) alone, neoantigen-specific IgE antibodies could not be detected. In contrast, when co-challenge with neoantigen and DEPs was performed, most of the subjects produced anti-neoantigen IgE [46]. The ability of DEPs to induce a primary IgE response to subsequently encountered antigen can be inhibited by thiol antioxidants [47] and induction of phase II antioxidant enzymes [48]. These previous findings, together with our current observations provide indirect evidence for the existence of a link between OGG1-BER and DC activation in the lungs.

To underpin our findings in animal experiments in a relevant *in vitro* human model, we treated human moDCs with free 8-oxoG. We found that 8-oxoG exposure alone was able to activate human moDCs that was reflected in the increased cell surface expression of maturation/costimulatory/antigen-presenting molecules and enhanced production of pro-inflammatory cytokines. On the contrary, we could not detect any changes in the secretion of IL-10 upon 8-oxoG treatment suggesting that the DC-mediated anti-inflammatory responses are not affected. Previous studies have revealed that several guanosine derivatives, structurally related to 8-oxoG, and oligodeoxynucleotides containing 8-oxoG can be recognized by TLR7, TLR7/8 or TLR9, leading to activation of immune cells [29–32]. All of these endosomal TLRs utilize MyD88 adaptor protein to initiate downstream signaling cascades [49]. Blocking of TLR-mediated signals with a MyD88-specific inhibitory peptide (Pepinh-MYD) did not affect the 8-oxoG-induced activation of moDCs, which excludes the involvement of endosomal TLRs in 8-oxoG-triggered moDC activation. Based on this observations and prior findings we suppose that 8-oxoG-triggered DC activation occurs via OGG1-initiated activation of small GTPases. We have previously shown that 8-oxoG base in complex with OGG1 induces the activation of small GTPases triggering multiple cellular signal transduction pathways [14,16,18]. Small GTPases are also involved in the regulation of DC functions including differentiation [50,51], endocytosis [52], maturation [53,54], chemotaxis [52], antigen presentation [52], cross-presentation [55–57] and T cell priming [58]. All these observations imply that human DCs exhibit the molecular signaling network, which can mediate the 8-oxoG:OGG1 complex-triggered inflammatory responses. Furthermore, it has been found that small GTPases can play a positive or negative regulatory role in NF- κ B activation depending on the cellular context [59]. A growing body of evidences has revealed that small GTPases such as Rac1, RhoG regulate gene expression, primarily through the initiation of MAPK, PI3K cascades, and eventually culminate in the activation of NF- κ B signaling pathway [60–62]. We have recently reported that the OGG1-BER product 8-oxoG base also activates the canonical NF- κ B pathway via KRAS, PI3K, MAPK and mitogen-stress related kinase (MSK) and consequently increases the expression of inflammatory mediators in airway epithelial cells [15]. In the present study we show that the 8-oxoG-triggered moDC activation is completely abrogated by OGG1 depletion, suggesting that the presence and functional activity of OGG1 are essential in these processes. Presumably, due to the lack of OGG1, less 8-oxoG:OGG1 complex is formed in the cytoplasm of DCs that decreases the activation of small GTPase-mediated signaling pathways. Another possible mechanism is that OGG1 deficiency reduces association of NF- κ B with promoter sequences leading to decreased activation of DCs. This assumption is based on findings that OGG1 binding to its substrate in double-stranded DNA is able to function as an epigenetic regulator of gene expression in

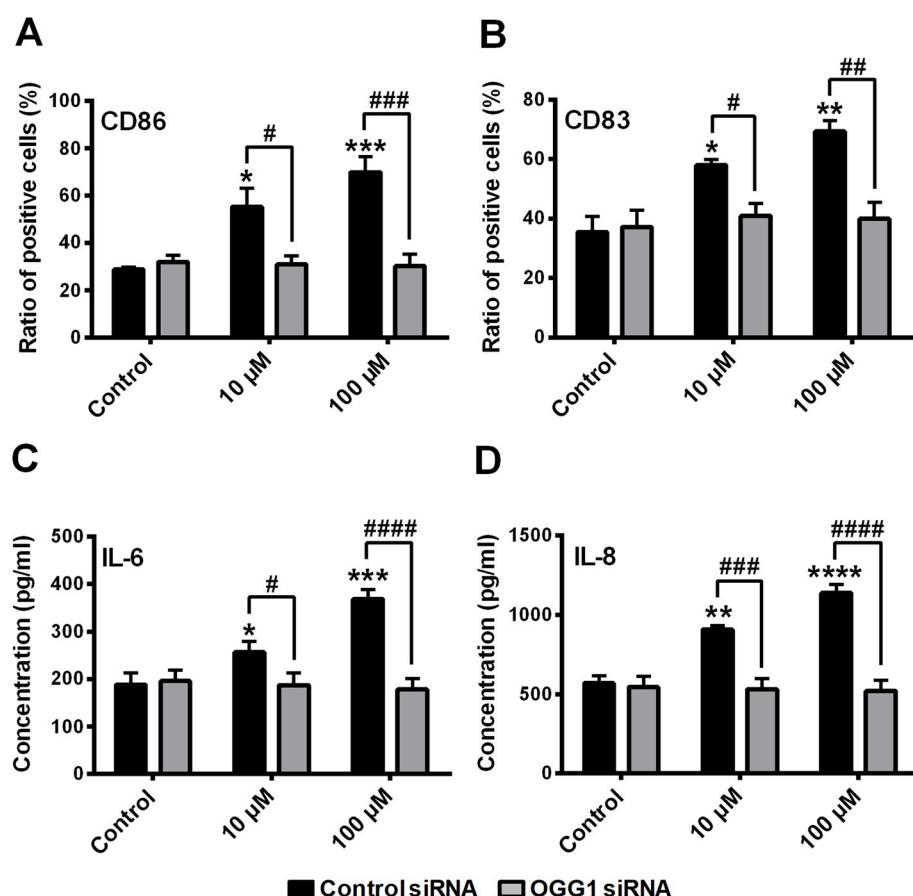


Fig. 7. Depletion of OGG1 prevents 8-oxoG-induced activation of human moDCs. Freshly isolated human monocytes (day 0) were electroporated with OGG1-specific or non-targeting (control) siRNA. On day 5, control and OGG1-specific siRNA-transfected moDCs were exposed to 10 and 100 µM of 8-oxoG for 24 h and the expression of CD86 (A) and CD83 (B) was determined by flow cytometry, whereas the levels of secreted IL-6 (C) and IL-8 (D) were measured by ELISA from cell culture supernatants. Data are shown as means $\pm$ SD from four independent experiments and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ vs control siRNA.

oxidatively stressed cells [63–65]. In particular, OGG1 depletion has been found to decrease both the association of NF- κ B with promoter sequences and cellular transcriptional response to TNF exposure. However, based on our observations that treatment of human moDCs with exogenous 8-oxoG does not induce oxidative stress in the cells or formation of 8-oxoG in their DNA, the potential mechanism that OGG1 binding to promoter sequences facilitates the initiation of gene expression can be ruled out. We would like to emphasize that in all of our experiments we used freshly solubilized 8-oxoG base proven to be oxidatively inert in various human cell cultures [66].

In conclusion, while playing an important role in maintaining genome integrity via excising 8-oxoG from DNA, the repair enzyme OGG1 has been associated to inflammation-related pathologies as well [67]. For instance, OGG1 signaling exacerbates antigen-driven allergic inflammation [68], whereas OGG1 gene polymorphisms have been reported to be associated with rheumatoid arthritis progression [69]. The results of the present study highlight for the first time the importance of OGG1-BER mechanism in DC activation. DCs play a major role in both initiating inflammation and linking innate and adaptive immune responses and they are the most potent targets among immune cells for immune-based therapies [70]. Therefore, we propose that a transient and selective modulation of OGG1 activity in DCs could decrease the severity of inflammation-associated diseases and might have clinical benefits.

Disclosure

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.freeradbiomed.2019.08.010>.

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