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Flow-cytometric Detection of Low-level Reactive Oxygen Species in Cell Lines and Primary Immune Cells

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[Abstract] Depending on its concentration and cellular origin the production of reactive oxygen species (ROS) in the organism serves a variety of functions. While high concentrations during an oxidative burst are used to fight pathogens, low to moderate amounts of ROS act as signaling molecules important for several physiological processes such as regulation of immune responses. The ROS-sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is an inexpensive and well-established tool for measuring intracellular ROS levels. However, it needs to be carefully controlled to be able to draw firm conclusions on the nature of ROS species produced and the cellular source of ROS generation such as the enzyme complex NADPH-oxidase 2 (NOX-2). In this protocol, a robust method to determine low intracellular ROS production using H_2DCFDA was validated by several ROS-specific as well as NOX-2-specific inhibitors. Cells were treated with inhibitors or control substances prior to treatment with the ROS-inducer of interest. H_2DCFDA was added only for the last 30 min of the treatment schedule. To terminate its conversion, we used a ROS-specific inhibitor until analysis by flow cytometry within the FITC-channel (Ex: 488 nm/Em: 519 nm). In summary, this protocol allows the detection of signaling-relevant intracellular ROS production in cell lines and primary immune cells (*e.g.*, Mono Mac 6 cells and Bone marrow-derived dendritic cells, respectively). Using this method in combination with specific inhibitors, we were able to validate even exceptionally low amounts of ROS produced by NOX-2 and relevant for immune-regulatory signaling.

Keywords: Reactive Oxygen Species (ROS), Bone Marrow-derived Dendritic Cells (BMDCs), Mono Mac 6 (MM6) cells, Jurkat T cells, NADPH-oxidase 2 (NOX-2)

[Background] Reactive oxygen species (ROS) can be generated in different cellular compartments such as peroxisomes, the cytosol, at mitochondrial membranes, or at the plasma membrane (Di Meo *et al.*, 2016). High concentrations of ROS can damage all major cellular constituents and are, thus, a lethal threat for living organisms. High amounts of ROS produced in a short period of time (often called oxidative burst) are important for the immune system to fight pathogens. Persistence of excessive ROS production is associated with several diseases such rheumatoid arthritis, diabetes mellitus and neurodegenerative diseases. Low concentrations of superoxide anions (O_2^-) and derivatives thereof, however, have been described to act as essential regulators in cellular signaling cascades (Dröge, 2002). The plasma membrane-bound complex NADPH-oxidase 2 (NOX-2) is one of the main O_2^- producers in phagocytes such as dendritic cells, macrophages, and neutrophils. The active NOX-2 complex is formed

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by 6 subunits (gp91phox, p22phox, Rac2, p40phox, p47phox and p67phox), which allow a tight regulation of NOX-2 activity. NOX-2 can produce both, low and high levels of O_2^- that are rapidly converted into hydrogen peroxide (H_2O_2; Giardino *et al.*, 2017; Belambri *et al.*, 2018). H_2O_2 is comparatively stable and is responsible for both, intra- and extracellular effector functions. High amounts of NOX-2-derived O_2^- produced by neutrophils in phagosomes are important to kill bacteria *via* direct oxidative damage. In contrast, low concentrations of H_2O_2 fulfill essential intra- as well as intercellular second-messenger functions for the regulation of, *e.g.*, immune responses (Bienert *et al.*, 2006; Holmdahl *et al.*, 2013). Thus, H_2O_2 were demonstrated to enhance the expression of interleukin-2 in activated T cells (Roth and Dröge, 1987) and to inhibit the secretion of pro-inflammatory cytokines in phagocytes (Jendrysik *et al.*, 2011; Sareila *et al.*, 2011; Singel and Segal, 2016; Bode *et al.*, 2019). Accordingly, loss of functional NOX-2 has been associated with dysregulated immune responses and several autoimmune diseases such as Lupus erythematosus and Crohn's-like inflammatory bowel disease (Lee *et al.*, 2011; O'Neill *et al.*, 2016; Rosenzweig, 2008; Singel and Segal, 2016).

Several protocols have been established for the detection of free radical production in cells which use either lucigenin, chemiluminescence of luminol, xylenol orange or 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) as tools for the detection of intracellular ROS production (Gyllenhammar, 1987; Dahlgren and Karlsson, 1999; Nourooz-Zadeh, 1999). H_2DCFDA is one of the most frequently used chemicals to investigate the intracellular redox state. Other sensitive and direct approaches for measuring H_2O_2 have also been described such as the *Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit* (Karakuzu *et al.*, 2019). However, this relatively expensive tool measures extracellular H_2O_2 and might not detect low amounts of intracellular ROS. In contrast, low cost, easy handling, high sensitivity, and the possibility to use it in kinetic approaches are important advantages of H_2DCFDA over other established tools. H_2DCFDA enters cells by diffusion and is converted into the non-permeable derivate H_2DCF, which can then be oxidized by ROS into fluorescent 2',7'-dichlorofluorescein (DCF). An alternative protocol for measuring ROS using H_2DCFDA has been published by Eruslanov and Kusmartsev (Eruslanov and Kusmartsev, 2010). Of note, H_2DCFDA lacks specificity for O_2^- radicals and instead reacts with several O_2^-/H_2O_2 conversion products such as alkoxyl, carbonate, peroxyl, NO_2^-, as well as OH^- radicals (Ischiropoulos *et al.*, 1999; Bilski *et al.*, 2002; Eruslanov and Kusmartsev, 2010). Moreover, H_2DCFDA is prone for artificial conversion mediated, *e.g.*, by light exposure or by direct or indirect oxidation *via* cytochrome c released during apoptosis (Kalyanaraman *et al.*, 2012). Hence, the detection of ROS by H_2DCFDA must be carefully controlled to confirm cell-dependent ROS production as well as the enzyme of origin such as NOX-2 (Bilski *et al.*, 2002; Ohashi *et al.*, 2002; Eruslanov and Kusmartsev, 2010). In order to further specify the source and species of the measured ROS, different ROS-specific inhibitors (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Trolox, N-Acetyl cysteine; NAC, and catalase) as well as NOX-2-specific inhibitors (sgp91 ds-tat and GSK2795039) are highly recommended.

The following protocol incorporates the use of specific inhibitors to enable a robust detection of low ROS levels in cell lines (Mono Mac 6 (MM6) cells, Jurkat T cells, *etc.*) and primary immune cells (Bone marrow-derived dendritic cells, *etc.*). Next to the use of inhibitors as pre-treatment to differentiate source

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and species mediated by the ROS-inducer of interest (see Table 1), the general ROS scavengers Trolox or NAC are used to stop the reaction of H_2DCF to DCF at the end of the experimental treatment. The latter ensures that only ROS which are produced during the experimental setting are measured and prevents unwanted conversion not related to the treatment such as responses to stress during washing and the final measurement. By using this protocol, highly reproducible detection of intracellular ROS production in signaling-relevant concentrations can be performed (Bode *et al.*, 2019).

Materials and Reagents

1. 48-well plates, non-treated (CytoOne, STARLAB GmbH, catalog number: CC7672-7548)
2. 48-well plates, tissue culture (tc) treated (greiner cellstar plate, Sigma-Aldrich, catalog number: M8937-100EA)
3. 96-well plate (U-shape, LIFE Technologies, catalog number: N8010560)
4. Jurkat E6.1 T cells (Schneider *et al.*, 1977, CVCL_0367); cultured in RPMI/10% FCS [v/v]
5. Mono Mac 6, MM6 (Ziegler-Heitbrock *et al.*, 1988, CVCL_1426); Dectin-1-overexpressing MM6 cells were generated previously (Bode *et al.*, 2019); cultured in RPMI1640/10% FCS/1% OPI [v/v]
6. Bone marrow-derived dendritic cells (BMDCs); See Bode *et al.* (2019) for further information BMDC generation
7. Bovine extracted catalase (Carl Roth, catalog number: 6025.1), storage at -20 °C, powder shelf-life ≥ 6 months, in solution only 1 day
8. Trolox (Th. Geyer, catalog number: CAY10011659-250), storage at 4 °C, powder shelf-life ≥ 6 months, in solution only ≤ 3 weeks
9. Dimethyl sulfoxide, sterile-filtered (BioPerformance Certified, Sigma-Aldrich, catalog number: D2438)
10. sgp91 ds-tat, scrambled (Anaspc, catalog number: AS-63821), storage at -20 °C, shelf-life ≥ 24 months, avoid repeated freeze and thaw cycles
11. gp91 ds-tat (Anaspc, catalog number: AS-63818), storage at -20 °C, shelf-life ≥ 24 months, avoid repeated freeze and thaw cycles
12. GSK2795039 (MedChem Express, catalog number: HY-18950), storage at -20 °C, shelf-life ≥ 3 months, avoid repeated freeze and thaw cycles
13. RPMI 1640 (Sigma-Aldrich, catalog number: R8758-500ML)
14. Fetal calf serum, FCS (Sigma-Aldrich, catalog number: 12133C-500ML), storage at -20 °C, shelf-life ≥ 12 months
15. OPI-supplement (Sigma-Aldrich, catalog number: O5003), storage at -20 °C, shelf-life ≥ 12 months
16. Polymyxin B sulfate salt (Sigma-Aldrich, catalog number: P4932), storage at 4 °C, powder shelf-life ≥ 12 months, in solution only ≤ 2 weeks

17. H_2DCFDA (Sigma-Aldrich, catalog number: D6883), storage at -20 °C, shelf-life ≥ 24 months, avoid repeated freeze and thaw cycles
18. N-acetyl cysteine, NAC (Sigma-Aldrich, catalog number: A7250), storage at 4 °C, shelf-life ≥ 6 months but in solution only 1 day
19. Phorbol-12-myristat-13-acetate (PMA; Sigma-Aldrich, catalog number: P8139-1MG), storage at -20 °C, shelf-life ≥ 6 months but in solution only 1 day, avoid repeated freeze and thaw cycles
20. Depleted zymosan (DZ; Invivogen, catalog number: tlrl-zyd), storage in solution at -20 °C, shelf-life ≥ 12 months, avoid repeated freeze and thaw cyclesGibco™ PBS, pH 7.4 (Thermo Fisher Scientific, catalog number: 11503387)
21. 500 mM H_2DCFDA solution (see Recipes)
22. 100 mM Trolox solution (see Recipes)
23. 100 mM NAC solution (see Recipes)
24. 1 mM Trolox stop solution (see Recipes)
25. 1 mM NAC stop solution (see Recipes)
26. Catalase mixture (*ca.* 10,000 U/ml) (see Recipes)
27. gp91 ds-tat, scrambled solution (see Recipes)
28. gp91 ds-tat solution (see Recipes)
29. GSK2795039 solution (see Recipes)

Equipment

1. Incubator (Heracell VIOS 160i, Thermo Fisher Scientific or similar product with the adjustment of 37 °C and 5% CO_2)
2. Centrifuge (Thermo Scientific Multifuge X1R Multi-Application, Thermo Fisher Scientific or similar product with 96-well inlay)
3. Single channel pipette, 2-20 µl (Gilson, catalog number: F144056M; or similar product), use appropriate standard pipette tips (sterilized)
4. Single channel pipette, 20-200 µl (Gilson, catalog number: F144058M; or similar product), use appropriate standard pipette tips (sterilized)
5. 8-channel pipette, 30-300 µl (Eppendorf Research, catalog number: 3125000052; or similar product), use appropriate standard pipette tips (sterilized)
6. Flow cytometer (FACSCanto II, BD Biosciences)

Software

1. Graph Pad Prism, version 6 (PrismGraphPad Software Inc., SCR_002798)
2. FlowJo, version 7.6.5 (BD Biosciences, SCR_008520)

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Procedure

A. Prepare stock solutions and reaction mixtures (sterile conditions)

B. Plate cells (sterile conditions) (see also representative pipetting scheme in Figure 1)
   1. Use non-treated 48-well plates for MM6 and Jurkat T cells and tc-treated plates for adherent cells such as BMDCs and primary monocytes.
   2. Plate 100,000 cells/well (inner 24 wells) in 140 µl RPMI1640/10% FCS by using a 200 µl single channel pipette.
   3. Incubate at 37 °C for 30 min.

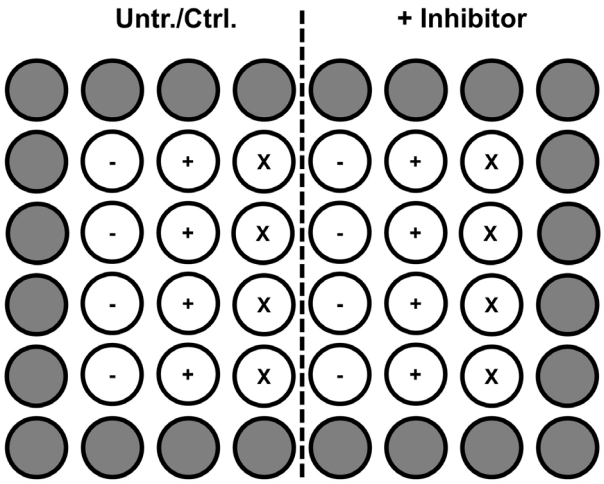

Figure 1. Representative pipette scheme. Fill the outer wells with approximately 200 µl medium (gray-filled circles) to prevent evaporation of sample wells. Plate the cells in the 24 inner wells (open circles). Add medium or the appropriate control to the 12 wells on the left and add the appropriate ROS inhibitor to the 12 wells on the right (separated by the dotted line). Add medium or appropriate reference control (-), a positive control such as Phorbol-12-myristat-13-acetate (PMA) or depleted zymosan (DZ) (+), and the ROS-inducer of interest (X) in quadruplicates (or at least in triplicates).

C. Add ROS-inhibitor or appropriate control to the cells (sterile conditions) (see also representative pipetting scheme in Figure 1 and detailed information of the ROS-inhibitor in Table 1)

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Table 1. Specifications of the used ROS-inhibitors

ROS-inhibitor	specificity	Interchangeable with
Catalase	Cell non-permeable; catalyzes specifically the decomposition of H_2O_2 to H_2O and O_2.	-
NAC	Cell permeable; the thiol group of NAC reduces various free radicals such as H_2O_2, hydroxyl radicals, and Hypochlorous acid.	Trolox
Trolox	Cell permeable; decreases intracellular peroxide formation, quenches superoxide and, thereby, increases H_2O_2 degradation.	NAC
gp91 **ds-tat**	Cell permeable; this NOX-2-specific peptide inhibitor is composed of a gp91phox (NOX-2) sequence linked to the human immunodeficiency virus-tat peptide. The tat sequence facilitates the entry of this peptide into all cells. gp91 ds-tat inhibits NOX-2 assembly.	GSK2795039
GSK2795039	Cell permeable; NOX-2-specific small molecule inhibitor.	gp91 ds-tat

1. Prepare ROS-inhibitor dilutions in RPMI1640/10% FCS
   a. Catalase: at least 2,000 U/ml final conc.; Reference control: RPMI1640/10% FCS.
   b. Trolox: 5 mM final conc.; Reference control: equal volume of DMSO.
   c. NAC: 5 mM final conc.; Reference control: RPMI1640/10% FCS.
   d. gp91 ds-tat: at least 10 µM final conc.; Reference control: gp91 ds-tat scrambled.
   e. GSK2795039: 1-4 µM final conc.; Reference control: equal volume of DMSO.
2. Place the plate with the seeded cells on a pre-warmed (37 °C) metal underlayment.
3. Add 10 µl inhibitor or control solution by using a 20 µl single channel pipette.
4. Place plate in cell culture incubator for 30 min at 37 °C.

D. Add ROS-inducing treatment or appropriate control to the cells (sterile conditions) (see Figure 1)
   1. Prepare treatment solutions such as PMA [100 ng/ml] in RPMI/10% FCS.
   2. Place the plate with the seeded cells on a pre-warmed (37 °C) metal underlayment.
   3. Add 50 µl treatment solution by using a 200 µl single channel pipette and incubate for 0.5-3 h in a cell culture incubator at 37 °C (the optimal incubation time depends on the individual treatments and needs to be empirically determined (*e.g.*, by a kinetic approach).

E. For the last 30 min of incubation add H_2DCFDA solution to the cells (sterile conditions)
   1. Prepare H_2DCFDA solution in RPMI/10% FCS.
   2. Place the plate with the seeded cells on a pre-warmed (37 °C) metal underlayment.

F. Add 10 µl H_2DCFDA solution (5 mM final concentration) by using a 20 µl single channel pipette and incubate exactly for 30 min in a cell culture incubator at 37 °C.

G. Finally, add Trolox or NAC solution to cells to terminate the reaction (sterile conditions)
   1. Place the plate with the seeded cells on a pre-warmed (37 °C) metal underlayment.
   2. Add 50 µl stop solution by using a 200 µl single channel pipette and incubate for 10 min at 37 °C.

H. Prepare cells for analysis
   1. Place the plate with the seeded cells at 4 °C in the dark for 10 min to slow down cell metabolism (during this step fluorescently-labeled antibodies against specific cell surface markers may be added to discriminate subpopulations; do not use FITC- or Alexa488-labeled antibodies).
   2. Carefully resuspend the cells by using a 200 µl single channel pipette (3-4 times) and transfer cells into a 96-well plate (U-shape).
   3. Spin down the cells at 300 *x g* for 5 min at 4 °C.
   4. Quickly remove supernatants.
   5. Resuspend cells in 300 µl ice-cold Trolox or NAC stop solution by using a 300 µl 8-channel pipette.

I. Analyze cells via flow cytometry within the FITC-channel (see also Figure 2)
   1. Keep cells on ice in the dark.
   2. Gate on living cells by morphology (FITC-positive events represent living and metabolically active cells).
   3. Exclude cell doublets by using the forward scatter (FSC)-A and FSC-H.
   4. Perform the measurement as soon as possible within 1 h after adding the stop solution.

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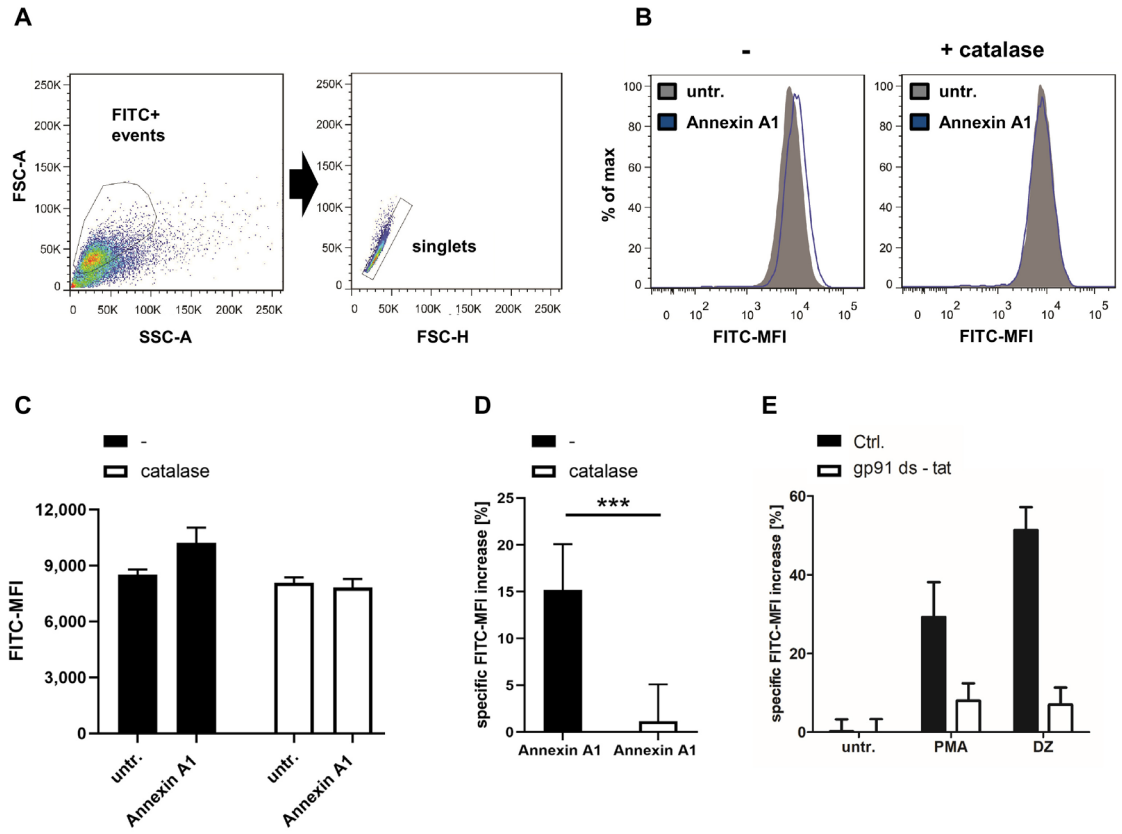

Figure 2. Gating strategy and data analysis of intracellular ROS detection experiments using H_2DCFDA. A. ROS production in cells leads to the conversion of H_2DCFDA into a green fluorescent compound which can be detected within the FITC-channel by flow cytometry. Gate on living cells (determined by morphology and/or by gating on FITC positive events; not shown) and exclude cell doublets by using the forward scatter (FSC)-A and FSC-H. B. Representative histograms depicting the ROS-induction in Dectin-1-overexpressing MM6 cells mediated by recombinant Annexin A1 (blue) compared to untreated (grey) in non-pre-treated MM6 cells (left histogram) or catalase pre-treated MM6 cells (right histogram; Ziegler-Heitbrock *et al.*, 1988). C. Quantification of one representative experiment measured in triplicates. The mean ± S.D. of the FITC-mean fluorescent intensity (MFI) is depicted. D. Statistical analysis of six independent experiments. The mean ± S.D. of the relative FITC-MFI increase in % of non-pre-treated MM6 cells compared to catalase pre-treated cells is depicted (MFI treated – MFI untreated)/MFI untreated * 100). This subfigure was taken from Bode *et al.*, 2019. ***P < 0.001 (paired two-tailed *t*-test). E. Representative ROS-induction by 100 ng/ml Phorbol-12-myristat-13-acetate (PMA) and 40 µg/ml depleted Zymosan (DZ) in Dectin-1-overexpressing MM6 cells using the NOX-2-specific peptide inhibitor gp91 ds-tat or the control peptide (Ctrl.) quantified as in panel D. The mean ± S.E.M. of triplicates (DZ) or quadruplicates (PMA) is shown.

Data analysis

Intracellular ROS induce conversion of H_2DCFDA into a cell-impermeable green fluorescent product

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which can be quantified by flow cytometry within the FITC-channel (Ex: 488 nm/Em: 519 nm) as mean/median fluorescent intensity (MFI). To determine the ROS signal induced by the treatment, the MFI should be normalized to untreated (or control) cells ((MFI treated–MFI untreated)/MFI untreated * 100). For every independent experiment, use at least triplicates for each condition. For statistical analysis perform at least 3 independent experiments and summarize the specific FITC-MFI increases in % relative to untreated or control treated cells as described above (see Figure 2) and use the paired two-tailed *t*-test to determine significant differences in ROS-production.

Notes

1. Add the H_2DCFDA in the same order to the wells as the treatments were added.
2. The use of freshly prepared Trolox and NAC solutions is strongly recommended.
3. Using over night air oxidized RPMI1640/10% FCS may enhance the sensitivity of the measurement.
4. Handle not more than 48 wells/reactions (2 plates) at the same time to ensure short assay times.

Recipes

1. 500 mM H_2DCFDA solution
   in DMSO
   Storage at -20 °C; shelf-life ≤ 24 months, avoid repeated freeze and thaw cycles
2. 100 mM Trolox solution
   in DMSO
   Storage at 4 °C; shelf-life ≤ 3 months
3. 100 mM NAC solution
   in PBS
   Adjust pH to 7.4
   Storage at 4 °C; shelf-life 1 day
4. 1 mM Trolox stop solution
   in RPMI1640/10% FCS [v/v]
   Storage at 4 °C; shelf-life 1 day
5. 1 mM NAC stop solution
   in RPMI1640/10% FCS [v/v]
   Storage at 4 °C; shelf-life 1 day
6. Catalase mixture (*ca.* 10,000 U/ml)
   in RPMI1640/10 % FCS [v/v]
   Storage at 4 °C; shelf-life 1 day; do not use a metallic spoon for transfer the catalase powder
7. gp91 ds-tat
   According to the manufacturer's instruction

Storage at -20 °C, shelf-life ≥ 24 months, avoid repeated freeze and thaw cycles

8. gp91 ds-tat, scrambled solution
   Scrambled according to the manufacturer's instruction
   Storage at -20 °C, shelf-life ≥ 24 months, avoid repeated freeze and thaw cycles
9. GSK2795039 solution
   According to the manufacturer's instruction, storage at -20 °C, shelf-life ≥ 3 months, avoid repeated freeze and thaw cycles

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Competing interests

The authors declare no conflict of financial or scientific interests.

Ethics

The generation of BMDCs was approved by the veterinary authorities (Regierungspräsidium Karlsruhe) of Baden-Württemberg (DKFZ346).

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