SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Flow cytometry methods. Each CellROX dye was dissolved in DMSO at a final concentration of 5 mg/ml and stored at −80°C. Using the stock solution, dyes were diluted with PBS to achieve final staining concentrations. To minimize fluorescent quenching between whole blood proteins and fluorochromes, 100 µl of freshly isolated whole blood was centrifuged at 300 g for 5 min and the cell pellets were resuspended in PBS. To avoid artificial oxidation and to also study COS in neutrophils, we avoided red blood cell lysis and density gradient centrifugation that may artificially generate ROS. Cell pellets were stained with 5 µM CellROX Deep Red Reagent or 5 µM CellROX Green Reagent at 37C in the dark for 30 min.

After centrifugation at 300 g for 5 min, the cell pellets were resuspended in PBS and single cell suspensions were incubated with viability dye. LIVE/DEAD Fixable Aqua (a viability dye) for 405 nm excitation was used to exclude dead cells from analysis (Thermo Fisher Scientific). Aqua dye was dissolved in DMSO and stored at −80°C, according to the manufacturer's instructions. Just before use, Aqua dye was diluted 1:10 with PBS and used for staining. Aqua viability dye staining was performed by adding 2 µl of a diluted viability dye to 100 µl of cell suspension and then incubated at RT for 20 min in the dark. After washing twice with PBS, cells stained with Aqua dye were resuspended in 100 µl of PBS. The cell suspension was blocked at room temperature for 5 minutes for non-specific binding of antibodies with 5 µl of Fc blocker (Human TruStain FcX™, Biolegend) and for non-specific binding of fluorochromes with True-Stain Monocyte Blocker (Biolegend).

Appropriate antibodies were added to each tube and incubated in the dark for 20 minutes on ice. The following human antibodies were used: Brilliant Violet 570™ anti-human CD3 (clone UCHT1), Brilliant Violet 711™ anti-human CD4 (clone OKT4), PE/Cy7 anti-human CD8 (clone SK1), PE anti-human CD14 (clone HCD14), Brilliant Violet 650™ anti-human CD15 (SSEA-1) (clone W6D3), Brilliant Violet 605™ anti-human CD16 (clone 3G8), Brilliant Violet 510™ anti-

human CD19 (clone HIB19), Brilliant Violet 785™ anti-human CD45 (clone HI30) and PE/Dazzle™ 594 anti-human CD56 (NCAM) (clone 5.1H11). All antibodies were obtained from Biolegend. We chose antibodies labeled with fluorochromes that have as minimal excitation and emission overlap with CellROX® Green Reagent and CellROX® Deep Red Reagent as possible. The antibody staining of cells was performed after washes and separately than the staining of whole blood cell pellet with CellROX dyes to minimize interactions between fluorochromes that may confound interpretation of data. After 30 min, the cells were washed twice with PBS. After a short spin, the cells were suspended in 200 μL of ice-cold PBS buffer and transferred to fresh tubes for FACS analysis. Samples were acquired using an LSR Fortessa flow cytometer and FACSDiva software (BD Biosciences). Instrument settings (cytosettings) for each protocol were tailored with unique voltage and compensation matrices. Verify tubes were used to track instrument settings over time. Data were analyzed using FlowJo software. At least 30000 cells were acquired for each analysis, and each representative flow plot was repeated more than 3 times. Only live and singlet cells were chosen for analysis and gating (i.e., dead cells and aggregates were excluded).

To address the possible modulation effect of biochemical interactions (e.g. variable autofluorescence and uptake of a given concentration of fluorochromes) on detection of ROS formation among different participants, the MFI (Mean Fluorescence Intensity) related to fluorescence emission of the CellROX® Reagents was measured in single stain controls and also in fluorescence minus one (FMO) controls in the presence of a given concentration of the antibody staining cocktail. Two readouts of COS were used: i) % of cells that were positive for the CellROX fluorochromes (that determines the total cellular content of ROS); ii) the median cellular amount of ROS per cell type [median fluorescence intensity (MFI) of CellROX Green per cell type per sample minus the MFI of negative staining control]. Cellular ROS was determined in neutrophils, monocytes, lymphocytes and NK cells that carry ROS and contribute to systemic

OS and disease. The difference in fluorescence intensity compared to the negative control (DMFI or% positive cells for CellROX of parent cell population) was reported for each donor. Flow cytometry data among donors were obtained in parallel to avoid batch and autoxidative "aging" effects in stock solutions of dyes.

Gating strategies for viability dye and antibody staining are shown in Supplemental Figure 1. Total leukocytes were gated from all CD45 positive cells and gated cells included lymphocytes (low SSC) and monocytes (medium SSC), $HLADR^{+}CD14^{+}$ monocytes, $CD19^{+}$ B cells, $CD3^{+}$ T cells, $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ T cells and $CD3^{-}CD19^{-}CD56^{+}$ NK cells. Granulocytes gated by high side scatter (SSC^{high}) that were negative for the CD14 monocyte marker and were assessed for CD15 and CD16 to enumerate neutrophils (SSC^{high}, $CD14^{-}$, $CD15^{+}CD16^{+}$). Monocytes were separated into (1) $CD14^{+}CD16^{-}$ classical monocytes, (2) $CD14^{+}CD16^{+}$ intermediate monocytes, and (3) $CD14^{lo}CD16^{+}$ nonclassical monocytes. In cases where the antigens are expressed at low levels or do not have clearly defined positive populations, the position of the positive/negative gate was placed based on either different cell populations within the tube that were clearly negative, or the use of a fluorescence minus one (FMO) control tube.

Rationale to use CellROX Green and CellROX Deep Red for flow cytometric determination of cellular oxidative stress (COS)

CellROX Green. Cellular oxidative stress (COS) was determined by the use of the CellROX Green Reagent (absorption and emission maxima at 485/530 nm) which is non-fluorescent while in a reduced state and upon oxidation by reactive oxygen species (ROS) and subsequent binding to DNA, exhibits bright green fluorescence; it is a measure of total (cytoplasmic and nuclear) cellular ROS. CellROX Green (Thermo Scientific) is a proprietary, permeable, non-fluorescent, oxidation-sensitive dye that becomes fluorescent upon binding to DNA after being

oxidized by superoxide (O_1^-) and hydroxyl radical (•OH) and other species such as high-valence Fe centers in live cells[12, 42]. The CellROX green labels a series of intracellular compartments, including cytoplasm, nucleus, and mitochondria but is considered primarily a nuclear probe. DNA damage may be caused ROS-induced modifications of other molecules, such as lipids. Retention of activated dye in cells by binding to DNA, prevents loss of activated probe through leakage through damaged cell membranes[13]. Limited evidence suggests that CellROX Green may be a better detector for superoxide rather than hydrogen peroxide-induced hydroxyl radicals[13]. CellROX green may be more sensitive for detection of hydrogen peroxide-induced hydroxyl radicals compared to other probes like DCFH[43]. The efficiency of CellROX Green to determine COS has previously been validated in macrophages[17], sperm[43], epithelial cells[44-48], neurons[49-51], bacteria[11, 13, 42] and melanoma cells[41]. However, the CellROX Green is insensitive to oxidative nitrogen-containing radicals, hydrogen peroxide (H_2O_2) or to a variety of other oxidants including peroxynitrite ($ONOO^-$), NO, and hypochlorite (OCl^-).

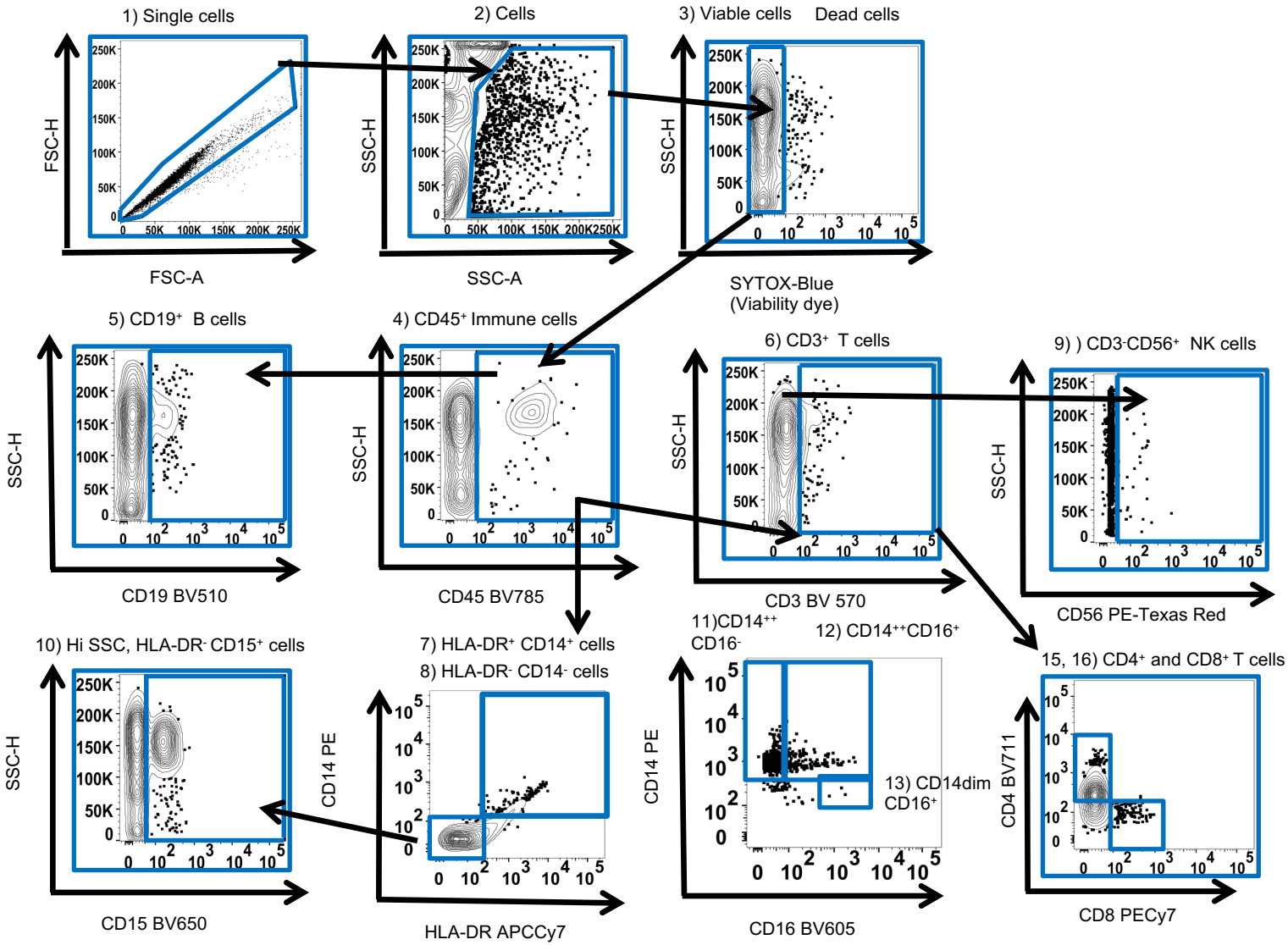
CellROX Deep Red. Cytoplasmic cellular oxidative stress was determined by the use of the CellROX Deep Red Reagent (absorption and emission maxima at ~644/665 nm) which is non-fluorescent while in a reduced state and upon exhibits bright fluorescence upon oxidation by ROS in the cytoplasm; it is a measure of cytoplasmic cellular ROS. In contrast to CellROX green that binds to DNA when it oxidizes, CellROX deep red detects cytoplasmic free radicals that may underlie several complex mechanisms, including membrane lipid peroxidation, protein denaturation, and DNA damage, which may in turn induce apoptosis[14]. The CellROX Deep Red can detect ROS in fresh and fixed cells and seems to be more specific in detecting superoxide anion, nitroxides[52] and hydroxyl radical compared to other fluorescent probes[53]. The efficiency of CellROX Deep Red to assess COS has previously been validated in sperm cells[43, 54-59], epithelial cells[52, 60-63], hepatocytes[64], neurons[65], cardiomyocytes[66], melanoma

cells[41], endothelial[67], immune (such as mast cells[15]) and bone marrow-derived mesenchymal stem cells[68]. The CellROX deep Red has been previously used to detect the *ex vivo* impact of cigarette smoke on cellular ROS by flow cytometry in spermatocytes[16]. Limited evidence suggests that the hydrogen peroxide interferes with the CellROX Deep Red probe[57] which is catalase sensitive[67] and mainly identifies superoxide that may reflect intense mitochondrial activity[54-56, 66].

Rationale to use both CellROX Green and Deep Red. CellRox dyes are proprietary probes with unknown chemical structures but seem to be more specific and less sensitive in detecting ROS compared to other fluorochromes like dichlorofluorescein (DCF), dihydrorhodamine 113 (DHR113), dihydroethidium (DHE) and CellRox Orange[58, 63]. The use of these fluorochromes for determination of COS in immune cells has previously been validated both *in vitro*[17] and *in vivo*[39]. The CellROX Deep Red and Green probes can detect, simultaneously, the presence of superoxide anion and hydroxyl radical. The CellROX ROS detection reagents are bright and stable ROS sensors that offer significant advantages over existing ROS sensors because they are compatible with labeling in different media and can be used with fixatives[40]. It has been proposed that the bias of each method to detect ROS could be overcome by the evaluation of OS by using more than one criterion[66, 69, 70]. Thus, the combined use of both CellROX Deep Red and Green may better reflect total COS rather than use of either one alone. CellROX Green and Deep Red probes can differentiate between mitochondrial and cytosolic ROS. This combined use has previously been described in non-immune cells[41].

Figure S1. Gating strategy in flow cytometry experiments to determine the percent of different immune cells types that were positive for a combination of specific cellular markers.

Fluorescence intensity of a positive cell population was compared to a negative cell population (fluorescence minus one negative control for staining). Representative data of gates are shown: **1)** Single cells; →**2)** Cells (FSC/SSC) to exclude red blood cells and debris. Red blood cell lysis was not performed to avoid artificial oxidative stress during RBC lysis; →**3)** Viable cells were gated as negative stain for the SYTOX™ Blue dead cell stain; **4)** Immune cells were gated as $CD45^+$ on gate 3. From gate 4 the following gates were created: **5)** for $CD19^+$ B cells; **6)** for $CD3^+$ T cells; **7)** HLA-DR^+CD14^+ cells (monocytes; to exclude granulocytes, lymphocytes and NK cells); **8)** HLA-DR^-CD14^- cells (to exclude monocytes). The following gates were also created: **9)** for $CD56^+$ NK cells (gated on $CD3^-$ cells on gate 6); **10)** for neutrophils (Hi SSC, $CD15^+$, $CD14^-$ HLA-DR^-; gated on 8); **11)** $CD14^{++}CD16^-$ classical monocytes; **12)** $CD14^{++}CD16^+$ intermediate monocytes; **13)** $CD14_{dim}CD16^+$ non-classical (patrolling or $CD14^+CD16^{++}$) monocytes; **14)** $CD14^+CD16^+$ total proinflammatory monocytes; **15)** $CD3^+CD4^+$ T cells (gated on 6); **16)** $CD3^+CD8^+$ T cells (gated on 6).


1) Single cells
FSC-H
FSC-A
2) Cells
SSC-H
SSC-A
3) Viable cells
Dead cells
SSC-H
SYTOX-Blue
(Viability dye)
5) CD19+ B cells
SSC-H
CD19 BV510
4) CD45+ Immune cells
SSC-H
CD45 BV785
6) CD3+ T cells
SSC-H
CD3 BV 570
9)) CD3-CD56+ NK cells
SSC-H
CD56 PE-Texas Red
10) Hi SSC, HLA-DR- CD15+ cells
SSC-H
CD15 BV650
7) HLA-DR+ CD14+ cells
8) HLA-DR- CD14- cells
CD14 PE
HLA-DR APCCy7
11)CD14++
CD16-
12) CD14++CD16+
13) CD14dim
CD16+
CD14 PE
CD16 BV605
15, 16) CD4+ and CD8+ T cells
CD4 BV711
CD8 PECy7