Chapter 4

Identification of ROS Using Oxidized DCFDA and Flow-Cytometry

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Abstract

Cells constantly generate reactive oxygen species (ROS) during aerobic metabolism. The ROS generation plays an important protective and functional role in the immune system. The cell is armed with a powerful antioxidant defense system to combat excessive production of ROS. Oxidative stress occurs in cells when the generation of ROS overwhelms the cells' natural antioxidant defenses. ROS and the oxidative damage are thought to play an important role in many human diseases including cancer, atherosclerosis, other neurodegenerative diseases and diabetes. Thus, establishing their precise role requires the ability to measure ROS accurately and the oxidative damage that they cause. There are many methods for measuring free radical production in cells. The most straightforward techniques use cell permeable fluorescent and chemiluminescent probes. 2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) is one of the most widely used techniques for directly measuring the redox state of a cell. It has several advantages over other techniques developed. It is very easy to use, extremely sensitive to changes in the redox state of a cell, inexpensive and can be used to follow changes in ROS over time.

Key words: Reactive oxygen species (ROS), Oxidative stress, 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 2′-7′-dichlorofluorescein (DCF), Flow-cytometry, Immature myeloid cells (ImC)

1. Introduction

Reactive oxygen species (ROS) are constantly generated under normal conditions as a consequence of aerobic respiration. ROS include free radicals such as the superoxide anion (O_2^-), singlet oxygen ($^1O_2$,) hydroxyl radicals (OH), various peroxides (ROOR′), hydroperoxides (ROOH) and the no radical hydrogen peroxide (H_2O_2). Despite on multiple redox modulation systems, a given proportion of ROS continuously escape from the mitochondrial respiratorial chain inducing a damage cells in various ways including

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numerous carcinogenic DNA mutations. The cell is equipped with an extensive antioxidant defense system to combat ROS, either directly by interception or indirectly through reversal of oxidative damage. When ROS overcome the defense systems of the cell and redox homeostasis is altered, the result is oxidative stress.

Free radicals and other "reactive oxygen (ROS)/nitrogen/chlorine species" are believed to contribute to the development of several age-related diseases, and perhaps, even to the aging process itself (1, 2) by causing "oxidative stress" and "oxidative damage." For example, many studies have shown increased oxidative damage to all the major classes of biomolecules in the brains of Alzheimer's patients (3–5). Other diseases in which oxidative damage has been implicated include cancer, atherosclerosis, other neurodegenerative diseases and diabetes (6–8). To establish the role of oxidative damage, it is therefore essential to be able to measure it accurately.

1.1. Sources of ROS and Antioxidant Defense Mechanisms

Major sources of ROS production include the mitochondria, endoplasmic reticulum, plasma membrane and cytosol. In normal resting cells, 1–2% of electrons carried by the mitochondrial electron transport chain (ETC) leak from this pathway and form the superoxide free radical O_2^- during respiration. The dismutation of O_2^- by superoxide dismutase (SOD) results in the generation of H_2O_2, which can then react with Fe^{2+} to form hydroxyl radicals via the Fenton reaction: Hydroxyl radicals can also be generated via the metal catalysed Haber–Weiss reaction (9, 10). Other sources of O_2^- include the enzymes xanthine oxidase in the cytosol, NADPH oxidase in the membrane and cytochrome *P*450 in the ER (11–13).

Under normal conditions, antioxidant systems minimize the adverse effects caused by ROS. Antioxidants can be divided into primary or secondary defence mechanisms. Components of the primary antioxidant defense function to prevent oxidative damage directly by intercepting ROS before they can damage intracellular targets. It consists of superoxide dismutase (SOD), glutathione peroxidase (Gpx), catalase and thioredoxin reductase. Four classes of SOD have been identified to date. These are Mn-SOD, Cu, Zn-SOD, Ni-SOD and extracellular SOD. All four SOD enzymes destroy the free radical superoxide by converting it to H_2O_2.

H_2O_2 is one of the major ROS in the cell. While low levels result in apoptosis, high levels can lead to necrosis or caspase-independent apoptosis (14, 15). The primary defence mechanisms against H_2O_2 are catalase (16) and glutathione peroxidase (GPx) through the glutathione (GSH) redox cycle (17). Catalase is one of the most efficient enzymes known and cannot be saturated by H_2O_2 at any concentration (18). It is present, only in the peroxisome fraction whereas the GSH redox cycle exists

in the cytosol and mitochondria. Catalase reacts with H_2O_2 to form water and molecular oxygen. Overexpression of catalase in cytosolic or mitochondrial compartments has been demonstrated to protect cells against oxidative injury (19).

The GSH system is probably the most important cellular defence mechanism that exists in the cell. The tripeptide GSH (γ-Glu-Cys-Gly), not only acts as an ROS scavenger but also functions in the regulation of the intracellular redox state. The system consists of GSH, glutathione peroxidase and glutathione reductase. Glutathione peroxidase catalyses the reduction of H_2O_2 and other peroxidases and converts GSH to its oxidized disulphide form (GSSG) as outlined below.

$$ROOH + 2GSH \xrightarrow{GPX\ ROH} + GSSG + H_2O$$

GSSG is then reduced back to GSH by glutathione reductase. The ability of the cell to regenerate GSH (either by reduction of GSSG or new synthesis of GSH) is an important factor in the efficiency of that cell in managing oxidative stress. The rate-limiting step for GSH synthesis is catalyzed by the enzyme L-γ-glutamyl-cysteine synthase. Inducers of this enzyme have been reported to prevent glutamate toxicity (20).

Thioredoxin reductase (Trx R)/thioredoxin (Trx) is another powerful system to protect cells against H_2O_2. Thioredoxin reductase (Trx R) utilizes NADPH to catalyze the conversion of oxidized Trx into reduced Trx. Reduced Trx provides reducing equivalents to Trx peroxidase, which breaks down H_2O_2 to water (21).

1.2. Role of Reactive Oxygen Species in Immune System and in Cancer

ROS generation plays an important role in the immune system. Phagocytes, including macrophages and neutrophils, are capable of generating large quantities of RNI and ROS, respectively. These free radicals are important for phagocytic anti-microbial and tumoricidal immune responses. Neutrophils have a short lifespan, usually about 24 h and ROS appear to play an important role in neutrophil survival (22). Activated neutrophils undergo spontaneous apoptosis shortly after producing an ROS burst against invading pathogens. Research suggests that this spontaneous apoptosis is mediated by endogenous ROS production (23). Survival of neutrophils incubated with antioxidants is greatly enhanced over 24 h in culture (24). Generation of ROS by TNF-α is critical for the phagocytic immune response against invading pathogens (25).

The data with knockout animals that lack antioxidant enzymes, supported by data from some animal knockouts of repair enzymes, strongly support the view that ROS contribute to the age-related development of cancer (26, 27). Direct damage to DNA is probably one key event, but it alone is insufficient to

produce cancer, suggesting that the ability of RS to suppress apoptosis, and promote proliferation, invasiveness and metastasis (and possibly angiogenesis) are also important (28, 29). The relative contributions of these various mechanisms are unclear. Cancer associated with chronic inflammation may also involve ROS (30, 31). The trend now in cancer treatment is to make therapies based on the gene expression, cell signaling and proteomic profiles of a tumor. Perhaps, we need to do the same for its "oxidative stress status".

It is well established that tumor growth is associated with accumulation of immature myeloid cells (ImC), which in mice are characterized by expression of Gr-1 and CD11b markers. They play an important role in tumor associated immune suppression (32).

ImC from tumor-bearing mice had significantly higher levels of reactive oxygen species (ROS) than ImC obtained from tumor-free mice. Hydrogen peroxide, but not superoxide radical anion was found to be a major part of this increased ROS production. In vitro experiments demonstrated that scavenging of hydrogen peroxide with catalase induced differentiation of ImC from tumor-bearing mice into macrophages. Thus, tumors may prevent differentiation of antigen presenting cells by increasing the level of endogenous hydrogen peroxide in immature myeloid cells (32, 33).

We demonstrated that implantation of human RCC tumor cells into athymic nude mice promotes the appearance of VEGF receptor 1 (VEGFR1)/CD11b double-positive myeloid cells in peripheral blood. Up-regulation of VEGFR1 by myeloid cells could also be achieved in vitro by short-term exposure of naive myeloid cells to oxidative stress. Furthermore, after exposure to oxidative stress, myeloid cells acquire immunosuppressive features and become capable of inhibiting T cell proliferation. Data suggest that tumor-induced oxidative stress may promote both VEGFR1 up-regulation and immunosuppressive function in bone marrow-derived myeloid cells (34).

ImC isolated from tumor-bearing mice but not their control counterparts were able to inhibit antigen-specific response of $CD8^+$ T cells. ImC did not produce nitric oxide, however, ImC obtained from tumor-bearing mice had significantly higher level of reactive oxygen species (ROS) than ImC isolated from tumor-free animals. Accumulation of H_2O_2, but not superoxide was a major contributor to this increased pool of ROS. It appears that arginase activity played an important role in H_2O_2 accumulation in these cells. Inhibition of ROS in ImC completely abrogated the inhibitory effect of these cells on T cells. This indicates that ImC generated in tumor-bearing hosts suppress $CD8^+$ T cell response via release of ROS (34).

1.3. ROS Detection

There are many methods for measuring free radical production in cells: Chemiluminescence of luminol (35, 36) and lucigenin (37),

cytochrome *c* reduction (35), ferrous oxidation of xylenol orange (38) and DCFH-DA (12, 39) have all been used successfully to detect ROS generation. The most straightforward techniques use cell permeable fluorescent and chemiluminescent probes. Flow cytometry or fluorimetry can be used for the detection of ROS with fluorescent probes.

2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) is one of the most widely used techniques for directly measuring the redox state of a cell.

DCFH-DA, a cell permeable, non-fluorescent precursor of DCF can be used as an intracellular probe for oxidative stress. It has many advantages over other techniques developed. It is very easy to use, extremely sensitive to changes in the redox state of a cell, inexpensive and can be used to follow changes in ROS over time.

Intracellular esterases cleave DCFH-DA at the two ester bonds, producing a relatively polar and cell membrane-impermeable product, H_2DCF. This non-fluorescent molecule accumulates intracellularly and subsequent oxidation yields the highly fluorescent product DCF. The redox state of the sample can be monitored by detecting the increase in fluorescence. Accumulation of DCF in cells may be measured by an increase in fluorescence at 530 nm when the sample is excited at 485 nm. Fluorescence at 530 nm can be measured using a flow cytometer and is assumed to be proportional to the concentration of hydrogen peroxide in the cells (40, 41).

DCF, the oxidized fluorescent product of $DCFH_2$, is membrane permeable and can leak out of cells over time. Detecting slow hydrogen peroxide production over time can be difficult (42). To enhance retention of the fluorescent product, various analogues (improved versions of H2DCFDA) have been developed. For instance, carboxylated H2DCFDA analog, which has two negative charges at physiological pH, and its di(acetoxymethyl ester), which should more easily pass through membranes during cell loading. Upon oxidation and cleavage of the acetate and ester groups by intracellular esterases, both analogs form carboxydichlorofluorescein, with additional negative charges that should impede its leakage out of the cell. There is another analog called 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA)-a chloromethyl derivative of H2DCFDA. The CM-H2DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields a fluorescent adduct that is trapped inside the cell, thus facilitating long-term studies.

The chemistry of the conversion is complex (Fig. 4.1) (43). Neither H_2O_2 nor $O_2^{\cdot-}$ can oxidize DCFH, but peroxyl, alkoxyl,

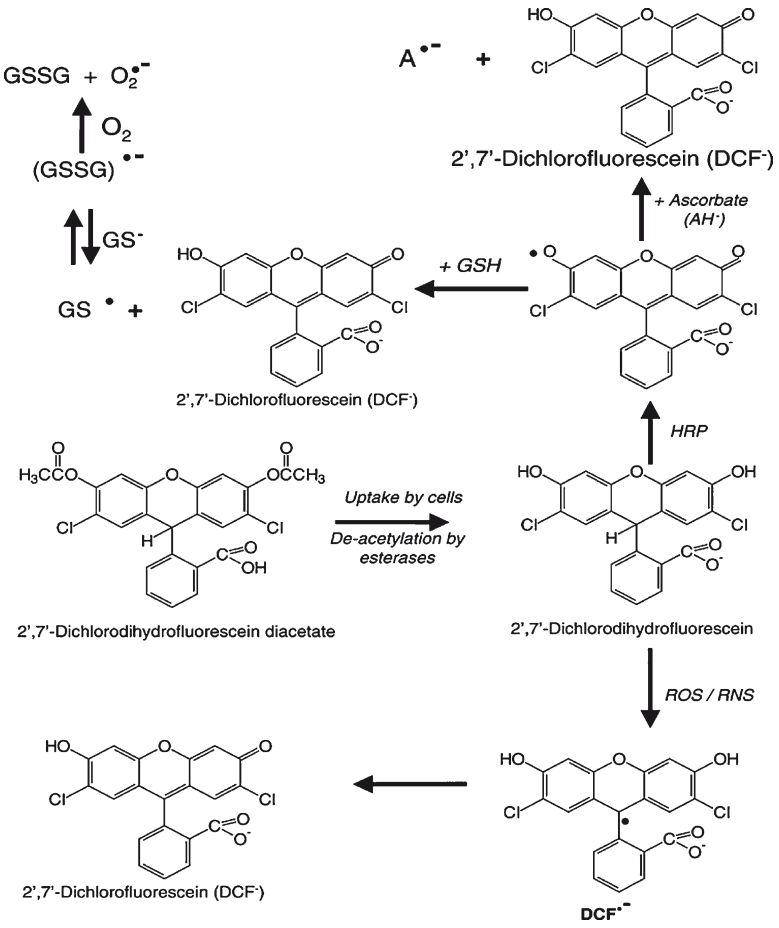

Fig. 4.1. (From Ref. 43). Conversion of DCFDA to a fluorescent product DCFDA is dichlorofluorescein diacetate, It is hydrolysed by cellular esterases to dichlorofluorescin (2′,7′-dichlorodihydrofluorescein), whose oxidation by several RS yields florescent DCF (dichlorofluorescein, more correctly called 2′7′-dichlorofluorescein) via an intermediate radical, DCF–. Peroxidases can also convert it into a phenoxyl radical that can interact with antioxidants such as ascorbate (AH–), reducing the phenoxyl radical and oxidizing ascorbate, or with GSH. GS resulting from the latter reaction can lead to O_2– generation. The phenoxyl radical can also be recycled by NADH (not shown), producing NAD radical, which reacts rapidly with O_2 to produce O_2–.

$NO_2^{\cdot}$, carbonate ($CO_3^{\cdot-}$) and $OH^{\cdot}$ radicals can as well as peroxynitrite (44–46). DCFDA can only detect cellular peroxides efficiently if they are decomposed to radicals, for example, by transition metal ions. For instance, in bovine aortic endothelial cells, the generation of a signal from DCFDA upon addition of H_2O_2 required the uptake of extracellular iron from the medium (47). Horseradish peroxidase, myeloperoxidase and other heme proteins can also oxidize DCFH in the presence of H_2O_2 (indeed, DCFDA was first used in biology as a detector for H_2O_2 by adding horseradish

peroxidase (48). Hence cellular peroxidase level and heme protein content are other variables to consider when interpreting studies with this probe (46, 48).

It follows that DCF fluorescence is an assay of generalized oxidative stress rather than of any particular RS, and is *not* a direct assay of H_2O_2, $NO^{\cdot}$, lipid peroxides, singlet O_2 or $O_2^{\cdot -}$. One-electron oxidation of DCFH by various radicals and heme proteins is likely to produce intermediate radicals (Fig. 4.1), including phenoxyl radicals that can interact with such cellular antioxidants as GSH and ascorbate and with NADH to create more free radicals (49–51). Lawrence et al. (52) pointed out that cytochrome *c* is a powerful catalyst of DCFH oxidation, and so use of DCFDA to probe oxidative stress during apoptosis should be approached with caution, as a rise in cytosolic cytochrome *c* levels could result in a bigger "signal" without any change in cellular peroxide levels. Chromium (V), pyocyanin, mitoxantrone and ametantrone can directly oxidize DCFH and cause an artifactual signal, and the possibility of such direct oxidations must always be checked before using DCFDA to measure oxidative stress in cells exposed to various toxins (53, 54). Variation in cellular esterase content could also conceivably affect the use of DCFDA as a probe, but this issue has not been explored in the literature.

Some cell types have low esterase activity. This can have important implications for detecting ROS because DCFH-DA needs to be hydrolysed to $DCFH_2$ by cellular esterases. Careful consideration must be given to the levels of esterase activity in cells before using this probe. In cells with low esterase activity or where the esterases are sequestered in inaccessible parts of the cell, using the deacetylated probe $DCFH_2$ or some other assay is advised. For example, luminol- and lucigenin-dependent CL possess many of the advantages of fluorescent probes such as DCFH-DA. They are easy to use, sensitive, and inexpensive and can measure ROS generation in intact cells over time. They possess advantages over DCFH-DA in that they do not need to be cleaved by esterases and can be used in systems with low esterase activity. Also, cytochrome *c* is not able to catalyse chemiluminescent reactions and cannot interfere with ROS measurements during apoptosis.

The simplest technique to read samples is the fluorescence microplate reader, where data are presented as increases or decreases in relative. Bottom-reading machines have the advantage that the cells can be measured in situ without the need for trypsinization or cell scraping, processes that themselves generate cellular oxidative stress and result in artifactual changes in fluorescence. Plate readers measure total fluorescence, that is, they do not distinguish between intracellular and extracellular fluorescence from chemical reactions in the culture medium. We have already alluded to this problem in the case of DCFDA.

Flow cytometry offers the advantage of being able to measure the intracellular fluorescence of cells in the culture media. Quantitative data on the numbers of cells emitting fluorescence can be obtained rather than just relative fluorescence units. However, cells are required to be in suspension and require either scraping or trypsinization, which induce oxidative stress. Control experiments to optimize assay conditions must always be conducted to limit this

Confocal microscopy is a powerful tool; cells can be loaded with fluorescent dyes and viewed in real time in situ in culture chambers at 37°C. The intracellular location of RS can be visualised, and the role of mitochondrial, endoplasmic reticulum or lysosomal events in oxidative stress may be visualised using counter stains.

Some simple principles can be used as guidelines in understanding oxidative stress/oxidative damage in cell culture. Hydrogen peroxide generally crosses cell membranes readily (55). Thus catalase added outside cells can exert both intracellular and extracellular effects on H_2O_2 level, the former by "draining" H_2O_2 out of the cell by removing extracellular H_2O_2 and thus establishing a concentration gradient. In contrast, $O_2^{\cdot-}$ does not generally cross cell membranes readily (56). Thus if externally added superoxide dismutase is protective against an event in cell culture, be wary of what this means; it could be indicative of extracellular $O_2^{\cdot-}$-generating reactions. Similarly, neither the iron-chelating agent deferoxamine (which suppresses most, but not all, iron-dependent free radical reactions) nor the thiol antioxidant GSH enter cells easily, so again be wary if they have protective effects in short-term experiments: this is suggestive of extracellular effects (56). As an example, Clement et al. (57) showed that GSH protects against the cytotoxicity of dopamine simply because it reacts with dopamine oxidation products generated in the cell culture medium.

2. Materials

2.1. Equipment

1. Table centrifuge.
2. Flow cytometer.

2.2. Reagents and Supplies

1. 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester CM-H2DCFDA. Catalog number C6827 (Invitrogen/Molecular Probes). Do not dissolve products until immediately before use. Extremely air and light sensitive.
2. High quality anhydrous dimethylsulphoxide (DMSO), dimethylform-amide (DMF), or 100% ethanol.

3. Loading buffer such as simple physiological buffer PBS or HBSS.
4. Hydrogen Peroxide or tert-butyl hydroperoxide (TBHP) to a final concentration 50 μM (increase or decrease based on the sensitivity and response of the cells).
5. Six well Ultra Low Cluster Plate, Ultra Low Attachment, (Costar, #3471).

3. Methods

It is important to understand the limitations of using this probe. Although DCFH-DA is used to measure the concentration of hydrogen peroxide in cells, superoxide and NO generation are also capable of oxidizing $DCFH_2$ (49). The presence of SOD in the cytosol (Cu,Zn-SOD), mitochondria (Mn-SOD) and extracellular space (Ec-SOD) all convert superoxide into hydrogen peroxide, resulting in the accumulation of DCF in the cells. Inhibitors of SOD can be used to eliminate this source of hydrogen peroxide production. $DCFH_2$ may also be oxidized independently of hydrogen peroxide. Nitric oxide reacts with superoxide producing peroxynitrite. $DCFH_2$ can be directly oxidized to DCF by peroxynitrite. Inhibitors of NOS should be used to prevent NO mediated $DCFH_2$ oxidation (50). Some cell types have low esterase activity. This can have important implications for detecting ROS because DCFH-DA needs to be hydrolysed to $DCFH_2$ by cellular esterases. Careful consideration must be given to the levels of esterase activity in cells before using this probe.

3.1. General Guidelines

1. Remove cells from growth media and wash out from the serum of culture medium (*see* Note 1), leave cells for 10 min in PBS. If cells have been isolated ex vivo transfer these cells directly in prewarmed loading buffer. To minimized extracellular hydrolysis of the dye medium should not contain culture serum, primary and secondary amines, phenol red or other colometric dyes before and throughout the assay or dymethylform-amide (DFM).
2. The CM-H2DCFDA should be reconstituted only in anhydrous dimethylsulphoxide (DMSO), or dimethylform-amide (DMF), or 100% ethanol (*see* Note 2). Working solutions should be freshly prepared (*see* Note 3). Keep the solution in the dark and tightly sealed until ready to use and discard excess diluted probe at the end of the work session. These probes oxidize more readily in solution, and the presence of moisture will facilitate the decomposition of the dye. To prepare 10 mM

concentrated stocks of CM-H2DCFDA (MW: 577.8) add 8.6 µl DMSO into the original vial with 50 µg dye.

3. Resuspend cells in pre-warmed loading buffer containing the probe to provide a final working concentration of 1–10 µM dye. Remember, that CM-H2DCFDA concentration should be kept as low as possible to reduce potential artifacts from overloading, including incomplete hydrolysis, compartmentalization, and the toxic effects of hydrolysis by-products. The optimal working concentration for your application should be empirically determined. Incubate cells in the 6 well Ultra Low Cluster Plate at the optimal temperature for the cells. Generally, a loading time of 30–60 min is sufficient.
4. Wash out the loading buffer and plate the cells back to medium and incubate at the optimal temperature to allow a short recovery time for cellular esterases to hydrolyze the AM or acetate groups and then let the dye to be responsive to oxidation in the particular experiment's condition.
5. It is recommended to use positive control. To create positive controls, oxidative activity may be stimulated with hydrogen peroxide or tert-butyl hydroperoxide (TBHP) to a final concentration of 50 µM or with PMA.
6. Examine the intensity of fluorescence by flow cytometry. The redox state of the sample can be monitored by detecting the increase in fluorescence. Accumulation of DCF in cells may be measured by an increase in fluorescence at 530 nm when the sample is excited at 485 nm
7. First, examine the fluorescence of negative control which is an untreated loaded with dye cells maintained in a buffer. In healthy cells, oxygen radicals are eliminated by cellular enzymes and/or natural antioxidant and because of that reason healthy cells should exhibit a low level of fluorescence.

3.2. Results

3.2.1. Detection of ROS

To measure ROS generation by myeloid cells we used two dyes: DHE and DCFDA. DHE is selectively oxidized by superoxide anion, while the fluorescence of DCFDA indicates oxidation by hydrogen peroxide, peroxynitrite or hydroxyl radical. Superoxide anions can also contribute to DCFDA oxidation albeit at a lesser degree.

Freshly isolated splenocytes from tumor-bearing or from tumor-free immunized mice were loaded with these dyes and then labeled with anti-Gr-1-APC and CD11b-PE antibodies. The fluorescence of those dyes was evaluated within the population of gated double positive Gr-1$^+$CD11b$^+$ myeloid cells (Fig. 4.2). No difference in superoxide production (DHE oxidation) was found between two groups of cells, whereas the level of DFCDA oxidation by ImC from tumor-bearing mice was significantly (threefold)

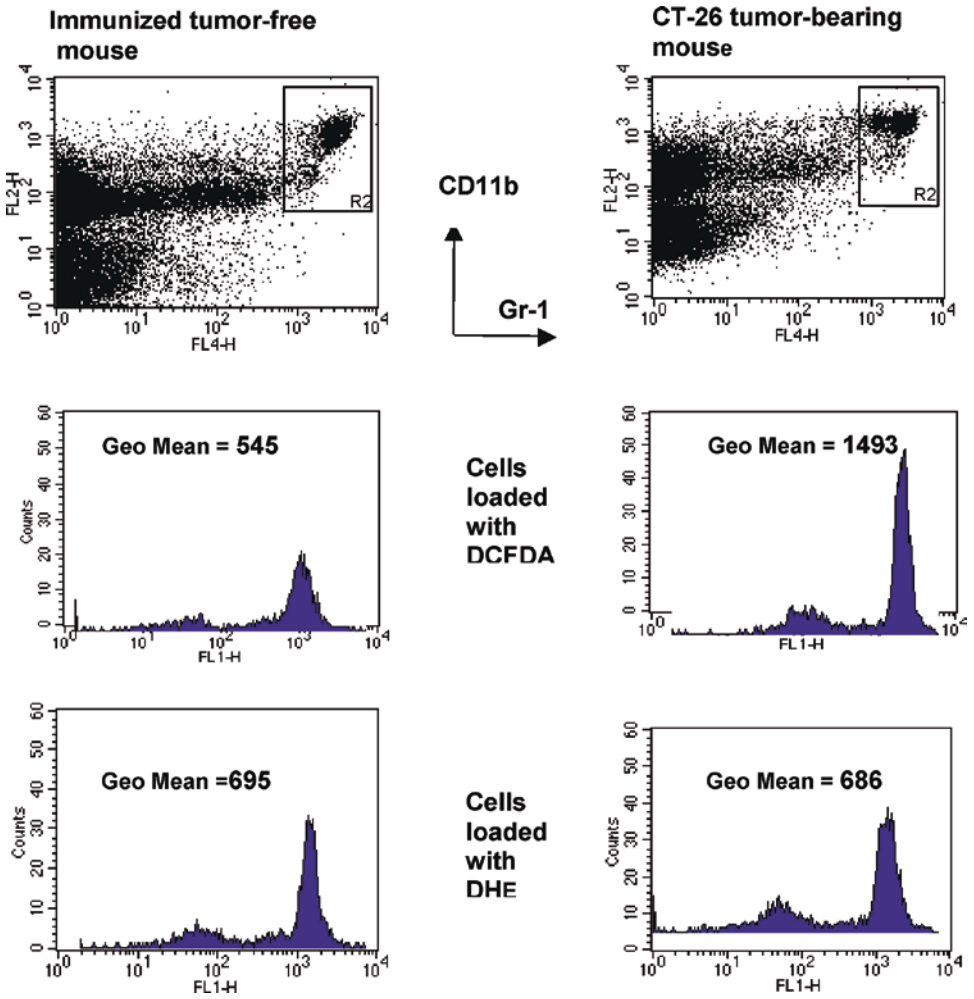

Fig. 4.2. Gr-1$^+$CD11b$^+$ myeloid cells from tumor-bearing mice demonstrate increased level of ROS. Splenocytes from tumor-bearing mice were incubated in serum-free medium at 37°C in the presence of DCFDA (2 μM, 30 min) or DHE (2 μM, 60 min), washed with cold PBS and then labeled with Gr-1-APC and CD11b-PE antibodies. After incubation on ice for 20 min cells were washed and analyzed by 3-color flow cytometry, using FACS Calibur (Becton Dickinson, Anaheim, CA). Two experiments with similar results were performed. The intensity of fluorescence (Geo Mean) in gated population of cells for each histogram is shown in Fig. 4.3.

higher than their counterparts from immunized tumor-free mice (Fig. 4.2). We compared the levels of DCFDA mediated fluorescence in Gr-1$^+$CD11b$^+$ ImC and Gr-1$^-$CD11b$^+$ macrophages in same spleens. ImC generated threefold to fourfold more ROS than Gr-1$^-$CD11b$^+$ macrophages.

When loaded with DCFDA Gr-1$^+$, splenocytes isolated from tumor-bearing mice demonstrated more than two-fold higher proportion of the cells with bright fluorescence and threefold higher intensity of total fluorescence than Gr-1$^+$ cells isolated from immunized mice even without additional stimulation with

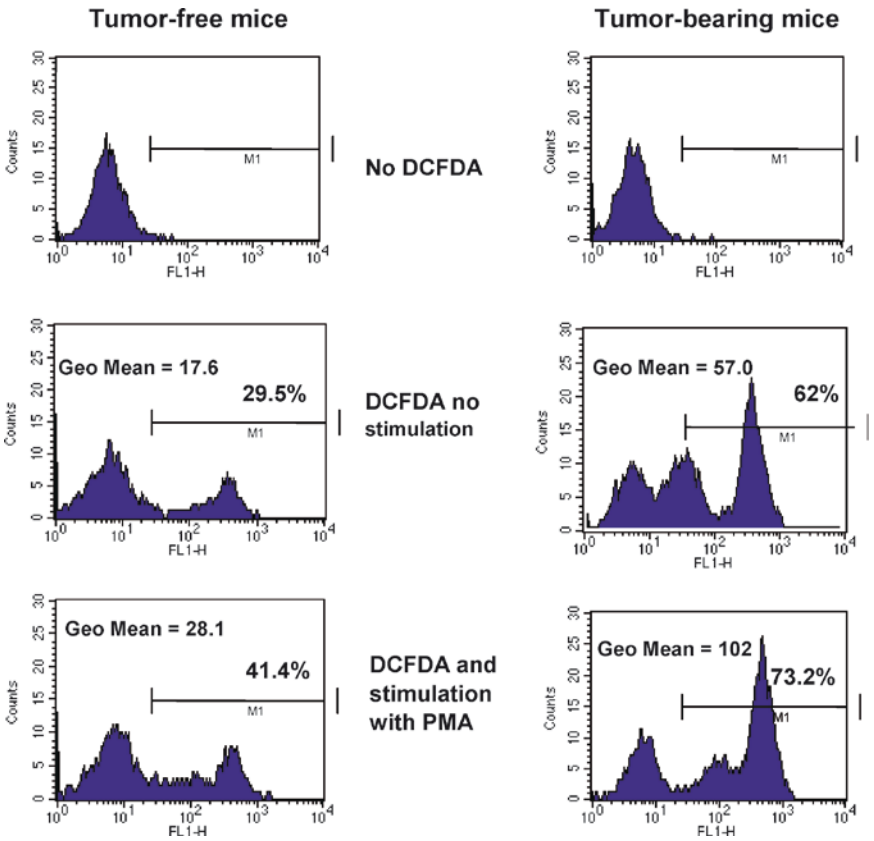

Fig. 4.3. Purified Gr-1⁺ splenocytes were incubated with 2 μM DCFDA for 15 min at 37°C, then washed twice with PBS. In the case of stimulation with PMA, cells were incubated at 37°C for 5 min with PMA (30 ng/ml), and then washed again with PBS. Intensity of fluorescence was measured by flow cytometry. Typical results of one of three performed experiments are shown. Proportion of cells with bright fluorescence was calculated. Geometric mean (Geo Mean) was used to calculate the total intensity of fluorescence.

PMA (Fig. 4.3). Activation of cells with PMA increased observed differences. Thus, Gr-1⁺ ImC from tumor-bearing mice had substantially higher levels of ROS production than ImC from tumor-free mice.

3.2.2. Nature of ROS

ROS may include different types of molecules from singlet oxygen to hydroxyl peroxide. We asked what type of ROS is produced by ImC. Gr-1⁺ cells were isolated from tumor-bearing mice and different oxygen species were neutralized using specific inhibitors or scavengers. The effect of these compounds was evaluated by flow cytometry using DCFDA. Catalase reduced ROS level in ImCs more than fourfold, indicating that H_2O_2 contributed greatly into overall level of ROS in these cells (Fig. 4.4). Uric acid had similar effects, suggesting that peroxynitrite could be a substantial part of ROS pool. However, the most noticeable differences were found in the effect of arginase inhibitor

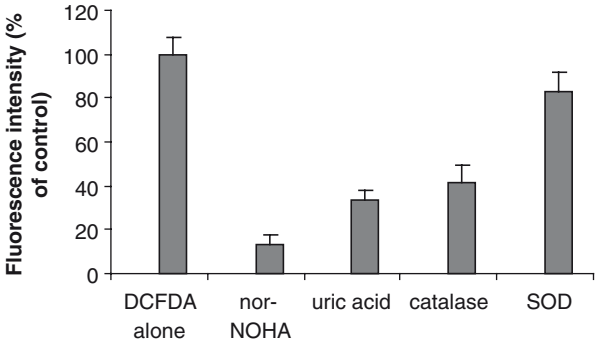

Fig. 4.4. Gr-1$^+$ myeloid cells were isolated from control and tumor-bearing mice as described in Methods. The oxidative sensitive dye DCFDA was used for the measurement of ROS production by these cells. To block ROS production, Gr-1$^+$ cells were incubated for 10 min at 37°C with different inhibitors followed by 20 min incubation at 37°C with DCFDA. The following reagents purchased from Calbiochem were used: Superoxide dismutase (SOD) – 200 units/ml, Catalase – 1,000 unit/ml, Peroxynitrite scavenger, Uric acid – 0.5 mM, Arginase inhibitor – Nor-NOHA (N-Hydroxy-nor-l-arginine, diacetate salt) – 2 μM. After incubation cells were washed in cold PBS and analyzing by flow cytometry. The level of fluorescence in ImC incubated in medium alone was used as a background.

Nor-NOHA. It decreased ROS levels in ImCs more than tenfold (Fig. 4.4). This strongly suggests that arginine metabolites play a critical role in generation of ROS in tumor-bearing mice derived ImCs. SOD did not significantly affect the levels of ROS (Fig. 4.4). This suggested a rather minor contribution of superoxide into the total ROS pool, which was consistent with lack of changes in DHE oxidation.

4. Notes

1. Serum-free media must be used since serum will contain endogenous esterase activity and de-esterified dichlorofluorescein (DCF) which is less permeable and will generate inconsistent data.
2. DCFDA enters cells and accumulates mostly in the cytosol. To avoid any cytotoxicity, cells should be loaded with DCFDA at low concentrations. With a variety of cell types, we have found loading at 1–10 μM for 45 min to 1 h is adequate. Higher levels of DCFDA or high light intensities can also result in an artifactual photochemical oxidation to fluorescent products that can be mistaken for ROS generation.
3. Because of oxidation, working solutions of CM-H2DCFDA should be freshly prepared right before performing the experiments.

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