The CD16<sup>−</sup>CD56<sup>bright</sup> NK Cell Subset Is Resistant to Reactive Oxygen Species Produced by Activated Granulocytes and Has Higher Antioxidative Capacity Than the CD16<sup>+</sup>CD56<sup>dim</sup> Subset

Helena Harlin, Mikael Hanson, C. Christian Johansson, Daiju Sakurai, Isabel Poschke, Håkan Norell, Karl-Johan Malmberg and Rolf Kiessling

*J Immunol* 2007; 179:4513-4519; doi: 10.4049/jimmunol.179.7.4513

http://www.jimmunol.org/content/179/7/4513

---

**References**

This article cites 50 articles, 26 of which you can access for free at:

http://www.jimmunol.org/content/179/7/4513.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
The Journal of Immunology

The CD16−CD56bright NK Cell Subset Is Resistant to Reactive Oxygen Species Produced by Activated Granulocytes and Has Higher Antioxidative Capacity Than the CD16+CD56dim Subset

Helena Harlin,2* Mikael Hanson,2* C. Christian Johansson,* Daiju Sakurai,† Isabel Poschke,* Håkan Norell,* Karl-Johan Malmberg,† and Rolf Kiessling3*

Human NK cells can be divided into CD56dim and CD56bright subsets. These two types of NK cells respond to different types of stimuli, with CD56dim NK cells having direct cytotoxic ability and CD56bright NK cells having mainly an immunoregulatory function. We show that the CD16+CD56dim NK subset is characterized by sensitivity to cell death induced by activated granulocytes. We identified hydrogen peroxide (H2O2) as the major effector molecule responsible for the cytotoxic effect of granulocytes on CD56dim NK cells, because the ability of granulocytes to kill CD56dim NK cells was completely abrogated in the presence of the hydrogen peroxide scavenger catalase. When exposing NK cells to H2O2, CD56dim NK cells showed rapid mitochondrial depolarization and down-regulation of activating NKRs, eventually resulting in cell death, whereas CD56bright NK cells remained unaffected. The difference in sensitivity to H2O2 was mirrored by a difference in intracellular oxidation levels between CD56dim and CD56bright NK cells, and cell lysates from the latter subset possessed a greater ability to block H2O2-mediated oxidation. Our data may explain the preferential accumulation of CD56bright NK cells often seen in environments rich in reactive oxygen species, such as at sites of chronic inflammation and in tumors. The Journal of Immunology, 2007, 179: 4513–4519.

Natural killer cells are lymphocytes that derive from the same common lymphocyte precursor as T cells. They play an important role in the early innate response against pathogens and may also be of importance in the recognition and clearance of cancer cells (1, 2). Human NK cells can be subdivided into two subsets based on their level of CD56 expression. CD56dim NK cells are potent cytotoxic cells, express high levels of granzymes and perforin, and can also mediate Ab-dependent cellular cytotoxicity through the FcR CD16. CD56bright NK cells have been described. CD56 bright NK cells preferentially accumulate in the T cell area of the lymph node, presumably through their selective expression of CCR7 and CD62L (4, 5). The developmental relationship between CD56dim and CD56bright NK cells is not clear, and these two subpopulations of NK cells may represent two developmentally distinct subsets. Alternatively, CD56bright NK cells have been considered to be immature NK precursor cells with a potential to differentiate into CD56dim NK cells (6).

There have been several reports of an increased ratio of CD56bright to CD56dim NK cells at sites of chronic inflammation, such as rheumatoid arthritis, tuberculosis, and pulmonary sarcoidosis (7–12). It is not clear whether this is due to increased recruitment, proliferation, or generation of CD56bright cells or due to preferential death of CD56dim cells. However, there is some evidence to suggest that the latter alternative may be the case, such as the finding that CD56dim cells preferentially undergo apoptosis upon incubation with tuberculous effusions (12). In addition, tumors are often themselves sites of chronic inflammation, and circulating CD56dim NK cells in cancer patients were shown to be preferentially targeted for apoptosis, leading to low levels of NK activity (13).

Reactive species that give rise to oxidative stress, such as the reactive oxygen species (ROS) (H2O2) and the reactive nitrogen species NO, are produced by activated granulocytes and macrophages during inflammation (14–16). Furthermore, H2O2 may be produced by the tumor cells themselves (17–19). This study was undertaken to address whether the decreased numbers of cytotoxic CD56dim NK cells seen during chronic inflammation may be due to selective cell death in response to exposure to ROS produced by granulocytes. Our data

*Department of Oncology and Pathology, Cancer Center Karolinska, Karolinska Institutet, Stockholm, Sweden; and †Department of Medicine, Center for Infectious Medicine, Karolinska Institutet, Stockholm, Sweden

Received for publication April 4, 2007. Accepted for publication July 12, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† This work was supported by grants to R.K. from the Swedish Cancer Society, the Cancer Society of Stockholm, the European Union, the Karolinska Institutet, an “ALF-Project” grant from the Stockholm City Council, and National Institutes of Health Grant CA102280. K.-J.M. was supported by the Royal Swedish Academy of Sciences, the Cancer Society of Stockholm, the Swedish Children’s Cancer Foundation, and the Tobias Foundation.

‡ H.H. and M.H. contributed equally to the work.

* Address correspondence and reprint requests to Dr. Rolf Kiessling, Department of Oncology and Pathology, Karolinska University Hospital, Solna, CCK 88:31, 171 76 Stockholm, Sweden. E-mail address: Rolf.Kiessling@ki.se

*Abbreviations used in this paper: ROS, reactive oxygen species; 7-AAD, 7-amino-actinomycin D; FSC, forward scatter; SSC, side scatter; CM-H2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; SNP, sodium nitroprusside; DCF, fluorescent dichlorofluorescein; HVA, homovanillic acid; DiOC6(3), 3,3′-dihexyloxocarbocyanine iodide.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00
suggest that CD56\textsuperscript{dim} cells, but not CD56\textsuperscript{bright} cells, are likely to succumb to cell death primarily as a result of exposure to H\textsubscript{2}O\textsubscript{2} produced by inflammatory cells. We further propose that the reason for the difference in sensitivity to H\textsubscript{2}O\textsubscript{2} between CD56\textsuperscript{dim} and CD56\textsuperscript{bright} cells is the lower antioxidative capacity found among CD56\textsuperscript{dim} cells.

### Materials and Methods

**Abs, staining reagents, and flow cytometry**

Cells were stained using anti-CD3-allophycocyanin (UCHT1), anti-CD56-PE (NCAM 16.2), anti-CD16-FTTC (3G8), anti-NKp46-allophycocyanin (9E2/NKp46), and anti-NKG2D-allophycocyanin (1D11). In addition, cells were labeled with 7-aminoactinomycin D (7-AAD) and/or Annexin VPE according to the manufacturer’s protocol. Viable cells were identified using 2-fold criteria: 1) no shift in the forward scatter/side scatter (FSC/SSC) profile and 2) no staining of 7-AAD and/or annexin V. All Abs and reagents above were obtained from BD Biosciences. To discriminate between sorted NK cells and granulocytes in coculture experiments, granulocytes were labeled with 5 µM CFSE (Invitrogen Life Technologies) for 10 min at 37°C, 7.5% CO\textsubscript{2} in RPMI 1640 serum-free medium (Invitrogen Life Technologies) and washed three times in AIM-V medium (Invitrogen Life Technologies). This enabled identification of NK cells (CFSE\textsuperscript{−}) and granulocytes (CFSE\textsuperscript{+}) according to their fluorescence intensity in channel 1 (FL-1). For investigating mitochondrial depolarization, cells were incubated with 20 nM 3,3′-dihexyloxacarbocyanine iodide (DiOC\textsubscript{6}(3); Invitrogen Life Technologies) in AIM-V for 15 min at 37°C, 7.5% CO\textsubscript{2} in the dark before FACS analysis. A decrease in DiOC\textsubscript{6}(3) fluorescence intensity in FL-1 corresponds to mitochondrial depolarization. For analyzing intracellular levels of oxidative stress, cells were labeled with 0.25 µM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H\textsubscript{2}DCFDA; Invitrogen Life Technologies) or the oxidized control 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (Invitrogen Life Technologies) in PBS for 15 min at 37°C, 7.5% CO\textsubscript{2} before FACS analysis.

**NK cell isolation, culture, and treatment**

PBMC were isolated from buffy coats from healthy blood donors (Karolinska University Hospital, Solna, Sweden) by density gradient separation through Ficoll-Paque (Amersham Biosciences). PBMC were then washed three times in PBS at 90 x g to remove platelets. NK cells were purified from PBMC by negative selection using the Human NK Isolation kit II (Miltenyi Biotec) according to the manufacturer’s protocol. The purity of the total NK cells was routinely >95%. For further separation into CD16\textsuperscript{bright} and CD16\textsuperscript{dim} subsets, CD16 microbeads (Miltenyi Biotec) were used according to the manufacturer’s protocol. The purity of CD16\textsuperscript{dim} cells was routinely >98% and the purity of CD16\textsuperscript{bright} cells was 47–60%. In some experiments, pure (>95%) CD16\textsuperscript{dim} and CD16\textsuperscript{bright} NK cell populations were isolated by cell sorting using flow cytometry. Purified NK cells were labeled with anti-CD56 and anti-CD3 Abs together with 7-AAD. The cells were then sorted into CD16\textsuperscript{bright} and CD16\textsuperscript{dim} subsets, CD16 microbeads (Miltenyi Biotec) were used according to the manufacturer’s protocol. The purity of CD16\textsuperscript{dim} cells was routinely >98% and the purity of CD16\textsuperscript{bright} cells was 47–60%. In some experiments, pure (>95%) CD16\textsuperscript{dim} and CD16\textsuperscript{bright} NK cell populations were isolated by cell sorting using flow cytometry. Purified NK cells were labeled with anti-CD56 and anti-CD3 Abs together with 7-AAD. The cells were then sorted into CD16\textsuperscript{dim} (CD16\textsuperscript{dim}, CD3\textsuperscript{−}, 7-AAD\textsuperscript{−}) and CD16\textsuperscript{bright} (CD16\textsuperscript{bright}, CD3\textsuperscript{−}, 7-AAD\textsuperscript{−}) NK cell populations by flow cytometry using a FACSaria (BD Biosciences). After separation, the cells were resuspended at 1 x 10\textsuperscript{6} cells/ml in AIM-V medium (Invitrogen Life Technologies). Cells were treated with gamma-radiation, sodium nitroprusside (SNP; Sigma-Aldrich) or hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}, 300 µM) CM-H\textsubscript{2}DCFDA or oxidized DCFDA in PBS at 37°C for 10 min. The cells were then washed and resuspended in fresh AIM-V. The subsets were cultured in AIM-V with or without addition of H\textsubscript{2}O\textsubscript{2} for 30 min at 37°C, 7.5% CO\textsubscript{2} and then either left solely CM-H\textsubscript{2}DCFDA labeled, or additionally stained with anti-CD56-PE. After verifying that compensation between FL-1 and FL-2 was not interfering with analysis of the data, total NK cells were also analyzed in the manner described, performing staining for CD56 expression along with CM-H\textsubscript{2}DCFDA labeling.

#### Antioxidative effect of cellular lysates on H\textsubscript{2}O\textsubscript{2}-dependent oxidation

To measure the total antioxidative levels of NK cell subsets, the ability of cellular lysates to inhibit H\textsubscript{2}O\textsubscript{2}-dependent oxidation of homovanillic acid (HVA; Sigma-Aldrich) was determined. HVA is oxidized by HRP (Sigma-Aldrich) and H\textsubscript{2}O\textsubscript{2} to form a highly fluorescent compound. Purified CD16\textsuperscript{−} (CD56\textsuperscript{dim}) and CD16\textsuperscript{−} (CD56\textsuperscript{bright}) NK cells were lysed at 10 x 10\textsuperscript{6} cells/ml in 50 mM phosphate buffer containing 1 mM EDTA (Sigma-Aldrich) and 1% Triton X-100 (Merck). Eighty microliters of a 1/2 dilution of each sample (cell lysate or PBS) was mixed with 15 µl of reaction mix (30 mM HVA, 1 U/ml HRP) and 5 µl of 6 mM H\textsubscript{2}O\textsubscript{2} (300 µM). The decreased production of oxidized substrate was measured with excitation at 355 nm and detection at 420 nm in a spectrophotometer (Victor\textsuperscript{2} 1420 Multilabel Counter, Wallac/PerkinElmer). Readings were made every 180 s for 30 repeats, and the average of peak repeats was analyzed (typically seven repeats, performed in duplicate).

#### Statistical analysis

The statistical analysis was done using the two-tailed paired t-test. Brackets in figures indicate which groups were compared and *,**,**,*** indicate values of p < 0.05, <0.01, and <0.001, respectively.

### Results

#### CD56\textsuperscript{dim} NK cells selectively undergo cell death when exposed to activated granulocytes

Several reports have shown increased CD56\textsuperscript{bright} to CD56\textsuperscript{dim} NK cell ratios at sites of chronic inflammation, including cancer (7–12). Activated granulocytes, associated with chronic inflammation, have been shown to suppress T and NK cell function and viability as well as the expression of NKRxs (20–23). We therefore asked whether the suppressive activity of granulocytes discriminates between the two NK cell subsets, which may explain the "skewed" CD56\textsuperscript{bright} to CD56\textsuperscript{dim} NK cell ratio seen in chronic inflammation.

To obtain CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cells, purified NK cells were separated using magnetic beads based on their expression of CD16. The CD16\textsuperscript{−} cell fraction routinely consisted of >98% CD56\textsuperscript{dim} NK cells, whereas the CD16\textsuperscript{−} cell fraction contained only 47–60% CD56\textsuperscript{bright} NK cells and were termed CD56\textsuperscript{bright}-enriched (remaining cells were CD56\textsuperscript{dim} NK cells). When CD56\textsuperscript{dim} NK cells were cocultured for 24 h with PMA-stimulated granulocytes from autologous healthy donors, a significant decrease in viability was found at a granulocyte-to-NK ratio of 0.5:1 (p = 0.025) (Fig. 1A). A tendency of decreased viability was also seen at a granulocyte-to-NK ratio of 0.25:1, but the difference did not reach statistical significance (p = 0.298). In contrast, the CD56\textsuperscript{bright}-enriched cells proved almost totally resistant to the granulocyte-induced cell death (Fig. 1A). It should be noted that because the purity of CD56\textsuperscript{bright}-enriched cells was never >60%, the actual difference between the subsets is likely even larger than...
what was observed. We conclude that granulocytes can induce significant cell death of CD56 dim but not CD56 bright NK cells. Altogether, this finding may explain the preferential accumulation of the more resistant CD56 bright NK cells in various inflammatory conditions where activated granulocytes are abundant. Next, we proceeded to investigate the mechanism responsible for this selective effect.

H2O2 is the major effector molecule responsible for the cytotoxic effect of granulocytes on CD56 dim NK cells

Activated granulocytes are known to produce a variety of molecules with a broad range of biological activities (see, for example, Ref. 24). An analysis of the underlying molecular mechanism behind their ability to induce cell death in the CD56 dim NK cell subset was therefore undertaken. The ability of PMA-activated granulocytes to kill CD56 dim NK cells was completely abrogated when catalase was present during the coculture period (Fig. 1B). In contrast, preincubation of the granulocytes with the inducible NO synthase inhibitor N G-monomethyl-L-arginine or the arginase inhibitor N 3-Hydroxy-nor-L-arginine had no or only marginal effects on the viability of this NK cell subset (data not shown). Thus, H2O2 plays a dominant role in granulocyte-mediated killing of CD56 dim NK cells.

H2O2 increases the CD56 bright to CD56 dim NK cell ratio in PBMC and sorted NK cells and preferentially kills isolated CD56 dim NK cells

Our results clearly indicated a dominant role for H2O2 in the observed cytotoxicity mediated by granulocytes on the CD56 dim NK cells. We therefore next performed a detailed analysis on the effect of H2O2 on this subset, analyzing the sensitivity of total PBMC or isolated NK cells to H2O2 as a model for exposure to ROS (Fig. 2A). Activated granulocytes can produce H2O2 at concentrations in the range of 5–50 μM (25), and we used a similar range of concentrations for our experiments to mimic physiological levels of H2O2 exposure. With increasing concentration of H2O2, the relative proportion of CD56 dim among total live NK cells decreased, as most of the CD56 bright NK cells were alive whereas a large proportion of CD56 dim NK cells were dead. At concentrations of 10 μM and above, the difference in proportion of live CD56 dim cells between untreated (0 μM) and treated NK cells reached statistical significance. Preferential survival of CD56 bright cells (p = 0.02 at

FIGURE 1. CD56 dim NK cells selectively undergo cell death upon coculture with granulocytes in a H2O2-dependent manner. Granulocytes and NK cells were isolated from buffy coats from healthy blood donors. Granulocytes were labeled with CFSE to discriminate between granulocytes and NK cells after coculture. NK cells were separated into CD16 (CD56 dim) and CD16− (CD56 bright-enriched) subsets. A, CD16+ or CD16− NK cells were cocultured for 24 h at the indicated ratios with PMA-stimulated granulocytes. Cell viability of NK cells (CFSE−) was determined by flow cytometry using 7-AAD as a cell death marker. Data are presented as the percent of dead cells at 1:0, 1:0.25, and 1:0.5 NK:granulocyte ratio divided by the percent of dead cells without granulocytes (1:0 ratio). B, CD16+ NK cells were incubated with the indicated ratios of PMA-stimulated granulocytes either in the presence or absence of H2O2 scavenger (catalase). The figure shows data from one representative experiment of three experiments.

FIGURE 2. CD56 dim NK cells undergo cell death upon exposure to low levels of H2O2. A, PBMC ( X) or purified NK cells ( ⋆) were incubated in the presence of increasing concentrations of H2O2 for 24 h and stained with anti-CD3 and anti-CD56. The percent of CD56 dim cells of total live NK cells (CD56+, CD3+, no FSC/SSC shift and 7-AAD−) are shown. Each line represents a separate donor and experiment and the bracket surrounding the p value indicates at what H2O2 concentration differences in viability between treated and nontreated cells reach statistical significance. B, NK cells were isolated and labeled with anti-CD56, anti-CD3 Abs, and 7-AAD. Pure (>95%) CD56 dim and CD56 bright NK populations were then separated by flow cytometry-based cell sorting. Cells were incubated in the absence or presence of increasing concentrations of H2O2 for 24 h. Dot plots indicating the percent of dead (7-AAD+) cells in each purified subset are shown. Results from one representative experiment of three are shown.
20 μM H₂O₂ was also seen when NK cells were separated into CD56dim and CD56bright subsets using flow cytometry-based cell sorting (>95% purity of each subset) and then exposed to H₂O₂ (Fig. 2B), similar to what was found following exposure to granulocytes as detailed above. To test whether H₂O₂, besides inducing death of CD56dim cells, may also have a direct effect on proliferation of CD56bright cells, CFSE-labeling experiments were performed. There was no induction of proliferation by H₂O₂ in our model system (data not shown). Thus, CD56dim cells were sensitive whereas CD56bright cells were largely resistant to cell death induced by physiological levels of H₂O₂ produced by activated granulocytes or added directly to the cells.

Selective death of CD56dim cells following H₂O₂ exposure is not observed with other apoptotic stimuli

The finding of selective death of CD56dim NK cells by H₂O₂ prompted us to explore whether this was restricted to H₂O₂ exposure, or whether other cell death-inducing factors would also preferentially kill this subset. Total PBMC were exposed to H₂O₂, the NO donor SNP, or gamma-radiation and the percent of CD56dim cells among the total live NK cells was determined under conditions where the overall lymphocyte cell death was 50–75%. There was a clear difference in relative sensitivity to cell death of CD56dim cells depending on the stimulus used. Exposure to H₂O₂ resulted in a significant decrease in the percent of CD56dim NK cells (median 92.7% without and 76.3% with H₂O₂ exposure) in the live lymphocyte population (Fig. 3). A minor decrease in the percent of CD56dim NK cells was also seen in cells exposed to SNP (median 94.6% without and 89.2% with SNP exposure). In contrast, CD56bright cells preferentially underwent cell death as a result of exposure to gamma-radiation. These data show that the CD56dim cells are not sensitive to apoptosis in a nondiscriminate fashion, hence supporting a unique role for H₂O₂ in preferentially inducing cell death of this subset of NK cells.

H₂O₂-treated NK cells display down-regulation of NKRs which correlates with depolarization of the mitochondrial membrane

Recent data has shown that the CD56dim, but not the CD56bright, NK subset down-regulates NKp46 and NKG2D upon exposure to ROS (21). We therefore asked to what extent these findings were related to the high sensitivity to H₂O₂ observed in the CD56dim NK subset in the present study, and also whether other cell surface receptors in this subset may be down-regulated by H₂O₂. NK cells were separated into CD56dim and CD56bright-enriched subsets and treated with H₂O₂ for a period of 15 h. Among CD56bright-enriched cells gated for CD56bright expression neither cell death nor down-regulation of cell surface receptor expression was seen following H₂O₂ exposure. However, among the viable CD56dim cells, we could confirm down-regulation of the NKp46 receptor (Fig. 4A). In contrast, CD16 was not down-regulated as a result of H₂O₂ treatment (Fig. 4A). Down-regulation of NKRs was not a general phenomenon, because NKG2A was expressed to the same extent on H₂O₂-treated and untreated cells, whether live or dead (data not shown). These data show that H₂O₂ treatment, besides triggering

---

**FIGURE 3.** CD56dim NK cells display a unique susceptibility to H₂O₂ exposure. PBMC were isolated from donor buffy coats and cells were exposed to increasing concentrations of H₂O₂, the NO donor SNP, or gammaradiation for 24 h. First, the total lymphocyte cell death was measured by FSC/SSC shift. To be able to compare the different cell death induction stimuli, the percent of CD56dim cells of live NK cells was analyzed when 50–75% total lymphocyte cell death was observed. Data from 10 (H₂O₂ and SNP) and 12 (gamma-radiation) independent experiments are shown.

**FIGURE 4.** NKp46 is down-regulated on H₂O₂-treated CD56dim cells before cell death in a manner that parallels mitochondrial membrane polarization. NK cells were purified fromuffy coat and separated into CD16⁺ (CD56dim) and CD16⁻ (CD56bright-enriched) subsets. Results from one representative experiment of three are shown. A, Cells were untreated (gray line) or treated with 20 μM H₂O₂ (black line) for 15 h and stained with anti-CD16 or anti-NKp46 ab, and 7-AAD. Live (no FSC/SSC shift and 7-AAD⁻) CD16⁺ or CD16⁻ cells were analyzed for expression of NKp46 or CD16. B, CD16⁺ NK cells (>95% CD56dim) were cultured in the absence or presence of 20 μM H₂O₂ for the indicated times. Cells were stained for NKp46 expression along with DiOC₆(3), as a marker for intact mitochondrial membrane potential. C, Cells shown in B stained with 7-AAD as a marker for cell death.
cell death of CD56\textsuperscript{dim} NK cells, also results in down-regulation of specific NK cell surface receptors.

Next, we asked whether the down-regulation of NKp46 in the CD56\textsuperscript{dim}, but not the CD56\textsuperscript{bright}, NK subset upon \( \text{H}_2\text{O}_2 \) exposure was related to the induction of cell death in these NK subsets. We therefore examined mitochondrial membrane depolarization as an early marker for cells targeted for apoptosis. Depolarization of CD56\textsuperscript{dim} cell mitochondrial membrane potential could be seen starting as early as 1 h after \( \text{H}_2\text{O}_2 \) exposure, peaking at \( \sim 6 \) h (Fig. 4B). NKp46 expression was reduced in a manner that closely paralleled that of mitochondrial membrane depolarization (Fig. 4B).

In contrast, cell death as determined by 7-AAD staining was absent in a majority of CD56\textsuperscript{dim} cells at 6 h, and was not detected to a large extent until after 15 h of \( \text{H}_2\text{O}_2 \) exposure (Fig. 4B). Our results show that there was an early down-regulation of NKp46 on the CD56\textsuperscript{dim} NK subset, preceding cell death.

**CD56\textsuperscript{bright} NK cells display higher intracellular antioxidative capacity than CD56\textsuperscript{dim} NK cells**

Several possible mechanisms may account for the observation that CD56\textsuperscript{dim} NK cells are more sensitive to \( \text{H}_2\text{O}_2 \) as compared with CD56\textsuperscript{bright} NK cells. We hypothesized that this may be due to differences in levels of cellular antioxidants. To directly test this possibility, we stained NK cells with CM-H\textsubscript{2}DCFDA and exposed the cells to \( \text{H}_2\text{O}_2 \), followed by assessment of levels of DCF fluorescence as a global measurement of oxidative stress (26). CD56\textsuperscript{dim} NK cells showed a higher level of intracellular oxidation when compared with the CD56\textsuperscript{bright} NK cell subset even in the absence of \( \text{H}_2\text{O}_2 \), and this difference was even more pronounced upon exposure to \( \text{H}_2\text{O}_2 \). Even though the level of oxidation increased in CD56\textsuperscript{bright} NK cells upon \( \text{H}_2\text{O}_2 \), exposure, the level did not exceed that seen in untreated CD56\textsuperscript{dim} NK cells (Fig. 5). To verify that this observation was not due to preferential uptake of the probe by CD56\textsuperscript{dim} NK cells, oxidized DCFDA was used as above. When the NK cells were labeled with oxidized DCFDA, there was no difference in fluorescence between the NK cell subsets. To further control for a possible artifact due to compensation settings used in the flow cytometric analysis, the two NK subsets were separated by CD16 expression into CD56\textsuperscript{dim} and CD56\textsuperscript{bright}, enriched fractions after labeling with CM-H\textsubscript{2}DCFDA, and were then cultured in the presence or absence of \( \text{H}_2\text{O}_2 \). The difference in DCF staining was still evident when comparing the enriched NK cell subsets not labeled by any other means (data not shown).

To confirm these observations, cellular lysates from purified CD56\textsuperscript{dim}- and CD56\textsuperscript{bright}-enriched NK cells were analyzed for their antioxidative ability in a fluorometric assay. Cell lysates from CD56\textsuperscript{bright} NK cells significantly reduced the level of HRP-mediated oxidation of the substrate to a greater extent than those of CD56\textsuperscript{dim} NK cells (Fig. 5C). Thus, the difference in sensitivity to \( \text{H}_2\text{O}_2 \) between CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cells is likely due to decreased levels of intracellular oxidation in the CD56\textsuperscript{bright} NK cells, most likely as a result of increased levels of cellular antioxidants.

**Discussion**

In this study, we report that CD56\textsuperscript{dim} NK cells are highly sensitive to activated granulocytes through a mechanism involving \( \text{H}_2\text{O}_2 \), while CD56\textsuperscript{bright} NK cells are almost completely resistant. The \( \text{H}_2\text{O}_2 \)-exposed CD56\textsuperscript{dim} NK cells showed down-regulation of the activating NKR NKp46 at an early time point, which correlated with mitochondrial membrane depolarization. The difference in sensitivity to \( \text{H}_2\text{O}_2 \) between CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cells may be related to the higher “baseline” levels of oxidative stress observed in CD56\textsuperscript{dim} NK cells and the higher antioxidative capacity of cell lysates from this subset. Collectively, our data suggest that CD56\textsuperscript{dim} NK cells present in an environment where \( \text{H}_2\text{O}_2 \) is produced, such as various inflammatory conditions, rapidly lose expression of receptors required for lytic function and then selectively undergo cell death.

We noticed rapid mitochondrial depolarization in the CD56\textsuperscript{dim} NK cells, followed several hours later by annexin V and 7-AAD
staining as well as by a shift in FSC/SSC profile consistent with cellular condensation. Cell death has classically been described as either apoptotic or necrotic, but more recent information has shown that a clear distinction between these two types of cell death is not always possible (for reviews, see Refs. 27–29). NK cell death induced by physiological levels of H$_2$O$_2$ is characterized by late caspase activation, DNA fragmentation, cellular condensation, and maintenance of intracellular ATP (data not shown and Refs. 23 and 30), more resembling apoptotic than necrotic cell death. To address the underlying mechanism behind the preferential cell death of CD56$_{dim}$ NK cells, we investigated the antioxidative capacity of the two NK subsets. Indeed, we and others have previously shown that death of total NK cells, typically consisting of a majority of CD56$_{dim}$ cells, can be blocked by agents that scavenge H$_2$O$_2$, such as serotonin and catalase (31–33).

Selective down-regulation of Nkp46 and NKG2D on CD56$_{dim}$ NK cells after exposure to ROS has recently been reported (21), although the correlation with eventual apoptosis of the CD56$_{dim}$ NK cells was not investigated in this system. Our results demonstrate that the decrease in Nkp46 expression strongly correlates with H$_2$O$_2$ treatment. Down-regulation of Nkp46 occurs in a manner that closely parallels that of mitochondrial membrane depolarization, several hours before cell death. Because Nkp46 is down-regulated very early, this seems to be an active process that would presumably quickly limit CD56$_{dim}$ NK cell’s ability to lyse Nkp46 ligand-expressing targets, such as tumor cells (34, 35). H$_2$O$_2$ exposure has been shown to inhibit NK cytotoxicity against K562 cells (31, 33, 36), and signaling through Nkp46 is very important for lysis of K562 cells by freshly isolated NK cells (33). Down-regulation of Nkp46 and other natural cytotoxicity receptors has been seen in NK cells from patients suffering from acute myeloid leukemia (37), chronic hepatitis C infection (38), and active HIV infection (39). Nkp46 associates with the ITAM-containing signaling molecules CD3ζ and FcεRI (40–42). CD3ζ chain has also been shown to be down-regulated in NK cells upon H$_2$O$_2$ exposure (33, 43), and a substance that up-regulates glutathione levels can protect from H$_2$O$_2$-mediated NK cell CD3ζ chain down-regulation (43). Thus, it seems possible that there is a connection between expression of CD3ζ and expression of Nkp46.

As a new and unexpected finding, our study shows that there is a difference in “baseline” oxidative levels between CD56$_{dim}$ and CD56$_{bright}$ NK cells even without exogenous exposure to H$_2$O$_2$, as measured by CM-H$_2$DCFDA conversion to DCF, and by the ability to block H$_2$O$_2$-mediated oxidation of HVA. We would thus propose that a small oxidative insult would be enough to push CD56$_{dim}$ NK cells into oxidative stress, whereas CD56$_{bright}$ NK cells have a buffer zone that allows them to absorb some level of oxidative insult without harm. It is not clear what form of ROS H$_2$O$_2$ is converted to during the in vitro culture, but it seems likely that the cells are not only exposed to H$_2$O$_2$, because H$_2$O$_2$ and O$_2$ do not oxidize H$_2$O$_2$ and O$_2$ do not oxidize H$_2$O$_2$. Peroxyl alcohol, NO$_2^-$, carbonate (CO$_3^-$) and OH$^-$ radicals do, as does peroxyxynitrite (26). Because NO does not preferentially induce cell death of CD56$_{dim}$ NK cells (Fig. 3), it is likely that the oxidative stress resulting from H$_2$O$_2$ exposure acts on one or several specific substrates, which remain to be identified.

In addition, the measurement of antioxidative ability of cellular lysates by CD56$_{dim}$ and CD56$_{bright}$ NK cells lends further support to the notion that differences in antioxidative function could explain the difference in sensitivity to H$_2$O$_2$ between these two NK subsets. Recently, expression of genes in CD56$_{dim}$CD16$^+$ and CD56$_{bright}$CD16$^-$ NK cell subsets was analyzed using a gene array approach (44). Several genes found to be differentially expressed between CD56$_{dim}$ and CD56$_{bright}$ NK cells may be candidates for antioxidation, as inferred from this study. In particular, paraoxonase 2, which has antioxidative ability (45), and lysozyme, which has been shown to be protective against oxidative stress by H$_2$O$_2$ and other oxidants (46), are two factors that were up-regulated at least 6-fold in CD56$_{bright}$ NK cells (paraoxonase 2 expression was below the limit of detection in CD56$_{dim}$ cells). Furthermore, glutathione peroxidase 1, an enzyme that catalyzes the reduction of H$_2$O$_2$, and organic hydroperoxides using glutathione, was found to be expressed to a higher extent in CD56$_{bright}$ cells (2.5-fold).

In conclusion, our data support a model where CD56$_{dim}$ NK cells have a high and selective sensitivity to H$_2$O$_2$ exposure due to low antioxidative ability. It is important to note, however, that our data do not distinguish between an effect on CD56$_{dim}$ NK cells mediated by “oxidative damage” caused by the externally added H$_2$O$_2$ on cellular components or, alternatively, by H$_2$O$_2$-triggering signaling events in the cell leading to receptor down-regulation and eventual cell death. H$_2$O$_2$ at low levels is known to act as an important “second messenger” during lymphocyte activation (see, for example, Ref. 47), although the exact molecular pathway involved has not been conclusively determined. H$_2$O$_2$ has been suggested to have both proliferative and apoptotic effects on T cells through various signaling pathways (48–50). Higher levels of antioxidative ability in the CD56$_{dim}$ NK cells could thus either have a direct effect through oxidative damage, or alternatively act through indirect activation of signaling pathways triggering NK cell death. Studies in progress will determine which one is the most important.

Our findings may be of a direct biological relevance to explain functional consequences in pathological conditions of inflammation, where activated granulocytes, monocytes, or myeloid-derived suppressor cells accumulate, leading to enhanced ROS levels. In these environments, our data suggest that CD56$_{dim}$ NK cells would rapidly undergo mitochondrial membrane depolarization, accompanied by down-regulation of activating NKRs such as Nkp46, and eventually undergo cell death. This may explain why CD56$_{bright}$ NK cells, with their relative insensitivity to low levels of H$_2$O$_2$, accumulate at disproportional levels at the cost of CD56$_{dim}$ NK cells during chronic inflammation (8–12). In addition, our data may explain the poor lytic function of NK cells frequently found in the tumor microenvironment (13).

Disclosures
The authors have no financial conflict of interest.

References


