Preferential Cell Death of CD8$^+$ Effector Memory (CCR7$^-$CD45RA$^-$) T Cells by Hydrogen Peroxide-Induced Oxidative Stress[1]

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T cells are used in many cell-based cancer treatments. However, oxidative stress that is induced during various chronic inflammatory conditions, such as cancer, can impair the immune system and have detrimental effects on T cell function. In this study, we have investigated the sensitivity of different human T cell subsets to H_2O_2-induced oxidative stress. We showed that central memory (CD45RA$^-$CCR7$^+$) and effector memory (CD45RA$^-$CCR7$^-$) T cells are more sensitive to H_2O_2 as compared with naive (CD45RA$^+$CCR7$^+$) T cells. Furthermore, the study showed that CD8$^+$ effector memory T cells are more sensitive to low levels of H_2O_2 (5 μM) compared with other types of T cells investigated. H_2O_2-exposed CD45RO$^+$ T cells showed mitochondrial depolarization prior to caspase 3 activity. Moreover, the pan-caspase inhibitor z-Val-Ala-Asp(OMe)-fluoromethylketone rescued cells from death. These experiments suggest that H_2O_2-induced cell death of CD45RO$^+$ T cells acts via the mitochondrial pathway and that caspase involvement is needed. This study suggests that oxidative stress in cancer patients can be disadvantageous for T cell-based adoptive cell transfer therapies, since effector memory T cells are the primary phenotype of the cells administered. *The Journal of Immunology,* **2005, 174: 6080–6087.**

Reactive oxygen species (ROS)[4], produced by granulocytes and macrophages, are important components of the innate immunity to invading microbes (1, 2). However, oxidative stress associated with chronic inflammatory conditions can have detrimental effects mediated by the ability of ROS to induce cell death in a number of different cell types, including β cells of the pancreas (3), neural cells (4), and lymphocytes (5). It was demonstrated in several model systems that exposure of T cells to physiological levels of oxidative stress leads to a suppressed signal transduction and transcription factor activity, a block in NF-κB activation, and decreased cytokine production in response to nonspecific and Ag-specific stimulation (6–9). The ability of ROS to suppress T lymphocyte functions may therefore be one important mechanism behind the hyporesponsiveness of the immune system often observed in various chronic inflammatory conditions, including rheumatoid arthritis (10–12), HIV infection (13), and cancer (14, 15).

In tumor immunology, the negative effect of NO and H_2O_2 produced from activated macrophages and granulocytes on T and NK cell functions is well established. Coculturing tumor-infiltrating macrophages and freshly isolated human T cells results in decreased TCR ζ expression and loss of Ag-specific T cell responses (16–18). Monocytes can inhibit in vitro human NK cell-mediated cytotoxicity via secretion of H_2O_2, leading to induction of cell death (19). In addition, macrophage-derived NO markedly reduces the phosphorylation and activation of JAK3/STAT5 signal transduction proteins, inhibiting the proliferative responses of T cells to IL-2 (20). Activated granulocytes and oxidative stress mediated by H_2O_2 in the circulation of patients with advanced cancer was also recently described (14). Taken together, H_2O_2 secretion by activated macrophages and granulocytes has been suggested as one possible mechanism behind the tumor-induced immune suppression with decreased signal transduction and poor effector functions of T cells and NK cells observed in cancer patients.

We have earlier described how cytokine production of human PBMC, upon stimulation with an HLA-A2-restricted influenza peptide and nonspecific receptor cross-linking, was reduced after exposure to micromolar levels of H_2O_2 (9). This reduction of primarily Th1 cytokines was predominantly observed in the memory/effector (CD45RO$^+$) T cell subset and correlated with a block in NF-κB activation. In this study, we confirm and extend these findings in a model where the sensitivity of various T cell subsets of unstimulated human PBMC to cell death induced by low doses of H_2O_2 has been investigated. We demonstrate that effector memory T cells (T_{EM}) (CCR7$^-$CD45RA$^-$) are particularly sensitive to low doses of H_2O_2, while central memory T cells (T_{CM}) (CCR7$^+$CD45RA$^-$) are significantly less sensitive. The pan-caspase inhibitor z-Val-Ala-Asp(OMe)-fluoromethylketone (z-VAD-FMK) was found to block the cell death of purified CD45RO$^+$ T cells. Time-kinetic experiments, where the

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[4] Abbreviations used in this paper: ROS, reactive oxygen species; T_{EM}, effector memory T cells; T_{CM}, central memory T cells; 7-AAD, 7-aminoactinomycin D; z-VAD-FMK, z-Val-Ala-Asp(OMe)-fluoromethylketone; TMRE, tetramethylrhodamine ethyl ester; FLICA, fluorochrome inhibitor of caspases; FSC, forward scatter; SSC, side scatter.

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mitochondrial membrane potential and caspase 3 activity were analyzed, suggested that the mitochondrial pathway is the primary cell death pathway for $CD45RO^+$ T cells exposed to low levels of H_2O_2.

Materials and Methods

Cells

PBMC were prepared from buffy coats from healthy blood donors admitted to the blood bank at the Karolinska Hospital by means of Ficoll-Paque (Amersham Pharmacia Biotech) density gradient centrifugation. Cell concentration was adjusted to 1×10^6/ml in AIM-V serum-free medium (Life Technologies). Cells were subsequently exposed to titrated doses (0–40 μM) of H_2O_2 (Sigma-Aldrich) for different time periods at 37°C in 7.5% CO_2. In some experiments, $CD45RO^+CD3^+$ cells and $CD45RA^+CD3^+$ cells were negatively selected using a miniMACS kit according to the manufacturer's protocol (Miltenyi Biotec). In short, $CD3^+$ cells were negatively selected from PBMC by a pan-T cell isolation kit. This was followed by negative selection of $CD45RO^+$ or $CD45RA^+$ cells using either anti-CD45RA or anti-CD45RO beads. The purity of the obtained cells was always above 95% in each separation step.

Abs and FACS analysis

Cells were stained with mouse mAb anti-CD3-allophycocyanin (UCHT1), anti-CD4-FITC (SK3; BD Biosciences), anti-CD8-FITC (SK1), anti-CCR7-unconjugated (2H4) followed by secondary anti-mouse IgG-PE polyclonal rabbit Ab (R0439; DakoCytomation), anti-CD45RO-Cychrome (UCHL1), and anti-CD45RA-Cychrome (HI100) at 4°C for 30 min. All Abs were purchased from BD Biosciences if not stated otherwise. Cells were analyzed on a FACSCalibur (BD Biosciences). When stated in the results and figure legends, live cell population was determined by forward/side scatter (FSC/SSC) of lymphocytes. A shift in FSC/SSC of lymphocytes corresponds to annexin V staining, thus the shift in FSC/SSC in the lymphocyte population indicates cell death.

Cell death assays

Using a flow cytometry-based method, cell death measurements were performed by the Annexin V^{PE} apoptosis detection kit according to the manufacturer's protocol (BD Biosciences). Cells were stained with Annexin V^{PE} and the vital dye 7-aminoactinomycin D (7-AAD). Early apoptotic cells were defined as annexin V^+ and 7-AAD^-, and late apoptotic cells were defined as annexin V^+ and 7-AAD^+. Live cells were defined as double negative for these markers. To determine caspase dependency of cell death, z-Val-Ala-Asp(OMe)-fluoromethylketone (z-VAD-FMK; Alexis Biochemicals), a pan-caspase inhibitor, was used to block caspase activity. In brief, $CD45RO^+$ or $CD45RA^+$ T cells were isolated and cultured in AIM-V serum-free medium. To block caspase activity, z-VAD-FMK (50 or 100 μM, final concentration) was added to the cell culture and incubated for 1 h at 37°C in 7.5% CO_2 pre-exposure to 5 or 20 μM H_2O_2 and subsequently cultured for different time points. Cells were then analyzed by flow cytometry. To assess the cell death pathway, the depolarization of the mitochondrial membrane potential and caspase 3 and 7 activity were measured at different time points (0–14 h). To measure mitochondrial membrane potential, the fluorescent mitochondrial probe tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) was used. Briefly, cells were cultured in AIM-V with or without 5 μM H_2O_2 for different time points. TMRE (final concentration 25 nM) was added to the cell suspension that was incubated for 20 min at 37°C in 7.5% CO_2. Cells were washed twice in PBS containing TMRE (25 nM) and subsequently analyzed in FACS. To measure caspases 3 and 7 activity, a kit providing fluorochrome inhibitor of caspases (FLICA) was used according to the manufacturer's protocol (Caspases 3 and 7 Detection kit; Immunochemistry Technologies). FLICA binds covalently to specific active caspases, thus the fluorochrome accumulates in cells having active caspases and may be detected in FACS. In short, FLICA specific for caspases 3 and 7 (FAM-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone) were used and FLICA solution was added to cell suspension and incubated for 1 h at 37°C in 7.5% CO_2. Cells were then washed in washing buffer included in the kit and further analyzed in FACS.

Results

Preferential cell death of $CD45RO^+$ T cells induced by hydrogen peroxide

In this study, the sensitivity of peripheral blood-derived T cells to low levels of H_2O_2 was tested using a modification of a previously established method (9). Here, we confirm that there is a dose-dependent and selective targeting of $CD45RO^+$ T cells by low levels (<40 μM) of H_2O_2. The percentage of $CD45RO^+$ T cells was significantly decreased in the live cell population following 1-day incubation of PBMC in medium containing H_2O_2 at a concentration of 5 μM or higher (Fig. 1*A*). This observation was confirmed by a corresponding increase in the percentage of $CD45RA^+$ T cells as tested using an anti-CD45RA mAb (Fig. 1*B*).

We next investigated whether this H_2O_2-induced shift in T cell subsets, leading to a decreased proportion of $CD45RO^+$ T cells in the viable cell population, was a result of differential proliferation or a consequence of enhanced cell death of this particular subset. To this end, we stained T cells exposed to titrated doses of H_2O_2 with the cell death marker annexin V. We found that $CD45RA^-$ T cells were more prone to cell death as compared with $CD45RA^+$ T cells following exposure to H_2O_2 (Table I). Of note, H_2O_2 induced cell death also in $CD45RA^+$ T cells, but to a lesser extent. Thus, we conclude that the decrease in the percentage of $CD45RO^+$ T cells is due to the enhanced sensitivity of this subset to H_2O_2-induced cell death.

Hydrogen peroxide selectively targets $CD45RO^+CD8^+$ T cells

We next asked whether there was a selective targeting of $CD4^+$ and $CD8^+$ T cells by H_2O_2. We found that $CD8^+$ T cells were significantly more sensitive to H_2O_2 as compared with $CD4^+$ T cells leading to an increased CD4:CD8 ratio (Fig. 2, *A* and *B*). The effect on the $CD45RO^+CD8^+$ T cell subset was even more pronounced (Fig. 2*C*), although the percentage of $CD45RA^+CD8^+$ T cells was also significantly reduced (Fig. 2*D*). Furthermore, the $CD45RO^+$ T cells displayed a significantly larger relative decrease

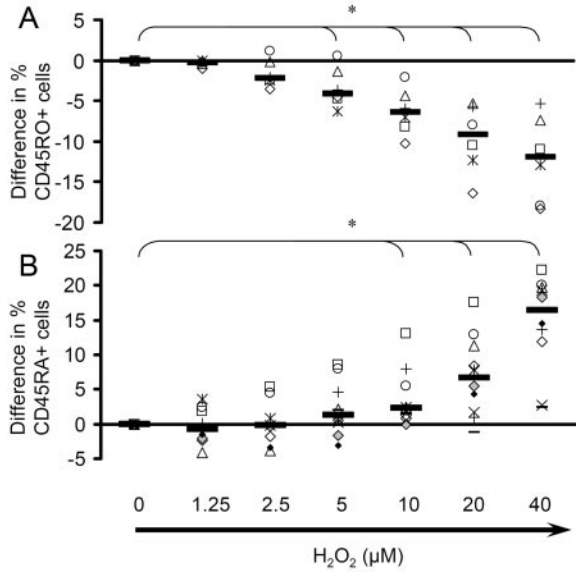

FIGURE 1. Selective cell death of $CD45RO^+$ T cells after H_2O_2 exposure. PBMC were exposed to H_2O_2 (0–40 μM) for 21 h. *A,* PBMC from six donors were stained with anti-CD3 and anti-CD45RO mAb, and the viable lymphocyte population was analyzed by FACS. *B,* PBMC from 10 donors was stained with anti-CD3 and anti-CD45RA mAb, and the viable lymphocyte population was analyzed by FACS. Each donor is indicated by an individual symbol. The difference in the proportion of $CD45RO^+$ T cells (*A*) and $CD45RA^+$ T cells (*B*) compared with unexposed cells (i.e., percent $CD45RO^+$ T cells at x μM H_2O_2 − percent $CD45RO^+$ T cells at 0 μM H_2O_2); *, $p < 0.05$ based on Wilcoxon's two-tailed matched pairs test. The brackets indicate the concentration of H_2O_2 where a significant difference is observed compared with unexposed cells. The horizontal bar indicates the median in each experimental group.

Table I. *Percent annexin V^+ $CD45RA^-$ or $CD45RA^+$ T cells after H_2O_2 exposure in PBMC of three donors*

Donor	Phenotype[a]	H_2O_2 (μM)[b] 0	1.25	2.5	5	10
1	RA^-	1%[c]	1%	1%	22%	45%
	RA^+	2%	2%	3%	4%	13%
2	RA^-	4%	9%	23%	42%	75%
	RA^+	3%	5%	10%	27%	53%
3	RA^-	1%	3%	3%	23%	39%
	RA^+	5%	5%	5%	7%	13%

[a] Cells were stained with anti-CD3, anti-CD45RA mAb, and PE-labeled annexin V and further analyzed by FACS.

[b] One million PBMC were incubated in AIM-V serum-free medium in the absence or presence of H_2O_2 (0–10 μM) for 21 h.

[c] The percent annexin V^+ cells in each cell population (i.e, annexin V^+ $CD45RA^+CD3^+$ cells/total $CD45RA^+$ $CD3^+$ cells).

of $CD8^+$ T cells as compared with $CD45RA^+$ T cells when exposed to 2.5 and 5 μM H_2O_2 (Fig. 2*E*). Importantly, the selective targeting of $CD45RO^+$ T cells cannot be explained by the higher sensitivity of $CD8^+$ cells to H_2O_2, as the proportion of $CD8^+$ cells is lower in the $CD45RO^+$ than in the $CD45RA^+$ T cell population (Fig. 2, *C* and *D*). Consequently, the higher sensitivity of $CD8^+$ T cells may not be explained by the enhanced sensitivity of $CD45RO^+$ T cells. Thus, the T cell sensitivity to H_2O_2-induced oxidative stress is dependent both on the CD4 or CD8 phenotype and on the differentiation stage of the T cell ($CD45RA^+$ vs $CD45RO^+$ cells).

Effector memory T cells are more sensitive to a low dose of H_2O_2 as compared with central memory T cells

We investigated the effect of H_2O_2-induced oxidative stress on two memory T cell subsets previously identified based on their anatomic compartmentalization and phenotypic profiles (21), i.e., T_{CM} ($CCR7^+CD45RA^-$) and T_{EM} ($CCR7^-CD45RA^-$). T_{CM} primarily traffic within lymphoid tissues and are distinguished by their expression of the lymph node homing receptors CD62 ligand and CCR7. In contrast, T_{EM} are found in peripheral tissues and do not express CCR7 and have heterogeneous expression of CD62 ligand (22). The sensitivity of T_{CM} and T_{EM} cells to H_2O_2 was analyzed in PBMC from eight different healthy donors. Interestingly, a low dose of H_2O_2 (5 μM) was found to selectively target $CD8^+$ T_{EM} (Fig. 3*A*). The same tendency was seen for $CD4^+$ T_{EM}, but this observation did not reach statistical significance ($p = 0.055$) (Fig. 3*B*). In contrast, neither $CD4^+$ nor $CD8^+$ T_{CM} cells demonstrated significant sensitivity to H_2O_2 after exposure to 5 μM H_2O_2 (Fig. 3). After culture in the presence of 20 μM H_2O_2, a significant decrease of both the $CD4^+$ and $CD8^+$ T_{EM} and $CD4^+$ T_{CM} cells was detected. However, no difference was observed in $CD8^+$ T_{CM} (Fig. 3). Thus, we conclude that $CD8^+$ T_{EM} cells are more sensitive to low doses of H_2O_2 as compared with other cell types investigated.

The pan-caspase inhibitor z-VAD-FMK blocks the induction of cell death of purified $CD45RO^+$ cells after H_2O_2 exposure

To investigate whether the $CD45RO^+$ T cell death, induced by H_2O_2 (5 and 20 μM), was dependent on caspase activities, PBMC from eight healthy donors were cultured 1-day in H_2O_2-containing medium, in the presence or absence of the pan-caspase inhibitor z-VAD-FMK. The PBMC were then stained with anti-CD3 and anti-CD45RO mAb and further analyzed by measuring the proportion of viable $CD45RO^+$ T cells in the in vitro culture. When caspase activity was inhibited by z-VAD-FMK (50 and 100 μM) in T cells exposed to 5 μM H_2O_2, the percentage of viable $CD45RO^+$ T cells was significantly increased (Fig. 4*A*). z-VAD-FMK pretreatment also increased the proportion of viable $CD45RO^+$ T cells that was exposed to 20 μM H_2O_2 (Fig. 4*B*), although not to the same extent as cells exposed to 5 μM H_2O_2. In conclusion, the ability of z-VAD-FMK to suppress the decline of $CD45RO^+$ T cells implies that H_2O_2-induced cell death is largely dependent on caspase activation.

Next, $CD45RO^+$ and $CD45RA^+$ T cells were isolated by negative selection with magnetic beads, resulting in >95% pure cell populations of the respective T cell subtype. These purified T cell subsets were then used to investigate whether a low dose of H_2O_2 (5 μM), at various time points, would result in cell death, as analyzed by flow cytometry viability staining (annexin V/7-AAD). The time-kinetic experiments revealed an increase in both early (annexin V^+, 7-AAD^-) and late (annexin V^+, 7-AAD^+) apoptotic cells in the $CD45RO^+$ T cell subset after 6 h of 5 μM H_2O_2 treatment, whereas no cell death was detected after 3-h in vitro exposure (Fig. 5*A*). Cell death of $CD45RO^+$ T cells gradually increased 12 h after H_2O_2 treatment, and after 20 h the majority of the cells in this subpopulation was in the late apoptotic stage. The inclusion of 100 μM z-VAD-FMK protected the $CD45RO^+$ T cells from cell death even when measured 20 h after H_2O_2 treatment, confirming the caspase dependency of H_2O_2-induced cell death in this model. In sharp contrast to the high sensitivity of the $CD45RO^+$ T cell subset, $CD45RA^+$ T cells were completely resistant to cell death induced by 5 μM H_2O_2 even 20 h after treatment (Fig. 5*B*). Of note, no major difference was apparent in the spontaneous cell death between $CD45RO^+$ and $CD45RA^+$ T cells after 72 h of in vitro culture (Fig. 5).

H_2O_2 induces cell death of $CD45RO^+$ T cells through the mitochondrial pathway

T cell death has been shown to be executed by either the cell surface death receptor pathway or the mitochondrial pathway (23). The cell surface death receptor pathway is characterized by activation of caspase 8 before caspase 3 activity, followed by mitochondrial depolarization and cytochrome *c* release resulting in cell death. In contrast, the mitochondrial pathway exhibits a depolarization of mitochondrial membrane before caspase activity and cell death.

To investigate the primary pathway of H_2O_2-dependent T cell death, negatively selected $CD45RO^+$ and $CD45RA^+$ T cells were exposed to 5 μM H_2O_2. The T cell subsets were then examined at different time points for caspase 3 and 7 (hereafter referred to as caspase 3 activity) activity, using caspase-specific fluorescent substrates (FLICA), and for mitochondrial membrane potential, using a mitochondrial specific dye (TMRE). In the $CD45RO^+$ T cell subset, the time-kinetic experiments showed a depolarization of mitochondrial potential at 8 h after H_2O_2 exposure (Fig. 6*A*) followed by an increase in caspase 3 activity at 10 h (Fig. 6*C*). The $CD45RA^+$ T cell subset did not show any mitochondrial depolarization or caspase 3 activity at any time point (Fig. 6, *B* and *D*). An activation of caspase 8 was also observed after 10 h in $CD45RO^+$ T cells, but not in $CD45RA^+$ T cells, implying a H_2O_2-induced caspase 3-dependent activation of caspase 8 in $CD45RO^+$ T cells (data not shown). The experiment suggests that the mitochondrial pathway is the primary pathway for $CD45RO^+$ T cell death exposed to 5 μM H_2O_2. Furthermore, the experiment indicates that the difference in cell death between the $CD45RO^+$ and $CD45RA^+$ subsets is upstream of the mitochondria in the cell death signaling pathway.

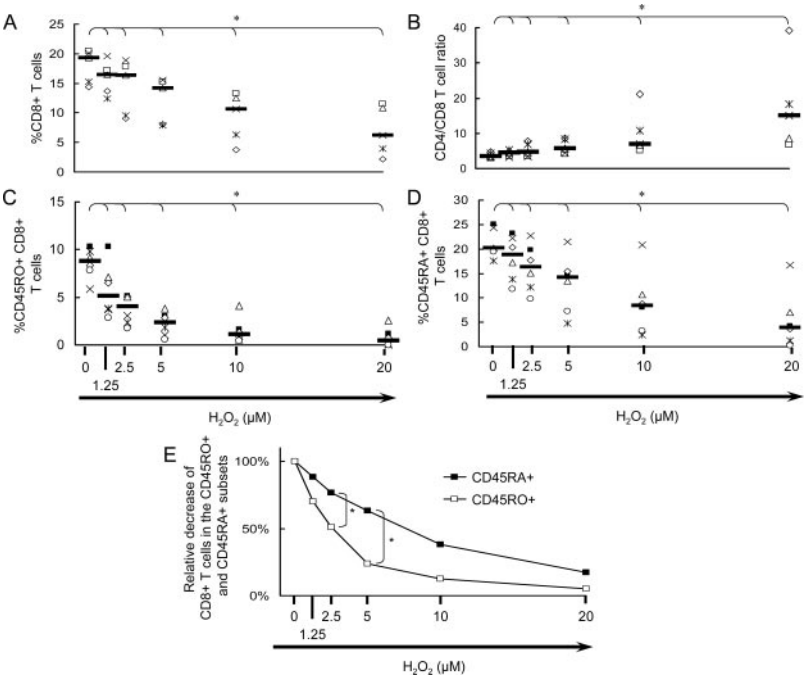

FIGURE 2. $CD45RO^+$ $CD8^+$ T cells display the largest sensitivity to H_2O_2-induced cell death. PBMC were exposed to H_2O_2 (0–20 μM) for 21 h. *A* and *B,* PBMC from five donors were stained with anti-CD3 and anti-CD8 or anti-CD4 mAb, and the viable lymphocyte population was analyzed by FACS. *A,* The proportion of $CD8^+$ T cells; *B,* the CD4:CD8 ratio after in vitro culturing. Each donor is indicated by an individual symbol. *C* and *D,* PBMC from six donors were stained with anti-CD3, anti-CD8, and anti-CD45RO or anti-CD45RA mAb and analyzed by FACS. The proportion of $CD45RO^+CD8^+$ (*C*) and $CD45RA^+CD8^+$ (*D*) T cells after in vitro culturing is shown. Each donor is indicated by an individual symbol. The horizontal bar indicates the median in each experimental group. *E,* The median of the relative decrease of $CD8^+$ T cells in the $CD45RO^+$ and $CD45RA^+$ subset compared with unexposed cells (i.e., percent $CD8^+$ cell at x μM H_2O_2/percent $CD8^+$ cell at 0 μM H_2O_2) for the donors in *C* and *D*; $*$, $p < 0.05$ based on Wilcoxon's two-tailed matched pairs test. The brackets indicate the concentration of H_2O_2 where significant difference is observed compared with unexposed cells (Figs. 2A-D) or the significant difference between the experimental groups (CD45RO+ vs CD45RA+) at different H_2O_2 concentrations in Fig. 2E.

Discussion

This study shows that human PBMC-derived T cells demonstrate a difference in their sensitivity to H_2O_2-induced cell death depending on their differentiation stage. Although the $CD45RO^+$ T cells, in particular the $CD8^+$ T_{EM} ($CCR7^-CD45RO^+$), were found to be most sensitive to cell death induced by low doses of exogenous H_2O_2, the $CD45RA^+$ T cell subset was relatively insensitive. Furthermore, we found that $CD45RO^+$ T cells exposed to low-dose H_2O_2 gradually started to die within a period of 6–8 h in a caspase-dependent manner where the mitochondrial pathway seemed to predominate.

The finding of $CD45RO^+$ T cells, in contrast to $CD45RA^+$ T cells, being more sensitive to cell death induced by H_2O_2, confirms and extends our previous study in which the capacity of stimulated T cells to produce cytokines under conditions of oxidative stress was analyzed (9). In this earlier study we noticed that the $CD45RO^+$ T cell subset lost the capacity to produce IFN-γ, TNF-α, and IL-2 after stimulation with PMA/ionomycin or anti-CD3 mAb if they were pre-exposed to H_2O_2, whereas $CD45RA^+$ T cells producing these cytokines were not affected. We speculated that the lost functional capacity of the H_2O_2-exposed $CD45RO^+$ T cell subset may reflect an early "preapoptotic" condition, although in this particular experimental setting of that study where the H_2O_2 was washed away following a 10-min exposure period, no significant cell death occurred in the lower mircomolar (<25 μM) H_2O_2 range. Motivated by this study, we therefore have changed the experimental setup and have found that if PBMC-derived T cells are subjected to a more sustained exposure to H_2O_2, where this molecule is present throughout the entire incubation period, a higher susceptibility in the $CD45RO^+$ T cell subset to H_2O_2-induced cell death can be demonstrated also in the lower micromolar range.

Exposure of cells to extracellular ROS is known to mediate cell death also in several other model systems, which include PMA-induced death of neutrophils, HIV-induced death of T cells, death of pancreatic β cells exposed to H_2O_2, and excitoxic neural cell death (3–5, 13, 24, 25). Also, others have studied this phenomenon in human T cell lymphomas (26–28), and several features of the H_2O_2-induced cell death observed here in freshly isolated $CD45RO^+$ human T cells are similar to those described in the human T cell lines Jurkat or CEM C7. These include induction of caspase 3 activity and the ability of z-VAD-FMK to prevent cell death (26–28). Also, in line with our findings on freshly isolated $CD45RO^+$ T cells is the previous finding of others that H_2O_2 can induce the loss of the mitochondrial membrane potential and release of cytochrome *c* (28, 29). Our results from the time-kinetic analysis demonstrated that the depolarization of mitochondrial membrane potential occurs before caspase activation, indicating that the mitochondrial pathway is predominant in H_2O_2-induced cell death of $CD45RO^+$ human T cells. Also, Dumont et al. (27) concluded, based on inhibition experiments with drugs, that the mitochondrial cell death pathway is predominant upon exposure of human T cell lines with 100 μM H_2O_2. The present report is however the first one to show that a similar mechanism of caspase-dependent H_2O_2-induced cell death also occurs in nontransformed human T lymphocytes.

These represent examples of cell death caused by H_2O_2, which is produced by cells other than the "target" cell in a "paracrine" fashion. In contrast to these findings and to our model described here, ROS have also been reported to mediate cell death in an "autocrine" fashion, acting as an internal messenger regulating signals involved in cell death of T cells. Thus, T cells activated in vivo through injection of mice with the superantigen staphylococcal enterotoxin A were shown to die via a Fas- and TNF-α-independent cell death (23). This activation-induced T cell death was characterized by caspase-independent loss of mitochondrial transmembrane potential, caspase-dependent DNA loss, and enhanced generation of ROS, and ROS was suggested to regulate both caspase activation and cell death in this model. Others have demonstrated that peripheral T cells cultured in the absence of survival factors accumulate ROS and up-regulate BIM (Bcl-2-interacting mediator of death) and inducible NO synthase expression, which culminates in Fas-independent "neglect-induced death" (30). Also in this phenomenon, antioxidants were shown to inhibit cell death, Bim induction, and caspase activation, implicating the direct role of ROS in cell death induction. A possible relationship between these sets of observations, where ROS

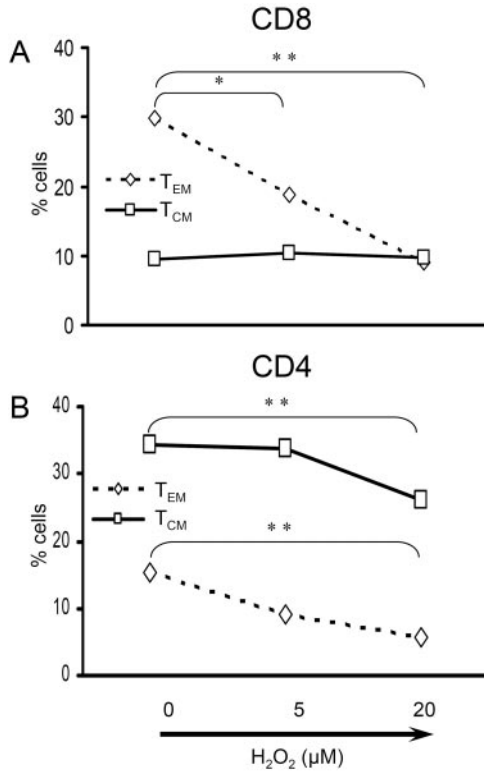

FIGURE 3. Selective targeting of T_{EM} within the CD8 T cell compartment. PBMC were exposed to H_2O_2 (0, 5, or 20 μM) for 21 h. Cells were stained with anti-CD3, anti-CD8, or anti-CD4, anti-CD45RA, and anti-CCR7 mAb, and the viable lymphocyte population was analyzed by FACS. T_{CM} cells are defined as $CD45RA^-CCR7^+$ cells; T_{EM} are defined as $CD45RA^-CCR7^-$ cells. The median of percent T_{EM} and percent T_{CM} cells for eight donors after 0, 5, and 20 μM H_2O_2 exposure in the $CD8^+$ (*A*) or $CD4^+$ (*B*) T cell subset is shown; *, $p < 0.05$; **, $p < 0.01$ based on Wilcoxon's two-tailed matched pairs test. The brackets indicate the concentration of H_2O_2 where a significant difference is observed compared with unexposed cells.

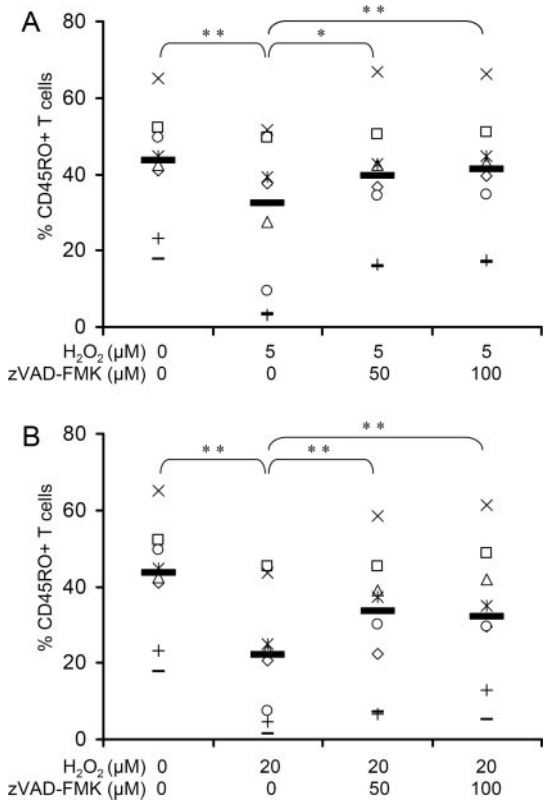

FIGURE 4. H_2O_2-induced cell death of $CD45RO^+$ T cells is caspase dependent. PBMC were incubated in AIM-V serum-free medium with or without z-VAD-FMK (50 or 100 μM) 1 h prior to H_2O_2 exposure (0, 5, or 20 μM) for 21 h. Cells were stained with anti-CD3 and anti-CD45RO mAb, and the viable lymphocyte population was analyzed by FACS. The figures show percent $CD45RO^+$ T cells in PBMC from eight donors after 5 μM (*A*) or 20 μM (*B*) H_2O_2 exposure and the effect of z-VAD-FMK (50 or 100 μM). Each donor is indicated by an individual symbol; *, $p < 0.05$ and **, $p < 0.01$ based on Wilcoxon's two-tailed matched pairs test. The brackets indicate the significant difference between unexposed cells and H_2O_2-exposed cells, and the significant difference between H_2O_2-exposed cells and H_2O_2-exposed cells pretreated with z-VAD-FMK. The horizontal bar indicates the median in each experimental group.

is produced and acting internally in T cells to induce cell death, and the phenomenon we have studied here, i.e., cell death induced by externally produced or added H_2O_2, remains to be elucidated. It is possible that the externally added H_2O_2 may penetrate the cell membrane of T cells, and thus trigger cell death by inducing molecules in a fashion comparable to that observed in the models above.

Furthermore, the disparity of sensitivity between $CD45RO^+$ and $CD45RA^+$ T cells and $CD8^+$ and $CD4^+$ cells may be due to altered antiapoptotic (e.g., Bcl-2 and Bcl-x) and proapoptotic molecule expression (e.g., Bax, Bak, and Bim) levels. Yokoyama et al. (31) showed that peripherally obtained $CD8^+$ T cells have significantly higher expression of Bcl-x and Bax than $CD4^+$ cells, suggesting that $CD4^+$ and $CD8^+$ cells may have a different sensitivity to activation-induced cell death (31). However, when these investigators activated T cells with Con A, there was no skewed survival of any of the subsets. In this study, we have demonstrated a difference in susceptibility of the H_2O_2-derived cell death of $CD8^+$ and $CD4^+$ T cells and it could be speculated that the differences of Bcl-x and Bax expression may play a role. Furthermore, others have shown that $CD45RO^+$ T cells express significantly less Bcl-2 than $CD45RA^+$ T cells (32) and that the levels of Bcl-2 and Bcl-x_L in T cells decreases upon activation (33–36). The decrease of these antiapoptotic molecules in activated T cells may explain the differences seen in this study regarding the enhanced susceptibility of $CD45RO^+$ T cells, and especially $CD8^+$ T_{EM}, to H_2O_2-induced oxidative stress. As antiapoptotic proteins are targeted by the nuclear transcription factor NF-κB (37–41), the previously described H_2O_2-induced down-modulation of NF-κB (9) may further decrease the expression of antiapoptotic molecules, leading to enhanced sensitivity to cell death.

A sustained exposure to oxidative stress could be the underlying mechanisms behind the immunosuppression generated in various pathological conditions, including cancer, autoimmune, and infectious diseases (11, 42–48). We found the $CD8^+$ subtype of the $CD45RO^+$ T cell compartment to be more sensitive to H_2O_2 as compared with the $CD4^+$ T cell subtype. It is of interest to consider whether this could be related to the observation of increased spontaneous cell death among $CD8^+$ T cells in PBL from cancer patients and in mice with experimental tumors (49–52). It has been suggested that cytokines, such as IFN-γ, produced by activated T cells, NK cells, or APCs may be the initial step in recruiting "regulatory" non-T cells that induce immune suppression and down-regulation of CD3 ζ expression (53). These regulatory cells may be ROS-producing monocytes/macrophages/granulocytes, as initially demonstrated with monocytes recovered by centrifugal elutriation from human PBMC (19), granulocytes, or immature myeloid cells in the tumor microenvironment (17, 52, 54, 55), or in advanced

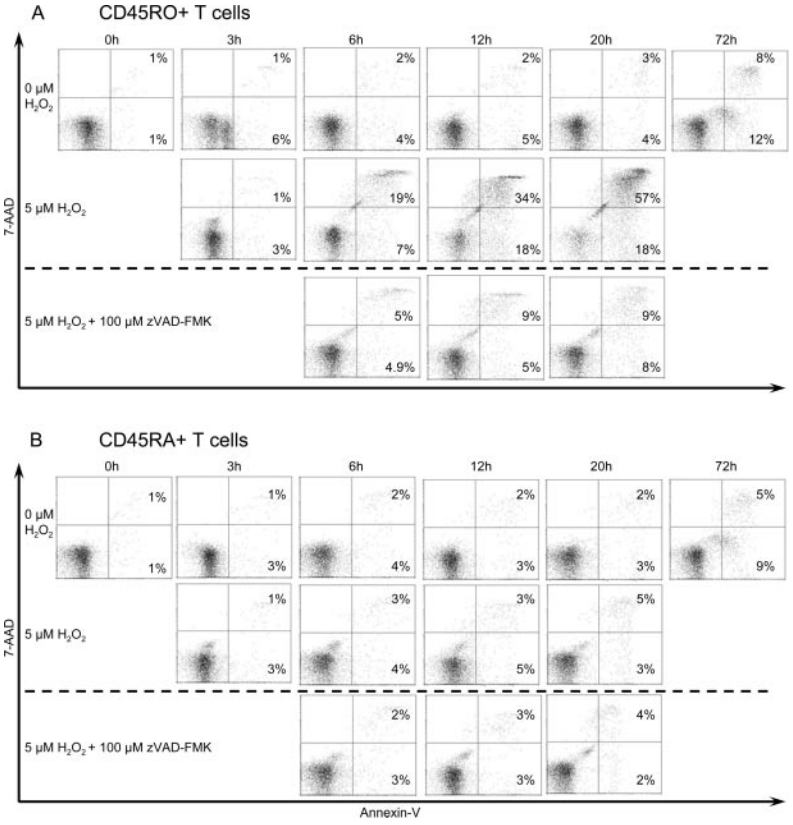

FIGURE 5. Time kinetics of H_2O_2-induced cell death of CD45RO+ T cells. CD3+, CD45RO+ or CD45RA+ T cells were isolated by negative selection using magnetic beads and cultured for different time points. The isolated cells were incubated with or without 100 μM z-VAD-FMK 1 h before 5 μM H_2O_2 exposure. After the incubation period, the cells were stained with 7-AAD and PE-labeled annexin V and further analyzed in FACS. Early apoptotic cells were defined as 7-AAD$^-$annexin V$^+$ cells; late apoptotic cells were defined as 7-AAD$^+$annexin V$^+$ cells. The percentage of early and late apoptotic cells is indicated in each dot plot. *A,* First row shows the cell death of unexposed CD45RO$^+$ T cells at different time points (0, 3, 6, 12, 20, and 72 h). Second row shows the cell death of CD45RO$^+$CD3$^+$ cells at different time points (3, 6, 12, and 20 h) exposed to 5 μM H_2O_2. Third row shows the cell death of z-VAD-FMK-pretreated CD45RO$^+$CD3$^+$ cells at different time points (6, 12, and 20 h) exposed to 5 μM H_2O_2. *B,* The cell death of CD45RA$^+$CD3$^+$ cells using the same setup as in *A*.

disease even in the peripheral circulation (14). This mechanism may initially serve to down-regulate an immune response that is potentially harmful, but when becoming chronic may itself cause injury and sustained immune suppression.

The phenomenon studied here could explain why various regimens of adoptive or active immunotherapy often fail to generate the desired clinical effects in the majority of treated cancer patients. The existence of ROS-producing cells within tumors or inflammatory foci or in the circulation of cancer-bearing individuals or patients with viral or bacterial infections may be particularly detrimental when considering adoptive immune therapy approaches. Tumor-specific T cell lines expanded in IL-2 and derived from tumor-infiltrating lymphocytes of patients with advanced cancer have been shown to predominantly have the CD8$^+$ memory effector T cell phenotype (56, 57), which we here demonstrate are highly sensitive to ROS. Therefore, one could predict that these cells upon injection into the circulation of patients with advanced cancer or when entering the microenvironment of tumors are

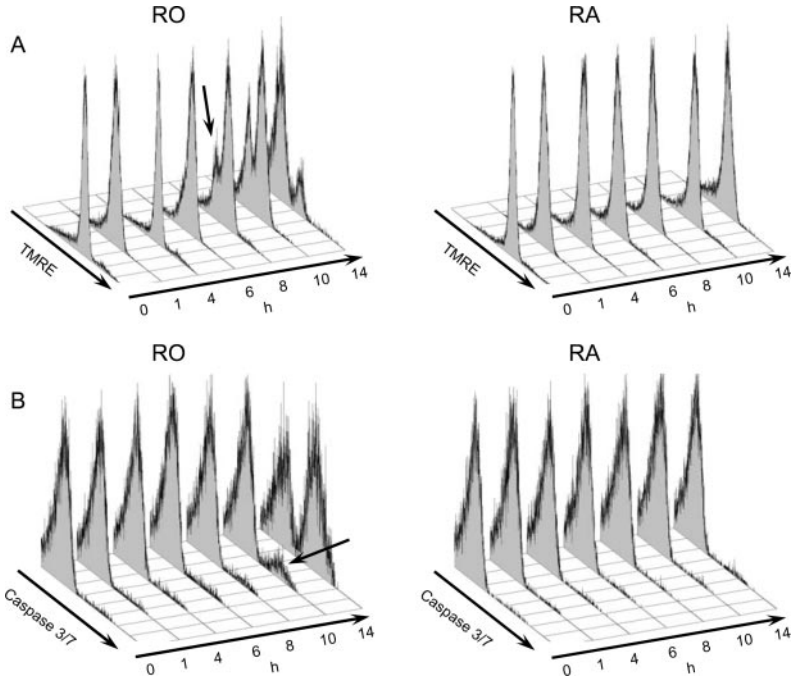

FIGURE 6. H_2O_2 induces cell death in CD45RO$^+$T cells via the mitochondrial pathway. Negatively selected CD45RO$^+$ and CD45RA$^+$ T cells were exposed to 5 μM H_2O_2 at different time points (0, 1, 4, 6, 8, 10, and 14 h) and analyzed in FACS. *A,* CD45RO$^+$ (*left panel*) and CD45RA$^+$ (*right panel*) T cells were labeled with the mitochondrial probe TMRE. A decrease in intensity of the staining corresponds to mitochondrial depolarization. *B,* CD45RO$^+$ (*left panel*) and CD45RA (*right panel*) T cells were labeled with caspase 3/7-specific fluorescent substrate. An increase in intensity of the staining corresponds to caspase 3/7 activity. Arrows indicate the earliest time point at which a difference is observed, compared with 0 h.

rapidly eliminated through the ROS-dependent mechanism described here. Combination of antioxidant treatment strategies, such as administration of high doses of vitamin E (58), with adoptive or active immunotherapy should therefore be considered in the treatment of cancer patients to enhance the function and survival of the injected T cells.

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Disclosures

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