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Inhibition of Activated/Memory (CD45RO⁺) T Cells by Oxidative Stress Associated with Block of NF-κB Activation¹

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Impaired immune responses in cancer patients have been associated with oxidative stress. Increased levels of reactive oxygen species released from activated, tumor-infiltrating macrophages or granulocytes may therefore constitute a hurdle for effective immunotherapy against cancer. In this study, we investigated functional consequences and molecular events in T cells exposed to low levels of oxidative stress. We observed that cytokine production of human PBMC, upon stimulation with an HLA-A*0201-restricted influenza peptide and nonspecific receptor cross-linking, was reduced after exposure to micromolar levels of H₂O₂. Functional impairment as measured by IFN-γ release occurred earlier and at lower doses of exogenously added H₂O₂ than required to induce apoptosis. This suggests that there is a dose window of oxidative stress leading to T cell unresponsiveness in the absence of apoptosis. The reduction of Th1 cytokines, induced by H₂O₂, was predominantly observed in memory/effector T cells and correlated with a block in NF-κB activation. IL-10 production was more profoundly influenced by low doses of H₂O₂ than IFN-γ, TNF-α, and IL-2. The influence of H₂O₂ on production of IL-10 was not significantly different between memory/activated and naive T cells. These observations suggest that Th1 and Th2 cytokines are differently regulated under conditions of oxidative stress. Taken together, these findings may explain why Ag-experienced, CD45RO⁺ T cells found in the tumor milieu are functionally suppressed. The Journal of Immunology, 2001, 167: 2595–2601.

H₂O₂ is physiologically produced in large amounts by granulocytes and macrophages as a mediator of innate immunity to invading microbes (1, 2). However, hydrogen peroxide and other reactive oxygen species (ROS)³ can mediate both beneficial as well as detrimental effects because ROS produced under chronic inflammatory conditions may severely impair the immune system. Long-standing and acute exposure of T cells to oxidative stress leads to the loss of transcription factor activity and diminished cytokine production in response to Ag stimulation (3–5). Coculturing tumor-infiltrating macrophages and freshly isolated human T cells result in a free radical-mediated decrease in TCR ζ expression and loss of Ag-specific T cell responses (6–8). Monocytes recovered from human PBMC can inhibit autologous NK cell-mediated cytotoxicity via secretion of H₂O₂, leading to induction of apoptosis (9). In addition, macrophage-derived NO reduces the phosphorylation and activation of Janus kinase 3/STAT5 signal transduction proteins, thus inhibiting the proliferative responses of T cells to IL-2 (10). The above findings lead to the hypothesis that local H₂O₂ or NO secretion by activated macrophages may be one mechanism behind tumor-induced immune suppression, as indicated by decreased signal transduction capacity, poor effector functions, and apoptosis of T and NK cells infiltrating the tumor lesion in cancer patients.

To further investigate the role of oxidative stress in relation to tumor immunology, we have examined how oxidative stress affected different subsets of T cells, with a particular focus on the cytokine production by cells expressing the memory/activation marker CD45RO. As this marker is commonly expressed by T cells infiltrating into tumors (11–13). We have also attempted to delineate whether loss of T cell function after H₂O₂ exposure is a predecessor to apoptosis of T cells or an early consequence following the initiation of the apoptotic process.

Adding to the complexity, H₂O₂ has been described to act as a second messenger leading to activation of NF-κB in T cells (14–16). NF-κB is activated by >150 different stimuli and regulates the transcription of >150 target genes, among which 27 are cytokines including IL-2, IFN-γ, and TNF-α (17). Block of NF-κB activation has been associated with T cell tolerance and decreased capacity to produce effector cytokines such as IFN-γ, IL-2, and IL-4 (18, 19). Thus, NF-κB is an important regulator of the human immune response. However, much of the evidence supporting H₂O₂ as an activator of NF-κB stems from studies in a particular T cell line (Jurkat Wurzburg), and the notion of ROS as an activator of NF-κB has been questioned by several recent reports (4, 5). Still, it remains a central question whether hydrogen peroxide acts as an activator or inhibitor of NF-κB in freshly isolated human T cells and what potential implications this has for the activity of the immune system.

In this study, we demonstrate that a dose window of oxidative stress exists where a state of unresponsiveness of T cells can be induced that is not followed by apoptosis. We also show that IL-10 production is much more sensitive to oxidative stress than the production of IL-2, IFN-γ, and TNF-α. Strikingly, the production of

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¹Abbreviations used in this paper: ROS, reactive oxygen species; 7-AAD, 7-amino actinomycin D.
these Th1 cytokines was more efficiently targeted in CD45RO+ (activated/memory) T cells, correlating with a more pronounced block of NF-κB activation, as opposed to CD45RO− (naïve) T cells.

Materials and Methods

Cells

PBMC were obtained from buffy coats from healthy blood donors admitted to the blood bank at the Karolinska Hospital by Ficoll-Hypaque (Amer sham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation for 20 min. CD3+ T cells were negatively selected using a MiniMACS kit (Miltenyi Biotec, Göteborg, Sweden). Selected cells were 95% T cells, as defined by FACS staining using a mAb against CD3. In some experiments, 25 ng/ml capturing Ab and anti-human IFN-γ (mAb 1-D1K; Mabtech, Stockholm, Sweden) were included isotype controls for both surface and cytoplasmic staining. The performance was checked daily with bead calibrators; QC Windows, CalIBRITE, and Q1000, and monthly with both beads and controls, as previously described (22). Data of at least 10,000 cells per sample were collected. Data analysis was done with CellQuest software (BD Biosciences), according to a standardized pattern-protocol. Gates were applied on all lymphocytes (light scatter cytogram) and all CD3+ lymphocytes (forward scatter vs F44), followed by subsequent gating on CD8+ and CD8- subsets (forward scatter vs F13). Each subset was further divided in two subpopulations; CD8+CD45RO+ and CD8+CD45RO−, or CD8-CD45RO+ and CD8-CD45RO−, respectively (F11 vs F13). The background fluorescence was determined with markers applied on the isotype control cytograms, and was in all cases <1%. As all analyzed cells were T cells (CD3+), the CD8- lymphocytes were considered CD4+, and the CD45RO− lymphocytes were considered CD45RA−.

ELISPOT assay

PBMC were exposed to hydrogen peroxide, washed, and stimulated with OKT-3 for 4 or 12 h. After two washes, nuclear extracts (5–10 μg) were prepared and preincubated with 1 μg poly(dI-dC) in binding buffer (10 mM Tris, 50 mM NaCl, 20% glycerol, 1 M DTT, and 0.5 mM EDTA) for 5 min at room temperature. Approximately 20,000 cpm of 3H-labeled DNA probe of the class I MHC NF-kB site was then added and allowed to bind for 20 min. The complexes were separated by 5% PAGE and detected by autoradiography.

Apoptosis analysis

Apoptosis measurements were performed using a flow cytometry-based method according to the manufacturer’s protocol (Nexin Research, Kat tendijkje, The Netherlands). Cells were stained for annexin V and the vital dye 7-amino actinomycin D (7-AAD) (Sigma, Stockholm, Sweden). Live cells were defined as double negative for these markers.

IFN-γ ELISPOT

IFN-γ ELISPOT was performed as previously described (20). Briefly, 96-well plates (MAIPS4510; Millipore, Molsheim, France) were coated with 2 μg/ml capturing Ab and anti-human IFN-γ (mAb 1-D1K; Mabtech, Stockholm, Sweden) at 4°C for 18 h and then blocked with AIM-V 2% human albumin (Baxter Medical, Stockholm, Sweden) for 1 h at 37°C. PBMC from healthy donors were then added to wells using 20,000 cells/well for OKT-3 (DAKO, Glostrup, Denmark) treatment and 7 × 105 cells/well for specific responses against the influenza virus matrix-derived peptide (MP1236, GILGFVFTL). OKT-3 was added at 25 ng/ml, and peptide was added at 2 μg/ml. Cells were incubated for 4 h in 37°C, 10% CO2. After six washes with 0.05% PBS-Tween 20 (Merck, Haar, Germany), anti-human IFN-γ (mAb 7B6-1-biotin; Mabtech) was added at 0.75 μg/ml and left for 2 h for incubation at 22°C. Another round of washing was done before streptavidin-alkaline phosphatase was added for a 1-h incubation. Spots were developed by addition of substrate (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; Life Technologies) and counted using an ELISPOT reader (KS ELISPOT; Zeiss, Oberkochen, Germany).

Lymphocyte activation for the cytokine-staining experiments

Cells exposed to H2O2 were washed, and the lymphocyte concentration was adjusted to 1 × 106 cells/ml. The cellular viability was in all experiments >97%, as determined with 7-AAD (Sigma). The lymphocytes were stimulated for 4 h at 37°C with FMA and ionomycin (Sigma) in the presence of 10 μg/ml brefeldin A (Sigma) (21).

FACS analysis

A four-color method was used. The cells were first stained for surface Ags (30 min at 4°C) with anti-CD45RO-FITC (DAKO), anti-CD8-PerCP, and anti-CD3-allophycocyanin (BD Biosciences, Stockholm, Sweden). Thereafter, the lymphocytes were permeabilized with FACS-lysing solution and with FACS-permeabilizing solution (BD Biosciences) and stained for 10 min at room temperature in the dark with PE-conjugated mAbs directed to IL-2, IFN-γ, IL-10, and TNF-α (BD Biosciences). The staining protocol included isotype controls for both surface and cytoplasmic staining. The stimulation and permeabilization procedures were checked by cytoplasmic staining for CD69 (BDIS Biosciences) and vimentin (Serotec, Oslo, Norway). After staining, the cells were fixed with CellFix (BD Biosciences). Acquisition was performed in less than 2 h. The flow cytometric measurements were performed on a FACS caliber (BD Biosciences). The instrument performance was checked daily with bead calibrators; QC Windows, CalIBRITE, and Q1000, and monthly with both beads and controls, as previously described (22). Data of at least 10,000 cells per sample were collected. Data analysis was done with CellQuest software (BD Biosciences), according to a standardized pattern-protocol. Gates were applied on all lymphocytes (light scatter cytogram) and all CD3+ lymphocytes (forward scatter vs F44), followed by subsequent gating on CD8+ and CD8- subsets (forward scatter vs F13). Each subset was further divided in two subpopulations: CD8+CD45RO+ and CD8+CD45RO−, or CD8-CD45RO+ and CD8-CD45RO−, respectively (F11 vs F13). The background fluorescence was determined with markers applied on the isotype control cytograms, and was in all cases <1%. As all analyzed cells were T cells (CD3+), the CD8- lymphocytes were considered CD4+, and the CD45RO− lymphocytes were considered CD45RA-.

INHIBITION OF CD45RO+ T CELLS BY OXIDATIVE STRESS

We developed a model to study the functional and molecular consequences of exposing T cells to oxidative stress. H2O2 dose and the exposure time needed for suppression of specific and nonspecific T cell responses was determined. A 10-min preincubation of PBMC to H2O2 at concentrations spanning from 25 to 100 μM was enough to significantly reduce peptide-specific and nonspecific (CD3 cross-linking) responses (Fig. 1A), as measured by an ELISPOT assay for the Th1 cytokine IFN-γ (p < 0.03). The presence of catalase during the H2O2 exposure completely abrogated the loss of function induced by H2O2 (Fig. 1B).

It was previously shown that triggering of T cells through the TCR is more sensitive to oxidative stress than triggering via crosslinking by anti-CD3 (6). We could not confirm this, as cells stimulated either by peptide or by anti-CD3 lost their capacity to produce cytokines when pre-exposed to H2O2 at similar doses (Fig. 1A).

Therefore, we conclude that a short exposure of PBMC to low concentrations of H2O2 severely impaired Ag-specific and nonspecific TCR-triggered cytokine production.

Loss of cytokine production is associated with block of NF-κB activation

Because IFN-γ and also many other Th1 cytokines, including IL-2 and TNF-α, are regulated by NF-kB, we were interested in studying the effects of oxidative stress on NF-kB in our model. Our results indicate that pretreatment of PBMC (data not shown) or of purified T cells with H2O2 at concentrations of 25 μM or more reduced the anti-CD3-induced NF-kB activation (Fig. 2), correlating with the dose needed to suppress cytokine production in experiments performed in parallel and with cells from the same donor (Fig. 1). Due to experimental variation, suppression of NF-kB was sometimes seen only at higher doses of H2O2, but always correlating with the dose needed to achieve down-modulation of NF-κB production. These results are compatible with the interpretation that the inhibitory effect of micromolar levels of H2O2 on cytokine production is related to inhibition of NF-κB activation.

The relationship between loss of cytokine production and apoptosis following exposure of T cells to oxidative stress

We next analyzed the possibility that the loss of function and block of NF-kB activation seen after treatment with H2O2 were consequences of apoptosis and cell death. To this end, the percentage of live cells, defined as double negative for annexin V and 7-AAD after exposure to various concentrations of H2O2 and subsequent stimuli with OKT-3, was measured. These experiments revealed...
that T cells (CD3+) died in a dose- and time-dependent manner following exposure to H$_2$O$_2$ (Fig. 3A). At 4 h after exposure of up to 100 µM H$_2$O$_2$, the majority of T cells did not show signs of apoptosis/cell death (Fig. 3A). When incubated for 15 or 60 h after H$_2$O$_2$ treatment, the majority of the cells exposed to 100 µM were apoptotic or dead. Loss of cytokine production was seen already at

4 h (Figs. 1 and 3B), when no signs of apoptosis were apparent. Importantly, most cells exposed to levels of H$_2$O$_2$ in the range of 25–50 µM, which effectively inhibited cytokine production following TCR stimulation, were still alive without signs of apoptosis even after 60 h (Fig. 3, A and B). In Fig. 3B, this is illustrated by calculating how many cells that were capable of producing IFN-γ per a fixed number of viable cells at 60 h. In conclusion, exposure to low doses of H$_2$O$_2$ resulted in a loss of T cell function without significant apoptosis, providing evidence that there is a dose window of exposure to oxidative stress in which cells enter an anergic rather than a preapoptotic state.

**The production of Th1 vs Th2 cytokines is differently affected by oxidative stress**

To characterize how H$_2$O$_2$ affects the spectra of cytokine responses in T cells, an intracellular staining protocol was used. In confirmation of, and extending the ELISPOT data shown in Fig. 1, this...
assay demonstrated how PMA/ionomycin-induced IFN-γ, IL-2, TNF-α, and IL-10 release from T cells was lost when cells were pre-exposed to H₂O₂ at micromolar concentrations (Fig. 4A). However, it is notable that the production of the Th2 cytokine IL-10 was significantly more sensitive to low levels (12.5–25 μM) of H₂O₂ than was the production of IFN-γ, IL-2, and TNF-α (Fig. 4A) (*p < 0.03). Confirming the intracellular staining results, IL-10- and IFN-γ-specific ELISPOT assays also demonstrated a more pronounced decrease in the number of T cells producing IL-10 as compared with IFN-γ in H₂O₂-exposed and OKT-3-triggered T cells (Fig. 4B). In conclusion, the production of the Th2 cytokine IL-10 seems to be more sensitive to oxidative stress than that of Th1 cytokines.

Loss of IL-10 occurs in all subsets of PBMC, whereas loss of IFN-γ, TNF-α, and IL-2 predominantly occurs in CD45RO+ cells

The phenotype of the cells that lost their capacity to respond to activation, when pre-exposed to H₂O₂, was measured by a protocol for intracellular staining of cytokines. We found that T cells with an activated/memory phenotype, as defined by CD45RO expression, lost their capacity to produce IFN-γ, TNF-α, and IL-2 when pre-exposed to 50 μM H₂O₂ before stimulation with PMA/ionomycin (Fig. 5, A–C) (*p < 0.05 for all cytokines and subsets except CD8+CD45RO+ cells producing IL-2). In contrast, no significant changes in the number of naive T cells, as defined by CD45RO negativity, were observed for the production of any of the three tested Th1 cytokines (Fig. 5, A–C) (*p > 0.2). This indicates that the mechanisms responsible for production of IFN-γ, TNF-α, and IL-2 are more sensitive to oxidative stress in activated/memory T cells, as compared with naive T cells. However, the difference between CD45RO+ and CD45RO− cells regarding production of IFN-γ, although statistically significant, remains somewhat unclear, because production of this cytokine was very limited by CD45RO− cells (Fig. 5C).

A completely different pattern appeared for T cells producing IL-10. At the doses of H₂O₂ used, there was a significant reduction in the number of cells producing IL-10 in all subsets of T cells examined (CD4/8+CD45RO−) (*p < 0.04), providing further evidence for a difference in the regulation of Th1 vs Th2 cytokine production under conditions of oxidative stress (Fig. 5D).

Importantly, these differences were not due to selective cell death of particular T cell subsets, as the percentage of cells belonging to different subsets remained stable throughout the experiment, with increasing concentration of H₂O₂ (data not shown).

**FIGURE 4.** IL-10 production is more sensitive to oxidative stress than IFN-γ, IL-2, and TNF-α production. Cells were exposed to H₂O₂ at different concentrations for 10 min, washed, and then stimulated by PMA/ionomycin (A) or OKT-3 (B). A. Cells were stimulated for 4 h in the presence of brefeldin A and then stained for cytokine production (IFN-γ, TNF-α, IL-2, and IL-10) using a protocol for intracellular staining, as described in Materials and Methods. The percentages of cells capable of producing the respective cytokines are shown. One representative experiment of three independent experiments is shown. *, A two-tailed Student’s t test showed statistically significant differences between the production of IFN-γ, TNF-α, and IL-2 compared with that of IL-10 (*p < 0.03). B. The y-axis represents the percentage of cells producing a cytokine in relation to maximum production (100%), as defined by the number of cells producing the cytokine when not exposed to H₂O₂ (0 μM). Cells treated as above were analyzed for cytokine production in a 4-h ELISPOT assay. One representative experiment of two is shown.
Blockade of NF-κB activation is more pronounced in CD45RO⁺ cells

Next, we analyzed the molecular explanation for the selective targeting of CD45RO⁺ cells by oxidative stress with respect to production of IFN-γ, TNF-α, and IL-2. CD45RO-positive and CD45RO-negative cells were isolated by positive selection of CD45RO⁺ cells before exposure to H₂O₂ and stimulation, and were then subjected to analysis of NF-κB activation. We observed that NF-κB was more strongly activated in CD45RO⁺ cells than in CD45RO⁻ cells (Fig. 6A, lane 6 compared with lane 1), possibly reflecting the fact that there are higher numbers of Th1 cytokine-producing cells within this subset (Fig. 5A–C). Also, lower doses of H₂O₂ (25 μM) were needed to substantially inhibit the NF-κB activation in CD45RO⁺ cells compared with CD45RO⁻ cells (Fig. 6A, lane 9 compared with lane 4). A densitometry of this experiment is shown in Fig. 6B. In parallel with the NF-κB analysis, IFN-γ ELISPOT assays were performed to confirm activation of T cells and inhibitory effects of H₂O₂. The loss of NF-κB always correlated with the loss of cytokine production (data not shown). In conclusion, these data are compatible with an effect of H₂O₂ on primarily CD45RO⁺ cells due to targeting of NF-κB in this subset.

Discussion

Although T cell responses to oxidative stress have been extensively studied, it still remains unclear whether the interaction between H₂O₂ and T cells results in activation or inhibition. In this study, we have used an in vitro model to examine alterations in T cell function following exposure to low levels of H₂O₂. The observed inhibition of T cell activity within 4 h after H₂O₂ exposure without measurable signs of apoptosis supports the conclusion that the observed loss of function is not merely due to T cell apoptosis or cellular toxicity. Higher concentrations, together with longer postexposure intervals, did indeed lead to T cell death, the rate of which correlated with time and dose of H₂O₂ (Fig. 3A). However, there exists a lower-dose threshold at which cells become unresponsive to stimuli without progressing to apoptosis up to 60 h postexposure (Fig. 3B).
A novel aspect of this study is the observed differential sensitivity to oxidative stress of Th1 cytokines vs IL-10 production. Although production of all the cytokines assayed (IL-2, IFN-γ, TNF-α, and IL-10) was generally reduced after a short exposure to H2O2, production of IL-10 was significantly more affected by this treatment (Fig. 4, A and B). T cell tolerance is usually associated with decreased IL-2 and IFN-γ production and enhanced production of IL-10 (23–25), which is why our data indicate that exposure of T cells to low levels of oxidative stress leads to a nonresponsiveness different from classical T cell tolerance. Although IL-10 is generally regarded as an immune-suppressive cytokine, this cytokine was also reported to promote T cell functions (26–29), which is why the functional consequences of decreased IL-10 production by T cells remain unclear.

The effect of H2O2 on NF-κB in T cells, in terms of activation or inhibition, remains controversial (for review, see Refs. 30 and 31). Studies in a subclone of Jurkat (Wurzburg) showed that H2O2 at micromolar concentrations activated NF-κB, an effect that could be blocked by antioxidants (14–16). However, in a more recent study (5), no activation of NF-κB in human PBL could be observed in response to hydrogen peroxide. Our results are compatible with studies on human PBL, because NF-κB activation was inhibited in response to hydrogen peroxide (3–5). We speculate that the effect of H2O2 on NF-κB may be cell type related and depend on differences between human T cells and Jurkat cells. Previous studies on Jurkat cells have reported that activation of NF-κB following exposure to H2O2 results in enhanced transcription of various genes, such as Fas ligand (32). Also, H2O2-induced apoptosis has been reported to result from activation of NF-κB and increased transcription of apoptosis-inducing genes (33). If NF-κB is inhibited by oxidative stress, as indicated by our data, alternative mechanisms for activation of apoptotic genes not affected by NF-κB may be responsible.

It is known that NF-κB regulates IL-2, TNF-α, and IFN-γ, but not IL-10 (reviewed in Ref. 17). In this study, the block of NF-κB activation was almost complete at doses in which changes in IFN-γ, IL-2, and TNF-α began to appear (25–50 μM) (Figs. 1 and 2 and 4, A and B). This indicates that loss of NF-κB activation might be the underlying mechanism for the impaired production of these cytokines. In contrast, IL-10 production was decreased already at doses of H2O2 below those needed to suppress NF-κB activation, suggesting that the loss of IL-10 production was not a result of impaired NF-κB activation. This observation is in line with recent reports, showing that induction of IL-10 production is inhibited in response to hydrogen peroxide (3–5), which is why our data indicate that exposure of CD4+ T cells in PBL from cancer patients and mice with experimental tumors (49–50). Mechanisms that may account for these immune-suppressive effects in tumor-bearing individuals include Fas-Fas ligand interaction leading to T cell apoptosis, shown to involve caspase 3-mediated cleavage of CD3 ε (51), as well as tumor-derived gangliosides inducing defective NF-κB activation in renal cell carcinoma lines (52). Also, release of H2O2 from activated macrophages derived from tumor lesions can result in loss of Ag-specific T cell functions and decreased expression of the CD3 ε molecule in T and NK cells (7, 8).

The data presented in this work suggest that increased amounts of ROS might be one mechanism explaining why activated/memory (CD45RO+) T cells recruited to inflammatory sites, including cancer lesions and other pathological conditions, often exhibit anergic properties similar to the ones found in this study. We speculate that oxidative stress might be one possible mechanism behind the defective NF-κB activation in T cells from tumor-bearing mice and cancer patients (44, 53). Thus, treatments aiming at reversing immune suppression, therefore, may target the altered redox status in cancer patients, and allow endogenous effector cells to function as desired.

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