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Differential effects on innate versus adaptive immune responses by WF10

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Abstract

Oxidative compounds that are physiologically generated in vivo can induce natural defense mechanisms to enhance the elimination of pathogens and to limit inflammatory tissue damage in the course of inflammation. Here, we have investigated WF10, a chlorite-based non-toxic compound for its functional activities on human PBMC in vitro. WF10 exerts potent immune-modulatory effects through generating endogenous oxidative compounds such as taurine chloramine. Proliferation and IL-2 production of anti-CD3 stimulated PBMC were inhibited by WF10, as was the nuclear translocation of the transcription factor NFATc. In PBMC and monocytes, however, WF10 induced pro-inflammatory cytokines like IL-1β, IL-8, and TNF-α. In the monocytic cell line THP-1, the activation of the transcription factors AP-1 and NFκB by WF10 was demonstrated. Inhibition of NFAT regulated genes in activated lymphocytes in concert with the induction of several myeloid cell associated pro-inflammatory genes in monocytes represents a novel mechanism of immune modulation.

Keywords: Inflammation; Cytokines; Gene regulation; Redox regulation

1. Introduction

Recent evidence changes conventional views regarding the role of oxidative compounds in the immune response and its inflammatory consequences. While such molecules have so far been widely considered major contributors to tissue damage, a number of reports have now demonstrated protective and anti-inflammatory activities, likely due to induction of endogenous defense pathways. For example, metabolites of the stress induced enzyme heme oxygenase I (HO-1)[1] such as carbon monoxide (CO) and biliverdin exert potent anti-inflammatory effects [1,2]. Similar cell-protective properties have been described for the redox-active molecule thioredoxin [3].

In an attempt to exploit the potential of oxidants for the treatment of inflammatory disorders in vivo, here we have investigated WF10, a chlorite-based compound, for its functional activities in vitro. After interaction with heme proteins the chlorite matrix of WF10 acquires oxidizing and chlorinating properties [4,5].

The data presented below demonstrate that WF10 exerts potent immunomodulatory effects most likely through generating physiologic oxidative compounds namely chloramines. The latter have been reported earlier to exert cell-protective and anti-inflammatory activities [6,7].

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[1] *Abbreviations used:* HO-1, heme-oxigenase 1; ACC 1, aminocyclopropanecarboxylic acid; Tau-Cl, *N*-chlorotaurine; TRX, thioredoxin; TR-1, thioredoxin-reductase 1, CO, carbon monoxide.

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Inhibition of adaptive immune responses along with an enhancement of innate immune mechanisms, as induced by WF10, represents a novel approach to immune modulation.

2. Materials and methods

2.1. WF10

WF10 is an aqueous solution of the chlorite drug substance solution OXO-K993 (referred to in the literature as tetrachlorodecaoxygen or TCDO). OXO-K993 is analytically characterized as a solution containing 4.25% chlorite, 1.9% chloride, 1.5% chlorate, 0.7% sulfate, and sodium as the cation. The active principle is defined by the chlorite ion content. WF10 solution contains 63 mmol/l of chlorite. WF10 is currently studied in the US, Europe, and Asia for treatment of late-stage HIV disease, as well as recurrent prostate cancer, late post-radiation cystitis, autoimmune disease, and chronic active hepatitis C [8]. WF10 solution was obtained from Dimethaid GmbH (Wanzleben, Germany).

2.2. Sample preparation

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were isolated on a Histopaque 1077 density gradient and 2×10^6 cells were resuspended in RPMI 1640 with 10% FCS. Cultures of PBMC were stimulated with anti-CD3 (OKT-3, 100 ng/ml) or with 10 ng/ml PMA and 0.5 μg/ml ionomycin (all reagents from SIGMA, Steinheim, Germany). Monocytes were isolated with CD14-microbeads on a Vario-MACS (Milteny, Bergisch-Gladbach, Germany) according to the manufacture's instruction. The monocytic cell line THP-1 was cultured in RPMI 1640 with 10% FCS. Unstimulated and stimulated PBMC or THP-1 cells were incubated in the presence or absence of either WF10—dependent on the experiment—at a final concentration of 200 or 600 μM ClO_2^-. After lysis with 300 μl of MagNAPure lysis buffer (RAS, Mannheim, Germany), the samples were frozen at −70 °C. After thawing, mRNA was isolated with the MagnaPure-LC device using the mRNA standard protocol for cells. The elution volume was set to 50 μl. An aliquot of 8.2 μl RNA was reverse transcribed using AMV-RT and oligo(dT) as primer (First Strand cDNA synthesis kit, RAS) according to the manufacture's protocol in a thermocycler. After termination of the cDNA synthesis, the reaction mix was diluted to a final volume of 500 μl and stored at −20 °C until PCR analysis.

2.3. LightCycler PCR

Target sequences were amplified using LightCycler Primer Sets (Search-LC, Heidelberg, Germany) with the LightCycler FastStart DNA Sybr Green I Kit (RAS) according to the manufacture's protocol. RNA input was normalized to the average expression of the two housekeeping genes β-actin and cyclophilin B. The transcript number was calculated from a virtual standard curve, obtained by plotting a known input concentration of a plasmid to the PCR cycle number (CP) at which the detected fluorescence intensity reaches a fixed value. The data are presented as adjusted transcripts/μl cDNA or as ratios to control values. For better visualization, a $\log_2$ transformation, of the ratio between WF10-treated and control cultures was calculated, as is common for gene expression studies [9].

2.4. Proliferation assay

Human PBMCs (5×10^4/well) were cultured in round-bottomed microtiter plates and stimulated with anti-CD3 (OKT-3, 100 ng/ml) in the presence or absence of WF10 at the indicated concentrations for 72 h at 37 °C in 7% CO_2. Individual wells were pulsed overnight with 1 μCi of [^{3}H]thymidine (Amersham, Braunschweig, Germany) harvested and [^{3}H]thymidine incorporation was measured in a liquid scintillation counter.

2.5. Cytokine release

Human PBMCs (2×10^6/ml) were stimulated with anti-CD3 (OKT-3, 100 ng/ml) or 10 ng/ml PMA and 0.5 μg/ml ionomycin, respectively. Unstimulated and stimulated cultures were incubated in the presence or absence of WF10 for 20 h at 37 °C in 7% CO_2. The supernatant was collected and frozen at −20 °C until analysis. The level of released cytokine was measured using commercial EIA-kits from Immunotech (Marseille, France) according to the manufacturer's protocol.

2.6. Chloramine formation

Ethylene formation from ACC represents a useful tool to detect halogenic oxidants in biological samples. Human PBMCs (4×10^6/ml) were washed in PBS and incubated with 5 mM 1-aminocyclopropanecarboxylic acid (ACC) (Sigma)/PBS in gas-tight sealed test tubes at 37 °C in a shaking water bath in the dark. WF10 was added at a final dilution of 2 mM ClO_2^-. To analyze the total accumulation of chloramines, cells were lyzed at the end of the indicated incubation time with NP40 (Sigma). At various time points, 1 ml of gas was taken from the headspace of the test tubes with gas-tight syringes (1 ml U-40; Becton–Dickinson, Heidelberg, Germany), which were stored until GC analysis for a maximum of 30 min. Ethylene analysis was performed with a Varian GC model 3400_{CX}, equipped with a deactivated aluminum oxide column (1/8 in. × 60 cm) and FID detection. The following temperatures were

adjusted: column 80 °C, injector 80 °C, and FID 225 °C. FID calibration was performed using a special ethylene gas standard containing 7 ng ethylene per milliliter synthetic air (Messer Griesheim, Olching, Germany).

2.7. Western blot analysis

THP-1 cells (10^6) were stimulated with WF10 (600 μM ClO_2^-) for 3 h at 37 °C in RPMI 1640 with 10% FCS in the presence or absence of 1 μg/ml LPS (added 15′ or 60′ till the end of the incubation). The cells were collected and lyzed in Laemmli sample buffer, sheared, and total lysates were subsequently subjected to SDS–PAGE (12.5%) and transferred to PVDF membranes. Filters were blocked in TBS containing 0.1% Tween 20 and 1% BSA.

After incubations with the primary antibody rabbit anti–IκBα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the secondary antibody peroxidase-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany), reactive signals were visualized by ECL (Amersham, Arlington Heights, IL). To control for the protein load the blots were stained with rabbit anti-actin antibodies (Sigma; A2066). The bands were scanned and analyzed with a GS-800 densitometer (Bio-Rad Laboratories, Munich, Germany). Data are presented as ratio between the optical density of the IκBα and the actin bands.

2.8. Transcription factor binding assays

To measure AP-1, NFκB, or NFAT activation, experiments with nuclear extracts prepared from THP-1 cells or from PBMCs of healthy donors were performed using the ELISA-based Trans-AM AP-1 family, NFκB family, and NFAT-c1 kits according to the manufacturer's protocol (Active Motif, Rixensart, Belgium).

Briefly, the Trans-AM kits employ 96-well microtiter plates coated with oligonucleotides containing the AP-1, NFκB, or NFAT consensus sequences. The active forms of the different subunits of AP-1, NFκB, and NFAT in nuclear extracts can be detected in a colorimetric reaction using first antibodies specific for the subunits (c-fos, phospho-c-jun, jun-B for AP-1; p50, p65 for NFkB; and NFATc1 for NFAT) followed by the addition of a secondary antibody conjugated to horseradish peroxidase. Photometric data were obtained using a Wallac Victor multilabel counter (Perkin–Elmer LAS GmbH, Rodgau-Jügesheim, Germany) at 450 nm.

Specificity of binding was determined by measuring the ability of soluble wild type or mutated oligonucleotides to inhibit binding.

Preparation of nuclear extracts was done immediately after the indicated stimulation using the nuclear extraction kit according to the manufacturer's protocol (Active Motif).

3. Results

3.1. Inhibition of T-cell responses by WF10

Previous studies suggested that WF10 reduces T-cell proliferation by inhibition of antigen presentation [10]. Whether WF10 directly influences T-lymphocytes was, however, not clear. In a first set of experiments, WF10 was added to isolated PBMCs in the presence of a monoclonal antibody known to mediate antigen-like effects towards the TCR/CD3 complex [11]. As shown in Fig. 1, WF10 exerts dose-dependent inhibitory effects on CD3-triggered T-lymphocyte proliferation in vitro. WF10 does not affect cell viability at the concentrations used for these experiments.

In order to study the effects of WF10 on antigen-receptor activated T-lymphocytes in more detail, we stimulated PBMCs with, respectively, CD3-antibodies and PMA/ionomycin in the presence or absence of WF10. As shown in Fig. 2, several genes encoding potent immunological response modifiers in activated T-cells including IL-2 and IL-4 are down-regulated upon exposure towards WF10.

In order to define the physiologic relevance of the observed changes in gene expression induced by WF10, we also investigated the amount of secreted IL-2 protein. As shown in Fig. 3, WF10 caused a strong inhibition of the amount of secreted IL-2 in both anti-CD3 and PMA/ionomycin stimulated cultures.

The observed expression profile as induced by WF10 suggested a suppression of NFAT regulated genes, as reported for hydrogen peroxide before [12]. Employing an ELISA system we measured NFATc binding in nuclear extracts of PMA/ionomycin stimulated PBMCs that were treated with WF10 for 3 h. As suggested from

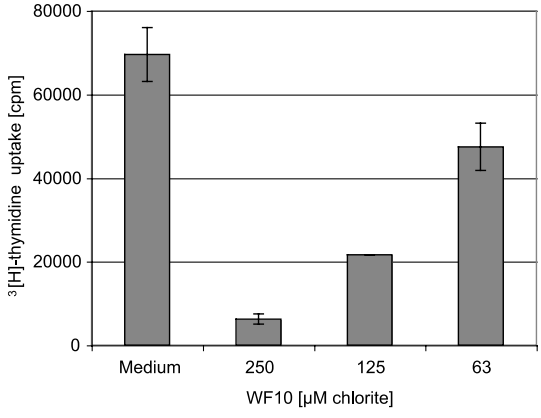

Fig. 1. Inhibition PBMC proliferation by WF10. PBMC from four healthy donors were isolated and stimulated with anti-CD3 antibodies in the presence of various concentrations of WF10 for 72 h. Data are presented as mean cpm ± SEM. The proliferation of PBMCs without CD3 stimulation was 3500 ± 1800 cpm and in the presence of 63 μM WF10 4300 ± 2100 cpm.

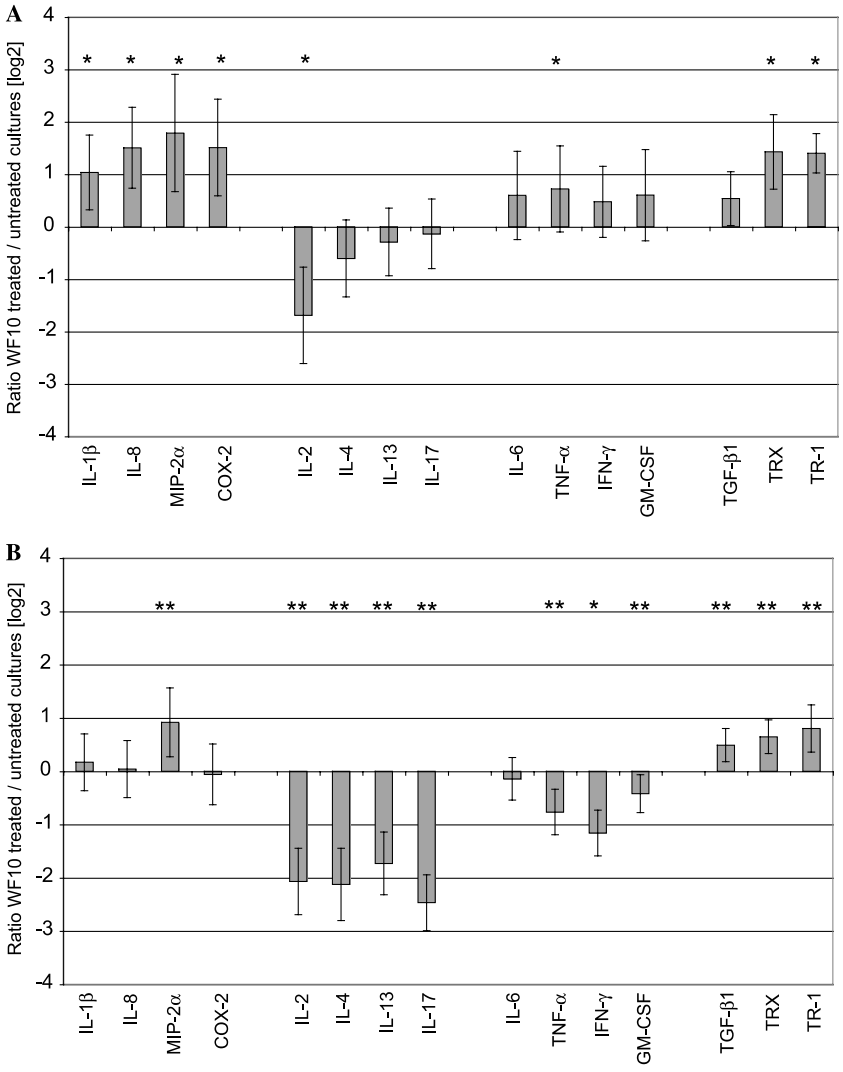

Fig. 2. WF10 induced changes in gene expression in stimulated PBMC. PBMC from healthy individuals were stimulated with anti-CD3 antibodies ($n = 9$) (A) or with PMA/Ionomycin ($n = 19$) (B) for 3 h in presence or absence of WF10 (200 μM ClO_2^-). Data are presented as mean and SEM of the $\log_2$ ratio between treated and untreated cultures. As indicated by the Wilcoxon signed rank test the values are statistically significant on either the $p < 0.05$ (*) or the $p < 0.001$ (**) level.

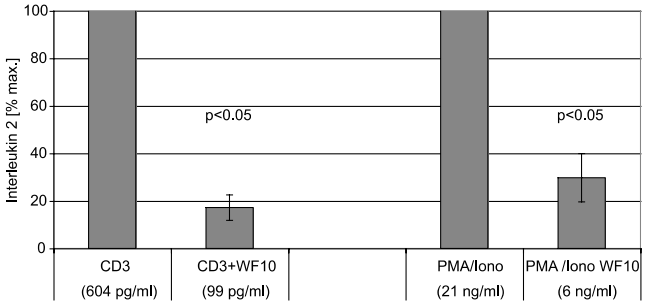

Fig. 3. WF10 induced changes in IL-2 protein secretion in PBMC. PBMC from eight healthy individuals were stimulated with anti-CD3 antibodies or PMA and ionomycin in the presence or absence of WF10 (200 μM ClO_2^-) for 20 h. Cytokine level were measured by ELISA. Data are presented as mean and SEM of the relative IL-2 secretion. The mean concentration of IL-2 is shown for each bar. The effect of WF10 is statistically significant on the $p < 0.05$ level as calculated by the Wilcoxon signed rank test.

the gene expression analysis, we observed a 50% reduced translocation of NFATc into the nucleus under the experimental conditions employed here (Fig. 4). For comparison, cyclosporin A (CsA), an inhibitor of calcineurin, caused a reduction of approximately 75% in this system. Unexpectedly, treatment with hydrogen peroxide, despite strong inhibition of IL-2, IL-4, and IFN-γ gene expression (data not shown), exerted a donor-dependent variable inhibitory effect on the nuclear translocation of NFATc.

3.2. Induction of pro-inflammatory genes in PBMCs and monocytes by WF10

Incubation of freshly isolated unstimulated PBMCs with WF10 for 20 h caused the secretion of several myeloid specific pro-inflammatory mediators including

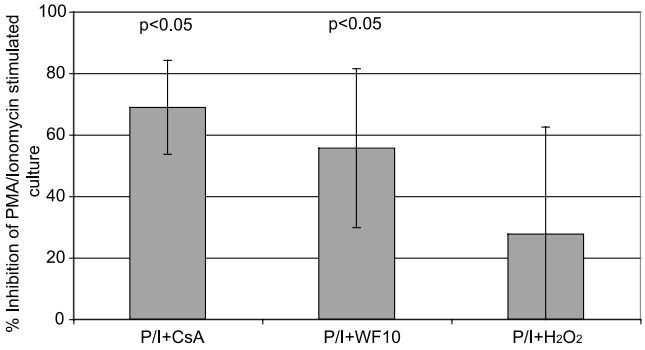

Fig. 4. Inhibition of NFATc nuclear translocation by WF10. NFATc activation was measured in nuclear extracts of PMA/ionomycin stimulated PBMC in the presence or absence of WF10 (600 μM ClO_2^-) or H_2O_2 (500 μM). As control the calcineurin inhibitor cyclosporin A (100 ng/mL) was used. Data are presented as mean of four independent experiments with a total of six donors. The effect of the CsA and WF10 treatments are statistically significant on the $p < 0.05$ level.

TNF-α, IL-1β, and IL-8 (Fig. 5). To study this phenomenon in more detail, we studied the gene expression of unstimulated PBMCs exposed for 3 h to WF10. WF10 induces the expression of a set of genes that are regulated by the transcription factors NFκB and AP-1; e.g., TNF-α and IL-8 (Fig. 6A). Importantly, protective genes such as thioredoxin (TRX), thioredoxin-reductase (TR-1), and HO-1 were also significantly induced by WF10.

Given the quantitative variability in responses by individual donors and the large number of cells required

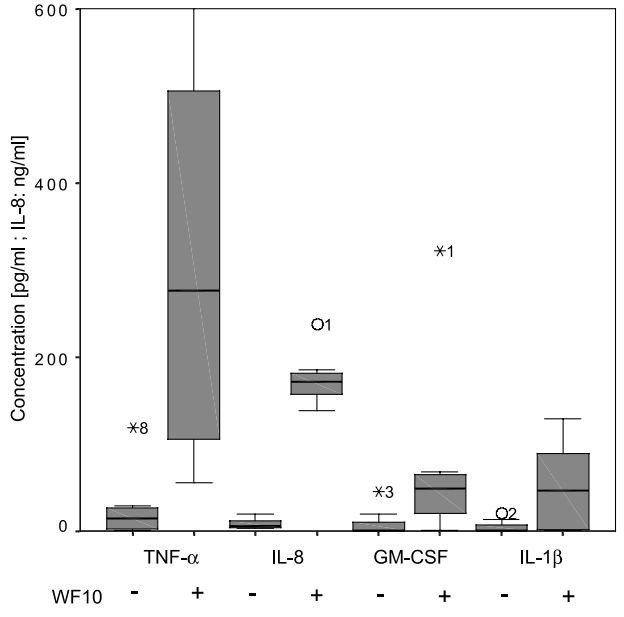

Fig. 5. WF10 induced changes in cytokine protein secretion in unstimulated PBMC. Freshly isolated PBMC from eight healthy individuals incubated in the presence or absence of WF10 (200 μM ClO_2^-) for 20 h. Cytokine level were measured by ELISA. Data are presented as Boxplot (median; 75% percentile). The differences between control and WF10 treated cultures are statistically significant ($p < 0.05$) as calculated with the Wilcoxon signed rank test.

to study intracellular signaling pathways we chose to employ for biochemical experiments a homogeneous cell population, namely the monocytic cell line THP-1. Note that THP-1 shows a similar gene expression profile as PBMCs or MACS-purified monocytes upon incubation with the compounds investigated here (Fig. 6B). To study the activation of NFκB and AP-1 by WF10, we employed a commercially available ELISA system that measures transcription factor binding to specific DNA sequences. Activation of transcription factors by WF10 was compared to the stimulatory capacity of LPS. As shown in Fig. 7A, WF10 treatment of THP-1 cells causes a significant activation of the AP-1 components c-jun, jun-B, and c-fos as well as the NFκB subunits p50 and p65.

Translocation of NFκB into the nucleus depends on the degradation of IκBα. Cellular stimulation causes the phosphorylation of IκBα, leading to its subsequent ubiquitinylation and degradation by the 26 S proteosome. This mechanism seems to be responsible for the effects of WF10 on NFκB activation as well, since Western blot analysis demonstrates the degradation of IκBα in THP-1 cells after WF10 treatment (Fig. 7B).

3.3. WF10 generates cell bound and soluble chloramines

Given the apparent stability of WF10 mediated effects we reasoned that based on its molecular composition and previously published results [5] WF10 could perhaps act through the generation of taurine chloramine (TauCl), a stable, non-toxic, physiologic oxidant which has been shown to exert cell-protective effects in various experimental models [13]. To investigate this possibility, PBMCs from three healthy donors were obtained and incubated with WF10 for different timepoints.

Subsequently, the formation of chloramines was monitored by the release of ethylene from ACC [14]. This effect was observed in either intact PBMCs in suspension as well as in their cellular lysates. As shown in Fig. 8, a time-dependent generation of chloramines was observed under these conditions. Notably, the generation of chloramines in this in vitro system was an exponential process: more than 60% of the newly formed chloramine was generated within less than 30 min of incubation. Maximum levels were reached after 120 min. Moreover, the oxidative capacity of the formed chloramines remained stable for the entire time period of investigation (>10 h). The continuous increase in the amount of ACC released ethylene after cellular lysis over the time of investigation argues for a de novo formation of chloramines. However, a stress-induced expression of heme-containing proteins and thereby increasing the formation of active chloroxygen species during the incubation period cannot be ruled out.

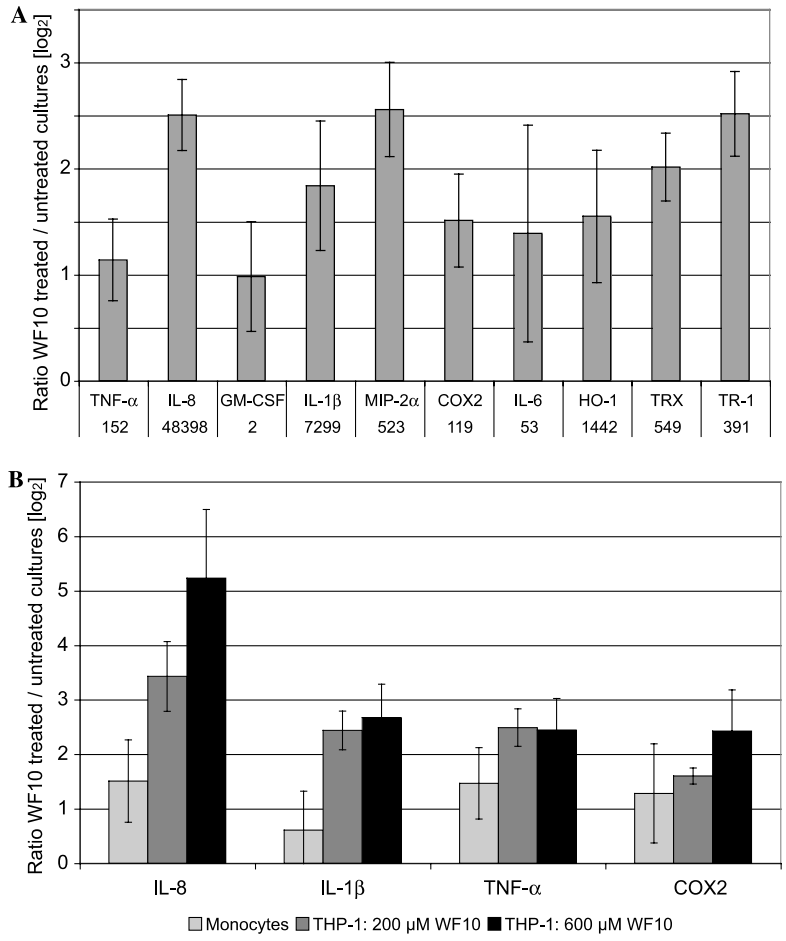

Fig. 6. WF10 induced changes in gene expression in PBMC (A), MACS isolated monocytes and THP-1 cells (B). Freshly isolated PBMC from 12 donors were incubated with WF10 (600 µM ClO_2^-) for 3 h. Monocytes from 13 healthy individuals were isolated with CD14-microbeads on a MACS device and cultured in the presence or absence of WF10 (200 µM ClO_2^-) for 18 h. THP-1 cells were cultured with WF10 (200 µM ClO_2^-) ($n = 2$) or (600 µM ClO_2^-) ($n = 4$) for 3 h. Data are presented as mean and SEM of the $\log_2$ ratio between treated and untreated cultures. The basic transcription in PBMC is shown as transcript $\times 10^{-3}$/sample. All effects shown in A and B are statistically significant ($p < 0.05$) as calculated with the Wilcoxon signed rank test.

4. Discussion

Pro-oxidative mediators are widely regarded as promoters of inflammatory tissue damage. However, increasing numbers of reports suggest that these same compounds can exert, under certain conditions, cell-protective activities as well. The latter view is profoundly supported by investigations on the "protective" enzyme HO-1. The catalytic products of HO-1, namely the oxidants CO, Fe^{2+}, and biliverdin, represent potent physiologic effector molecules and are capable of down-regulating inflammatory reactions [1,2].

Another stable natural oxidant, taurine-chloramine (TauCl), is produced by activated neutrophils in the course of inflammation and was shown to possess cell-protective as well as anti-inflammatory activities [15,16]. Its precursor, taurine, represents the most abundant free amino acid not incorporated into proteins in the cytosol of leukocytes. Taurine acts as a scavenger for HOCl, a microbicidal agent produced physiologically by the myeloperoxidase–hydrogen peroxide–halide system of activated neutrophils and monocytes during the oxidative burst [17]. The reaction of taurine and HOCl forms taurine-chloramine, a long-lived oxidant that is much less toxic than HOCl. Although the detoxification of HOCl/OCl by taurine has been proposed to account for its protective effects, recent studies imply that TauCl itself represents a potent biologic effector molecule, which contributes to self-limitation of inflammatory processes [7,15,16].

An attractive concept to explain how the antigen driven T-cell-response is eventually limited in vivo could be that physiologic anti-inflammatory compounds such as TauCl are generated for its physiologic termination. Such a view is supported by the fact that oxidation appears to represent a central component of natural mucosal immunoregulation in the intestine, a site where it is crucial to prevent adaptive T-cell responses towards

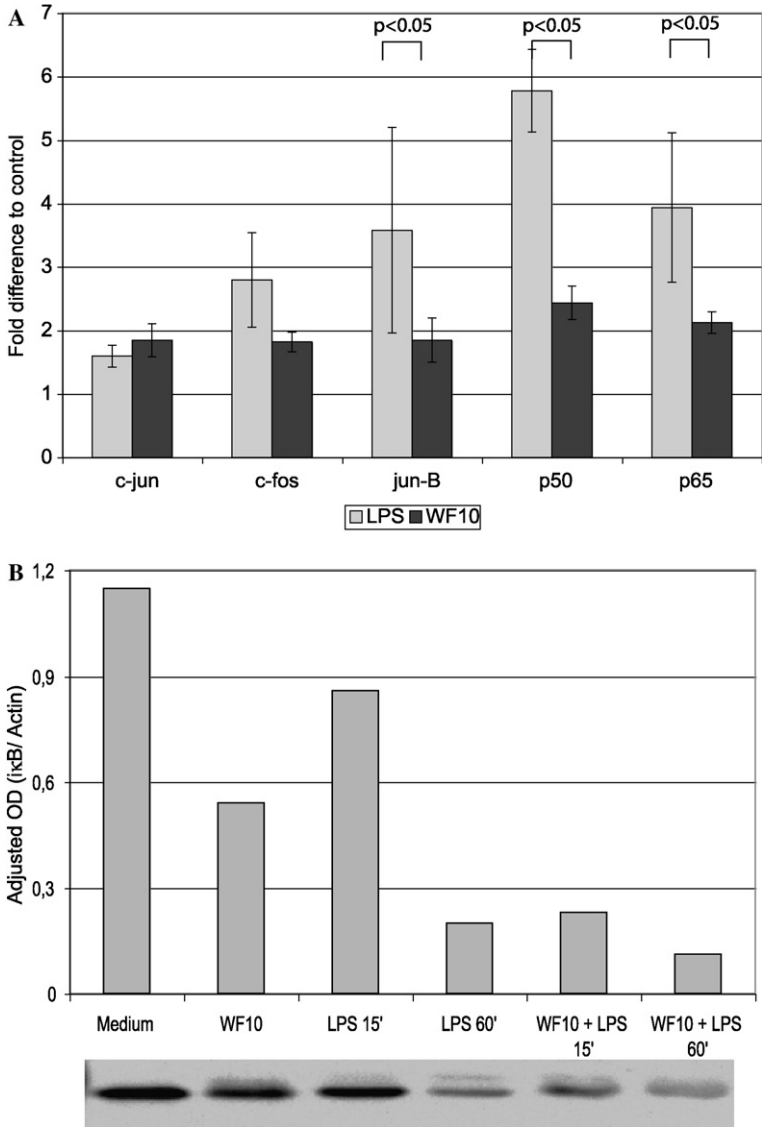

Fig. 7. Effects of WF10 on activation of transcription factors in THP-1 cells. THP-1 cells were incubated for 3 h in presence or absence of WF10 (600 μM ClO_2^-). For control purposes THP-1 cells were stimulated with 1 μg/ml LPS for 60 min. Nuclear extracts were prepared using a commercially available kit and activation of AP-1 and NFκB (A) was measured with an ELISA system. Data from four independent experiments are shown as mean ratio to untreated samples ± SEM. All LPS and WF10 induced changes are statistically significant ($p < 0.05$) as calculated with the Wilcoxon signed rank test. The degradation of IκB (B) was measured by Western blot analysis. THP-1 cells were incubated for 3 h in presence or absence of WF10 (600 μM ClO_2^-). For control purposes THP-1 cells were stimulated with 1 μg/ml LPS for the last 15 or 60 min. Data are shown as band intensity normalized to actin.

the enormous load of foreign luminal antigens. Note that T-lymphocyte activation is highly susceptible to inhibition by oxidants since T-cells possess an intrinsically low plasma membrane transport capacity for cystine [18]. Cystine transport is crucial for the generation of glutathione, the most potent intracellular anti-oxidant. It seems, therefore, an efficient strategy to shut off the antigen-driven proliferative T-cell response by means of exploiting this particular sensitivity to inhibition by oxidation.

In view of the known functional activities of the physiologic oxidant TauCl in vivo [19,20], we attempted to identify pharmacological compounds that would enable to intentionally generate TauCl in vivo. To this end, we chose to investigate the chloride based compound WF-10 with regard to its influences on cellular immune responses at a molecular level. After its activation by heme-containing proteins, WF10 chlorinates cellular proteins and free sulfur containing amino acids like taurine [5]. The present investigations demonstrate that this is indeed the case: incubation of PBMCs with WF10 in vitro leads to a rapid and stable generation of chloramines. It is experimentally difficult to estimate whether the detected chloramines are formed in intracellular or

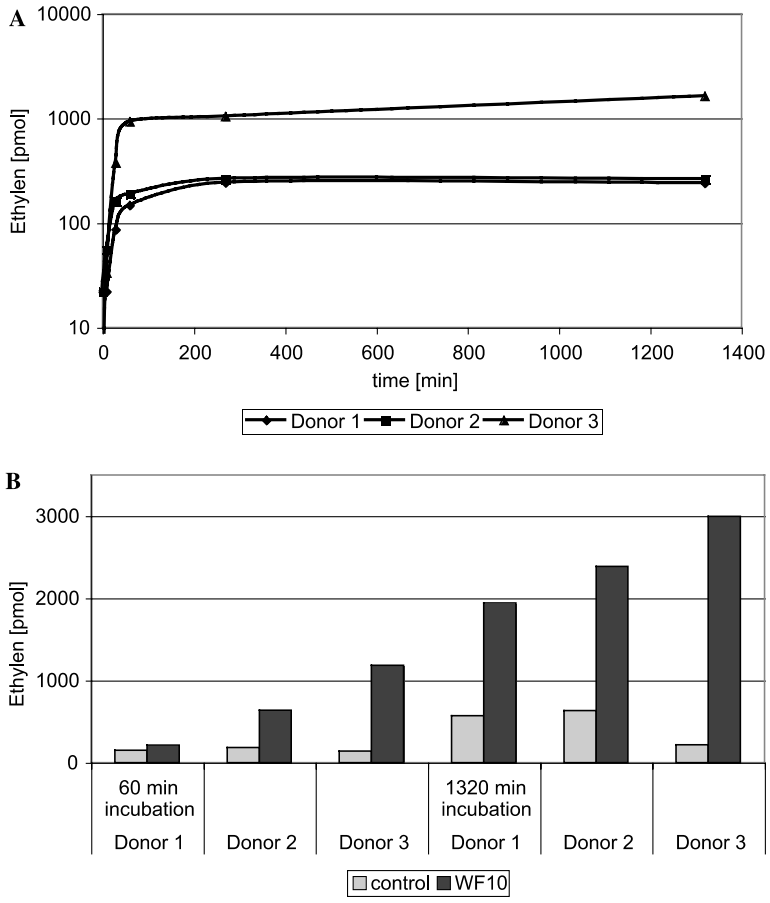

Fig. 8. Release of ethylene from chloramine oxidized ACC after in vitro treatment of PBMCs with WF10. The kinetics of chloramine formation in the PBMC suspension of three individual donors is shown in A. In B the cells were lyzed after either 1 or 22 h incubation with WF10 and the total accumulation of chloramines in the cellular lysates was measured by the formation of ethylene from oxidized ACC.

extracellular compartments. A possible explanation could be the formation of heme-containing proteins released by cells. Nevertheless, these experiments clearly show that WF10 generates chloramines in cultures of PBMCs. Although TauCl was not directly measured with the method employed here, given the uniquely high intracellular concentration of the amino acid taurine and the unique stability among other *N*-chloroamino acids, it seems justified to assume that TauCl represents the most relevant functional product that is formed under the influence of WF-10.

TauCl possesses the ability to regulate the production of macrophage derived pro-inflammatory mediators such as NO, IL-1, TNF-α, and PGE_2. The mechanisms involved include transcriptional as well as post-translational regulation [7,21].

The activities of WF10 as observed in this study, however, seem to be more complex than the previously described in vitro effects of taurine chloramine. This may in part be due to the fact that the latter, a hydrophilic compound, was added to cell suspensions in vitro whereas WF10 induces chloramines endogenously (see Fig. 8) and that the effects of TauCl were investigated on pre-stimulated cells. Moreover, differences in local concentrations and/or availability in cellular sub-compartments may explain these discrepancies.

Shifting the redox-balance towards an oxidative milieu leads to the activation of transcription factors like NFκB and AP-1 [22]. With regard to NFκB, reactive oxygen intermediates stimulate IκB-kinase, leading to phosphorylation, ubiquitination, and degradation of IκB and the subsequent rapid translocation of NFκB into the nucleus. Here, we show that WF10 causes degradation of IκB (Fig. 7B) and activation of the p50 and p65 members of the Rel-family (Fig. 7A).

Conversely, binding of transcription factors to responsive elements in the promoter regions of genes is negatively regulated by a pro-oxidative milieu. Thus, finetuning of the oxidative milieu between the cytosolic and nuclear compartments determines how a cell responds towards oxidative stress. For example, the rapid translocation of thioredoxin, a small endogenous reducing molecule, into the nucleus is necessary for gene activation after induction of transcription factors [3]. Importantly, WF10 induces both thioredoxin and thioredoxin reductase (Figs. 2 and 6A).

The observed induction of pro-inflammatory genes in unstimulated cultures of PBMCs seems to be a reflection of the direct influence on gene transcription by oxidation induced activation of transcription factors.

Cells of the monocyte lineage, unlike T-lymphocytes, are themselves physiologically well protected against oxidative stress. Their enhanced activity in a pro-oxidative milieu seems to be a natural way to enhance the elimination of pathogens and subsequently to limit inflammatory reactions. In the acute pathogen driven response, oxidative factors act synergistically. Under physiologic conditions, however, an oxidative milieu can also attenuate the response towards microbial organisms, e.g., by limiting signaling through pattern recognition receptors, like those for LPS. In this regard, macrophages of the intestinal lamina propria have a profoundly reduced expression of CD14 and TLR4 genes and are, therefore, insensitive towards LPS stimulation [18,23]. We have observed a clear reduction in the expression of CD14 protein on monocytes after exposure to WF10 (data not shown).

Another direct effect of pro-oxidative substances on transcriptional activities has been described for the NFAT species of transcription factors. The nuclear translocation of NFAT requires their dephosphorylation by the Ca^{2+}/calmodulin dependent serine/threonine phosphatase calcineurin. The phosphatase activity of calcineurin is redox sensitive, likely due to oxidation of the catalytic metal center and/or the formation of a disulfide bridge between two cysteine residues in the catalytic subunit [24,25]. Our investigations on gene expression profiles in stimulated PBMCs by WF10 strongly support these investigations: WF10 is able to inhibit antigen receptor driven lymphocyte proliferation, expression of NFAT regulated genes is strongly suppressed by WF10, and the nuclear translocation of NFATc is inhibited. Preliminary results in our laboratory indicate that this effect is synergistic with the action of the calcineurin inhibitor cyclosporine A.

Changes in biological cell responses as demonstrated here are likely due to influences on a number of additional processes including protein turnover, particularly with regard to disulfide-linked molecules whose composition and stability depends heavily on the respective intracellular redox state. Thus, direct influences of oxidative compounds on signaling components of the TCR complex, like the ζ-chain or LAT have been described earlier and might further modulate T-cell reactivity in an oxidative milieu [26,27]. Moreover, the costimulatory capacity of dendritic cells may be affected [28].

The WF10 associated inhibition of NFAT regulated genes in activated T-lymphocytes in concert with the induction of several monocyte associated pro-inflammatory genes suggests a novel mechanism of immune modulation. Activation of the innate myeloid functions concomitant with the inactivation of adaptive proliferative lymphocyte responses by administration of WF10 may represent a promising approach of targeting redox-regulation for the treatment of inflammatory disorders.

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