



The Journal of Immunology

This information is current as
of April 1, 2010

Mitochondrial Reactive Oxygen Species Control T Cell Activation by Regulating IL-2 and IL-4 Expression: Mechanism of Ciprofloxacin-Mediated Immunosuppression

Marcin M. Kaminski, Sven W. Sauer, Claus-Detlev
Klemke, Dorothee Süss, Jürgen G. Okun, Peter H.
Krammer and Karsten Gülow

J. Immunol. published online Mar 25, 2010;
doi:10.4049/jimmunol.0901662

Supplementary Data

[http://www.jimmunol.org/cgi/content/full/jimmunol.0901662/D
C1](http://www.jimmunol.org/cgi/content/full/jimmunol.0901662/D
C1)

Subscriptions

Information about subscribing to *The Journal of Immunology* is
online at <http://www.jimmunol.org/subscriptions/>

Permissions

Submit copyright permission requests at
<http://www.aai.org/ji/copyright.html>

Email Alerts

Receive free email alerts when new articles cite this article. Sign
up at <http://www.jimmunol.org/subscriptions/etoc.shtml>



Mitochondrial Reactive Oxygen Species Control T Cell Activation by Regulating IL-2 and IL-4 Expression: Mechanism of Ciprofloxacin-Mediated Immunosuppression

Marcin M. Kamiński,* Sven W. Sauer,[†] Claus-Detlev Klemke,[‡] Dorothee Süss,* Jürgen G. Okun,[†] Peter H. Krammer,* and Karsten Gülow*

This article shows that T cell activation-induced expression of the cytokines IL-2 and -4 is determined by an oxidative signal originating from mitochondrial respiratory complex I. We also report that ciprofloxacin, a fluoroquinolone antibiotic, exerts immunosuppressive effects on human T cells suppressing this novel mechanism. Sustained treatment of preactivated primary human T cells with ciprofloxacin results in a dose-dependent inhibition of TCR-induced generation of reactive oxygen species (ROS) and IL-2 and -4 expression. This is accompanied by the loss of mitochondrial DNA and a resulting decrease in activity of the complex I. Consequently, using a complex I inhibitor or small interfering RNA-mediated downregulation of the complex I chaperone NDUFAF1, we demonstrate that TCR-triggered ROS generation by complex I is indispensable for activation-induced IL-2 and -4 expression and secretion in resting and preactivated human T cells. This oxidative signal (H_2O_2) synergizes with Ca^{2+} influx for IL-2/IL-4 expression and facilitates induction of the transcription factors NF- κ B and AP-1. Moreover, using T cells isolated from patients with atopic dermatitis, we show that inhibition of complex I-mediated ROS generation blocks disease-associated spontaneous hyperexpression and TCR-induced expression of IL-4. Prolonged ciprofloxacin treatment of T cells from patients with atopic dermatitis also blocks activation-induced expression and secretion of IL-4. Thus, our work shows that the activation phenotype of T cells is controlled by a mitochondrial complex I-originated oxidative signal. *The Journal of Immunology*, 2010, 184: 000–000.

T cells are activated upon triggering of the TCR by APCs. T cell activation induces proliferation and differentiation of naive, resting T cells into different classes of effectors. These processes are governed by autocrine and paracrine actions of proteins secreted by activated lymphocytes. Among them, IL-2 and -4 are of major importance. IL-2, an Ag-nonspecific proliferation factor for T cells, induces cell cycle progression in resting T cells and clonal expansion of activated T cells (1). Being produced mainly by Th1 effector cells, IL-2 also plays a role in shaping the immune response. Differentiation of resting, naive T cells into Th2 effector cells is driven by IL-4. Furthermore, IL-4 produced by Th2 cells has a crucial role in humoral immunity: it promotes B cell activation and isotype switching to IgG₁ and IgE (2). Thus,

IL-4 production plays an important role in the pathogenesis of allergic inflammation. In atopic dermatitis, a chronic allergic skin disease, elevated levels of IL-4 coincide with increased IgE levels mediating hypersensitivity reactions (3, 4).

IL-2 and -4 expression is essentially controlled by three transcription factors: NF-AT, NF- κ B, and AP-1 (2, 5). The TCR activation-induced response is mediated by two secondary messengers: inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). On one hand, IP_3 binding to IP_3 -gated Ca^{2+} channels at the endoplasmic reticulum induces Ca^{2+} release and the depletion of intracellular Ca^{2+} stores. This leads to opening of the Ca^{2+} release-activated Ca^{2+} channels in the plasma membrane and, consequently, to activation of Ca^{2+} -dependent transcription factors (e.g., NF-AT). On the other hand, DAG binds to C1 domain-containing DAG receptor proteins. The most important T cell DAG receptors, protein kinase C θ and RasGRP family members, function as starting points for the signaling cascades that lead to activation of NF- κ B and AP-1, respectively (6). Thus, full T cell activation and activation-induced gene expression can be achieved by simultaneous treatment with the Ca^{2+} ionophore ionomycin (Iono) and the DAG mimetic PMA (7).

Our previous work demonstrated that expression of the activation-induced T cell death (AICD) mediator, CD95 (Apo-1, Fas) ligand (L), in preactivated T cells could only be triggered by the simultaneous presence of an IP_3 /Iono-introduced Ca^{2+} signal and a DAG/PMA-introduced H_2O_2 signal (8). Neither second messenger is sufficient by itself. Moreover, we showed that the H_2O_2 -mediated oxidative signal results from protein kinase C θ -dependent production of reactive oxygen species (ROS) by the mitochondrial electron transport chain (ETC) respiratory complex I (NADH: ubiquinone oxidoreductase) (9).

Ciprofloxacin, as well as other members of the fluoroquinolone group of antibiotics, is characterized by immunomodulatory properties of an unknown mechanism (10). The effects of ciprofloxacin

*Division of Immunogenetics, Tumor Immunology Program, German Cancer Research Center; [†]Division of Inborn Metabolic Diseases, Department of General Pediatrics, Ruprecht-Karls-University Children's Hospital, Heidelberg; and [‡]Department of Dermatology and Allergy, University Medical Centre Mannheim, Ruprecht-Karls-University of Heidelberg, Mannheim, Germany

Received for publication May 27, 2009. Accepted for publication February 17, 2010.

This work was supported by the Wilhelm Sander Stiftung (2007.126.1), the Deutsche Forschungsgemeinschaft, and the Helmholtz Alliance on Immunotherapy of Cancer (HA 202).

Address correspondence and reprint requests to Dr. Karsten Gülow, Tumor Immunology Program, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany. E-mail address: k.guelow@dkfz.de

The online version of this article contains supplemental material.

Abbreviations used in this paper: AICD, activation-induced T cell death; Cipro, ciprofloxacin hydrochloride; CSA, cyclosporin A; DAG, 1,2-diacylglycerol; DN-JNKK, dominant-negative form of human SEK1 kinase; EB, ethidium bromide; ETC, electron transport chain; Fluo-4-AM, fluo-4-acetoxymethyl ester; GOX, glucose oxidase; H_2DCF -DA, dichlorodihydrofluorescein diacetate; Iono, ionomycin; IP_3 , inositol 1,4,5-trisphosphate; L, ligand; MFI, mean fluorescence intensity; mtDNA, mitochondrial DNA; NAC, N-L-acetylcysteine; PI, propidium iodide; ps- p^0 , pseudo- p^0 phenotype; RC, respiratory chain; ROS, reactive oxygen species; Rot, rotenone; siRNA, small interfering RNA; U+P, uridine and pyruvate.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/\$16.00

on T cell activation-induced gene expression remain vague. Numerous conflicting reports stated that ciprofloxacin activates or inhibits T cell activation-induced gene expression (e.g., for IFN- γ , TNF- α , IL-2, and IL-4) (11–14). Interestingly, as an inhibitor of bacterial topoisomerase II and an inducer of DNA double-strand breaks, ciprofloxacin was also shown to deplete the mitochondrial DNA (mtDNA) content, thus leading to mitochondrial dysfunction and retarded cellular growth (15–17).

In this article, we show that prolonged ciprofloxacin treatment of preactivated human T cells leads to a loss of mtDNA content. This was accompanied by impaired activity of the mtDNA-encoded mitochondrial enzymes, such as complex I, whereas the activities of the nuclear-encoded mitochondrial enzymes, complex II (succinate dehydrogenase) and citrate synthase, were unaffected. In addition, prolonged ciprofloxacin treatment results in a dose-dependent inhibition of the T cell activation-induced oxidative signal, as well as IL-2 and IL-4 gene expression. Furthermore, by using various experimental models, such as ethidium bromide (EB)-induced mtDNA depletion, inhibition of complex I, or small interfering RNA (siRNA)-mediated knockdown of the complex I chaperone NDUFAF1, we demonstrate that TCR-triggered ROS generation by the mitochondrial complex I is indispensable for T cell activation-induced IL-2 and -4 expression in resting and preactivated human T cells. IL-2 and -4 expression requires a synergistic action of the Ca^{2+} signal and the mitochondrial complex I-derived oxidative signal in the form of H_2O_2 . The oxidative signal facilitates activation of the redox-dependent transcription factors NF- κB and AP-1. Moreover, using T cells isolated from patients with atopic dermatitis, we show that the inhibition of mitochondrial complex I leads to a significant decrease in spontaneous hyperexpression, as well as TCR-induced expression of IL-4. Prolonged ciprofloxacin treatment of T cells from patients with atopic dermatitis also blocked the activation-induced expression and secretion of IL-4. Thus, the current study demonstrates for the first time that mitochondrial complex I-derived ROS control T cell activation. Blocking mitochondrial ROS generation or the application of prolonged ciprofloxacin treatment opens new possibilities for the treatment of allergic inflammation and Th2-mediated diseases. Moreover, our results postulate a detailed analysis of the T cell activation phenotype in patients with mitochondrial complex I dysfunctions or mtDNA deletions.

Materials and Methods

Chemicals

Dichlorodihydrofluorescein diacetate ($\text{H}_2\text{DCF-DA}$), fluo-4-acetoxymethyl ester (Fluo-4-AM), CFSE, and BAPTA-AM were obtained from Invitrogen. Iono and cyclosporin A (CsA) were purchased from Merck (Darmstadt, Germany), and ciprofloxacin hydrochloride (Cipro) was purchased from AppliChem (Darmstadt, Germany). *N*-L-acetylcysteine (NAC), PMA, glucose oxidase (GOX), rotenone (Rot), and all other chemicals were supplied by Sigma-Aldrich (Munich, Germany). FITC-conjugated anti-CD3 Ab was purchased from BD Biosciences (Heidelberg, Germany), and cross-linking polyclonal goat anti-mouse Ab was obtained from Southern Biotechnology Associates (Birmingham, AL). The monoclonal mouse Abs (OKT3) against human CD3 and human CD28 (I5E8) were prepared as described (8).

Patients

T cells isolated from nine patients (experiments with Rot; Fig. 7) or from seven other patients (experiments with ciprofloxacin; Figs. 8, 9, Supplemental Fig. 2) with acute exacerbations of long-standing atopic dermatitis were investigated. Blood was drawn before the initiation of therapy. T cells from normal, age-matched healthy donors were used as controls. Informed consent was obtained from all subjects before inclusion in the study. The study was conducted according to the ethical guidelines of the German Cancer Research Center and the Helsinki Declaration, and it was approved by the ethics committee II of the Ruprecht-Karls-University of Heidelberg, Germany.

Isolation of human peripheral T cells

Human PBLs were purified as described (8). Homogeneity of the prepared T cells was verified by staining with FITC-conjugated anti-CD3 Abs followed by FACS analysis, and it was estimated to be >90%.

Cell culture

Jurkat J16-145 cells were derived from the human lymphoblastoid cell line Jurkat J16 (8). Jurkat cells were cultured in IMDM, 10% FCS. Freshly isolated resting ("day 0") or activated ("day 6") peripheral human T cells were cultured at a concentration of 2×10^6 cells/ml in RPMI 1640 (+ L-glutamine), 10% FCS. For activation, "day 0" T cells were treated with 1 $\mu\text{g/ml}$ PHA for 16 h, washed, and subsequently cultured in the presence of 25 U/ml IL-2 for 6 d ("day 6" T cells) or 7 d (ciprofloxacin treatment).

Determination of ROS generation

Cells were stained with $\text{H}_2\text{DCF-DA}$ (5 μM) for 30 min. Next, cells were divided and stimulated with plate-bound anti-CD3 Ab (30 $\mu\text{g/ml}$) or PMA (10 ng/ml). Treatment was terminated by ice-cold PBS, and ROS generation was determined by FACS analysis. If not stated otherwise, ROS generation was quantified as the increase in mean fluorescence intensity (MFI), calculated according to the following formula: increase in MFI (%) = $[(\text{MFI}_{\text{stimulated}} - \text{MFI}_{\text{unstimulated}}) / \text{MFI}_{\text{unstimulated}}] \times 100$ (9).

Assessment of intracellular Ca^{2+} concentration

Cells were stained with 1 μM Fluo-4-AM, a fluorometric Ca^{2+} indicator, for 30 min. Thereafter, cells were treated, and Ca^{2+} influx into the cytosol was monitored by real-time FACS (8).

Assessment of cell death

Cell death was assessed by propidium iodide uptake and/or a decrease in the forward-to-side scatter profile compared with living cells and recalculated to "specific cell death," as described previously (8).

Assessment of proliferation

After overnight (18 h) incubation with PHA, activated human T cells were washed, stained with CFSE (1 μM) according to the manufacturer's instructions, and treated with different amounts of ciprofloxacin for 7 d. The proliferation was assessed by FACS measurement and calculated as a percentage of the living cells showing reduced CFSE staining ("CFSE low") due to proliferation-induced dilution of the dye compared with non-proliferating cells ("CFSE high").

Generation of mtDNA-depleted cells (pseudo- p^0 cells)

Cells depleted of mtDNA were generated as described previously (9). Briefly, Jurkat J16-145 cells were cultured in IMDM supplemented with EB (250 ng/ml). The amount of mtDNA was assessed after isolation of total cellular DNA and PCR with primers specific to the mitochondrial heavy-strand origin of replication (mito-ori): sense, 5'-GAAAACAAAATACTCAAATGGGCC-3'; anti-sense, 5'-CCTTTTGATCGTGGTGATTTAGAGGG-3'; β -actin: sense, 5'-TG-ACGGGGTCACCCACACTGTGCCCATCTA, anti-sense, 5'-CTAGAATTTGCGGTGGACGATGGAGGG. mtDNA-depleted cells were further cultured in IMDM supplemented with EB, uridine (50 $\mu\text{g/ml}$), and pyruvate (110 mg/ml).

Isolation of total cellular DNA

Cells were lysed for 1 h at 55°C in 0.2 M sodium acetate, 6.25% SDS solution containing 250 $\mu\text{g/ml}$ proteinase K. Total cellular DNA was isolated by phenol/chloroform extraction and precipitated with cold absolute ethanol. Traces of EB were removed from DNA samples by extraction with an equal volume of *n*-butanol.

Luciferase reporter assay

Firefly luciferase reporter constructs containing the IL-2 (−300/+47) promoter, the IL-4 promoter (−269/+11), three copies of the AP-1 binding site from the SV40 enhancer (CGGTGCTGACTAATTG), four copies of the NF- κB consensus sequence (GGAAATTCCTCC), or three copies of the human IL-2 NF-AT (−280/−250) element (GAAAGGAGGAAAAA-CTGTTTCATACAGAAGGC) in the pTATA-Luc vector were kindly provided by M. Li-Weber (German Cancer Research Center, Heidelberg, Germany). pcDNA3 expression plasmids harboring human I $\kappa\text{B}\alpha$ or the dominant-negative form of human SEK1 kinase (DN-JNKK) under control of the CMV promoter were kindly provided by A. Pappa (Medac, Hamburg, Germany) and P. Angel (German Cancer Research Center, Heidelberg, Germany), respectively. A *Renilla* luciferase-expression reporter pRL-TK plasmid (Promega, Mannheim, Germany) was used as an internal

control of transfection efficiency. Jurkat T cells were transfected with 2 μ g pRL-TK plasmid, 5 μ g firefly luciferase reporter construct, or 5 μ g protein-expression plasmid (where indicated) by electroporation, as described (18). After overnight recovery, cells were divided, pretreated with NAC or BAPTA-AM for 30 min, and treated with PMA (10 ng/ml) and/or Iono (1 μ M) for 7 h. Luciferase activity was determined as described (8).

siRNA transfection and knockdown

siRNA oligonucleotides used for transfection were as follows: control (unlabeled "AllStars" nonsilencing, validated siRNA, Qiagen, Hilden, Germany) or specific for human NDUFAF1, reported previously (19) (oligo#1 anti-sense strand, 5'-UAAACUAUACAUCUGAUUCGdTdT-3'; oligo#2 anti-sense strand, 5'-ACUACAUCAGGCUUCUCCdTdT-3'). Jurkat T cells were transfected by lipofection (HiPerfect, Qiagen) or nucleofection (Cell Line Nucleofector kit V, Amaxa, Cologne, Germany). Lipofection was performed by incubating 2×10^5 cells in 90 μ l FCS-free media with 9 μ l HiPerfect and 150 nM siRNA oligonucleotides, according to the manufacturer's instructions. After 6 h, cells were resuspended in 500 μ l media containing 10% FCS. Nucleofection was performed with 250 nM siRNA oligonucleotides, according to the manufacturer's instructions.

RNA isolation and semiquantitative RT-PCR

RNA was isolated with TRIzol reagent (Invitrogen) or RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. Total RNA (1 or 5 μ g) was reverse-transcribed with an RT-PCR kit (Applied Biosystems, Foster City, CA). For semiquantitative PCR, aliquots were amplified as

described previously (8). Primers applied for the detection of β -actin and NDUFAF1 were reported previously (9). Primers used for the amplification of IL-2 and -4 were as follows: IL-2 sense 5'-ATGTACAGGATGCACTCCTGTCTT-3', anti-sense 5'-GTCAGTGTGAGATGATGCTTTGAC-3'; IL-4 sense 5'-ATGGGTCTCACCTCCCACTGCT-3', anti-sense 5'-CGAACACTTTGAATATTCTCTCTCAT-3'.

Quantitative real-time PCR

Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems). Gene expression was analyzed using the 7500 Real-Time PCR Systems and Sequence Detection Software, version 1.2.2 or 2.0.2 (Applied Biosystems). IL-2 and IL-4 gene expression levels were normalized using GAPDH expression levels as an endogenous reference. mtDNA content was estimated by gene copy number of the mitochondrial 12S rRNA gene and normalized to the gene copy number of nuclear GAPDH. Induction ratios (X) were calculated using the formula $X = 2^{-\Delta\Delta Ct}$, where Ct stands for cycle threshold and $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{reference gene}}$. $\Delta\Delta Ct$ is the difference between the ΔCt values of the "induced" samples and the ΔCt of the corresponding "noninduced" sample. The mean induction ratios were calculated. The relative IL-4 basal expression levels in T cell samples from acute atopic dermatitis patients and healthy donors were compared using factor $Y = 2^{-(Ct_{\text{gene of interest}} - Ct_{\text{GAPDH}}) \times 1000}$ (20). The ranges of values obtained for experiments with Rot (Fig. 7) and ciprofloxacin (Supplemental Fig. 2) differ because of alternative versions of the software and different amounts of RNA applied (1 or 5 μ g). The following primers were used for gene expression analysis: GAPDH, sense 5'-GCAATTCATGGACCG-3', anti-sense

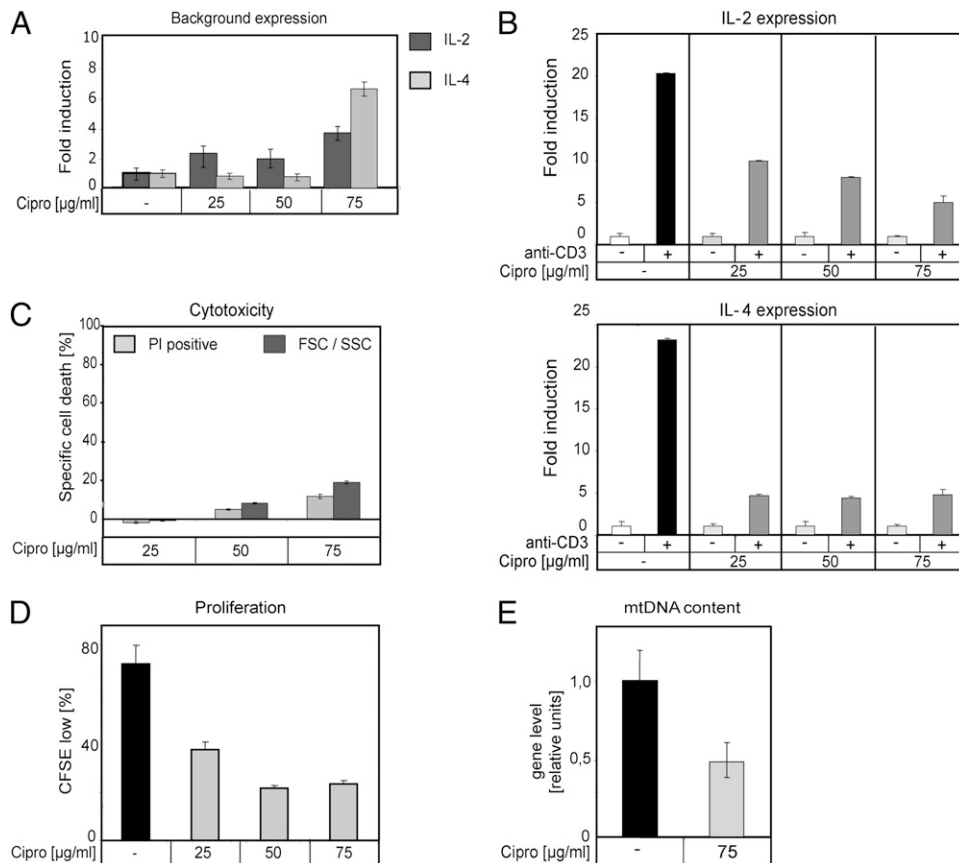


FIGURE 1. Prolonged ciprofloxacin treatment differentially affects preactivated human T cells (i.e., induces loss of mtDNA and blocks CD3-triggered IL-2 and -4 expression). *A*, PHA-preactivated primary human T cells were incubated with Cipro for 7 d. Background gene expression levels for IL-2 and -4 were analyzed using quantitative real-time PCR and normalized to GAPDH expression. *B*, After Cipro treatment (7 d), PHA-preactivated primary human T cells were stimulated with plate-bound anti-CD3 agonistic Ab (30 μ g/ml) for 1 h. Expression of IL-2 and -4 was analyzed using quantitative real-time PCR and normalized to GAPDH expression. Data are shown as fold increase of gene expression, where anti-CD3-activated cells are compared with respective unstimulated controls. *C*, Prolonged ciprofloxacin treatment shows low cytotoxicity. Cell death of ciprofloxacin-treated preactivated T cells (day 7) was analyzed by a decrease in the forward scatter/side scatter profile or by propidium iodide (PI) uptake in comparison with living cells. The data were recalculated to "specific cell death," as described previously (8). *D*, Prolonged ciprofloxacin treatment inhibits proliferation of mitogen-activated peripheral human T cells. After overnight activation (18 h) with PHA, T cells were stained with CFSE and treated with different doses of Cipro for 7 d. Next, T cell proliferation was assessed by FACS and quantified as a percentage of living cells with diminished CFSE staining intensity (CFSE "low") due to proliferation-induced dilution. *E*, Total cellular DNA was isolated from PHA-preactivated T cells on day 7 of Cipro treatment. An estimate of mtDNA content was obtained from the gene expression ratio of mitochondrial 12S rRNA gene and nuclear GAPDH using quantitative real-time PCR.

5'-TCGCCCCACTTGATTTTGG-3'; IL-2, sense 5'-CAACTGGAGCATT-TACTGCTG-3', anti-sense 5'-TCAGTTCTGTGGCCTTCTTGG-3'; IL-4, sense 5'-CACAAGCAGCTGATCCGATTC-3', anti-sense 5'-TCTGGTTG-GCTTCCTTCACAG-3'; NDUFAF1, sense 5'-GCAGTTTCTGGCACAT-GG-3', anti-sense, 5'-AAAGTAAGTTTCTTCTCTGGGCTA-3'; CD95L, sense, 5'-AAAGTGGCCCATTTAACAGGC-3', anti-sense, 5'-AAAGCAG-GACAATTCCATAGGTG-3'. Primers used for estimation of mtDNA content: 12S rRNA, sense 5'-GACGTTAGGTCAAGGTGTAG-3', anti-sense 5'-CAACTAAGCACTCTACTCTC-3'; GAPDH, sense 5'-GACCCCTTCATT-GACCTCAAC-3', anti-sense 5'-CTTCTCCATGGTGGTGAAGA-3'.

Determination of IL-2 and -4 secretion

IL-2 and -4 concentrations were measured by ELISA (BD OptEIA Set Human IL-2/IL-4, BD Biosciences). Resting ("day 0") T cells with or without Rot (10 µg/ml, 15 min pretreatment) or preactivated peripheral human ("day 6") T cells with or without ciprofloxacin (50 µg/ml, 7 d) were stimulated with plate-bound anti-CD3 mAb (30 µg/ml) and soluble anti-CD28 mAb (1 µg/ml) for 4 h ("day 0" T cells) or 16 h ("day 6" T cells). Next, the supernatants were cleared by centrifugation, and the measurements were performed according to the manufacturer's instructions.

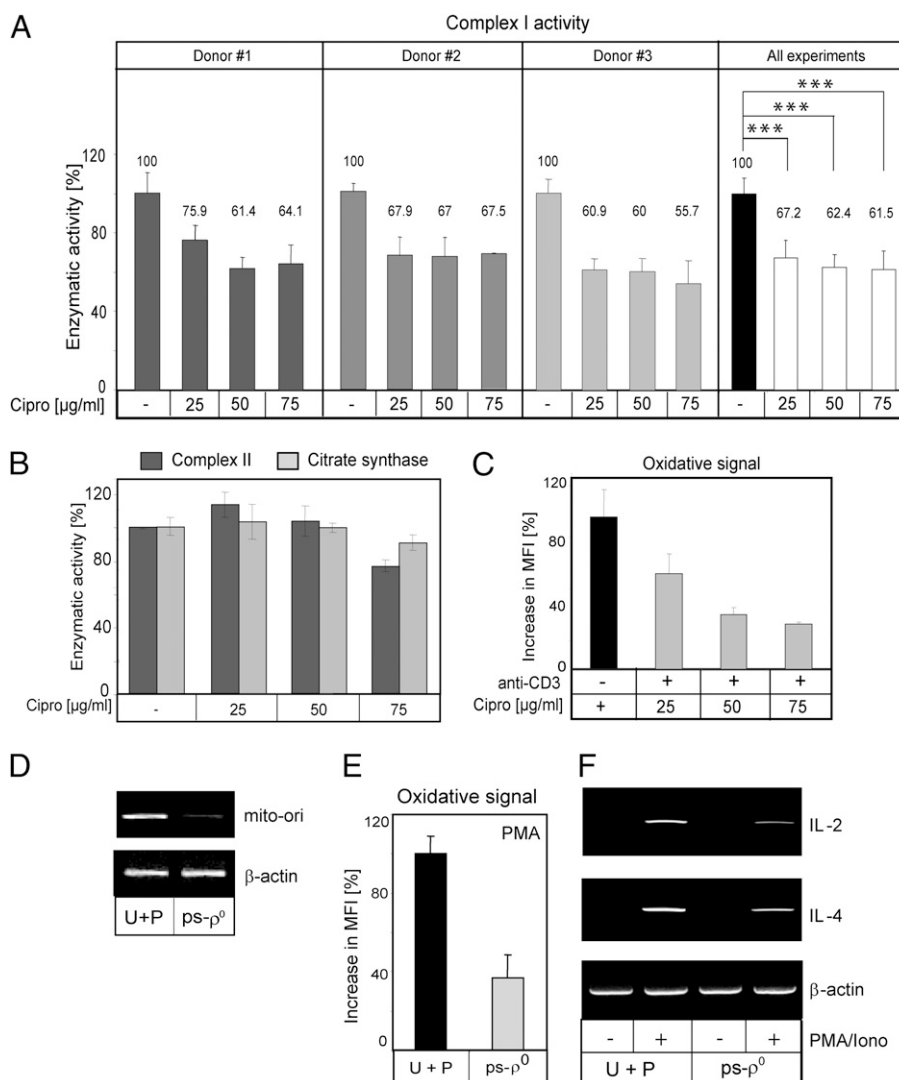


FIGURE 2. Ciprofloxacin-induced impairment of mitochondrial function inhibits T cell activation-induced ROS production and IL-2/IL-4 expression. **A**, Prolonged ciprofloxacin treatment affects enzymatic activity of mtDNA-encoded respiratory complex I. After the depletion of dead cells and adjustment to equal cell number in each sample, PHA-preactivated and Cipro-treated T cells (day 7) were shock-frozen in liquid nitrogen. Next, the activity of mitochondrial respiratory complex I was measured by real-time spectrophotometry and normalized to total protein content. The data were obtained by triplicated measurements of cells from three different donors. The average values of enzymatic activity $\pm$ SD of triplicated separate experiments, as well as interexperimental comparison, are presented (untreated control = 100%; the activities measured in cells treated with Cipro are depicted above each bar). *** $p < 0.0001$; Student t test. **B**, Prolonged ciprofloxacin treatment does not significantly affect enzymatic activity of the non-mtDNA-encoded mitochondrial enzymes: citrate synthase and complex II. Cells were treated and prepared as described in **A**. Enzymatic activities were measured by real-time spectrophotometry and normalized to protein content. Results of representative experiments performed in triplicate $\pm$ SD are presented. **C**, After PHA preactivation and 7 d of Cipro treatment, T cells were stained with H₂DCF-DA and subsequently activated via plate-bound agonistic anti-CD3 Ab (30 µg/ml). The level of activation-induced ROS was assessed by FACS 1 h after activation and calculated as the percentage increase in MFI (untreated control set to 100%). **D** and **E**, Jurkat T cells depleted of mtDNA show impaired activation-induced oxidative signal and express lower levels of IL-2 and -4 upon activation. **D**, Total cellular DNA was isolated from parental Jurkat T cells cultured in the presence of uridine (50 µg/ml) and pyruvate (110 mg/ml) (U+P) and Jurkat T cells cultured in the presence of U+P and EB (250 ng/ml) (ps-p⁰). For PCR amplification of the origin of replication of mitochondrial heavy strand (mt-ori), 100 ng of DNA template was used (upper panel). Amplification of a β-actin gene fragment was used as a loading control (lower panel). **E**, Cells depleted of mtDNA show impaired activation-induced ROS levels. Parental Jurkat T cells cultured in medium supplemented with U+P or ps-p⁰ cells were stimulated with PMA for 30 min, stained with H₂DCF-DA, and analyzed by FACS. The ROS levels were calculated as the percentage increase in MFI (untreated control set to 100%). **F**, J16-145 cells cultured in the presence of U+P and ps-p⁰ cells were treated with PMA (10 ng/ml) and Iono (1 µM) for 1 h. RNA was isolated, reverse transcribed, and amplified using IL-2- and -4- and actin-specific primers.

Measurement of enzymatic activity of mitochondrial enzymes

Activities of the respiratory chain (RC) single-enzyme complexes I and II, as well as of citrate synthase, were measured as described previously, with minor modifications (21, 22). Ciprofloxacin-treated cells were depleted of dead cells via Biocoll (Biochrom, Berlin, Germany) gradient centrifugation. The cell number in different batches of ciprofloxacin-treated cells was equalized. For measurement of the enzymatic activities, samples were prepared as described previously (23), with minor modifications. A total of 4×10^7 cells were washed with PBS, shock-frozen in liquid nitrogen, and thawed on ice. Next, cells were permeabilized by a 15-min incubation with 1 ml 0.015% digitonin (w/v) in RC buffer (250 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 20 mM Tris-HCl [pH 7.4]), washed with RC buffer, and centrifuged at 8000 rpm for 5 min at 4°C. For a single data point, the activity measurement was performed three times in triplicate, and the average value was calculated. Steady-state activity was recorded in a 96-well plate spectrophotometer using a thermostated chamber and a final volume of 300 μ l. Enzymatic activities of complex I and complex II were recorded as NADH oxidation at 340–400 nm and as succinate oxidation at 610–750 nm, respectively. Citrate synthase activity was detected after two additional freeze/thaw cycles as 5,5'-dithiobis-(2-nitrobenzoic acid) reduction at 412 nm.

Results

Prolonged ciprofloxacin treatment inhibits TCR-induced expression of IL-2 and -4 in preactivated human T cells

Ciprofloxacin treatment was shown to exert various effects on activation-induced gene expression in T cells (10). Stimulatory effects of

immediate ciprofloxacin treatment (incubation time up to 72 h) on basal expression of IL-2, TNF- α , or IFN- γ in mitogen-activated T cells have been reported (11, 12, 24). In contrast, other studies showed inhibitory effects of immediate ciprofloxacin treatment on cytokine expression (incubation time up to 48 h) (13, 14). We decided to investigate the effects of long-term ciprofloxacin pretreatment on TCR-induced IL-2 and -4 expression in preactivated T cells. To this end, isolated human peripheral blood T cells were preactivated by an overnight treatment with PHA. The T cells were subsequently expanded in the presence of exogenous IL-2 and different amounts of ciprofloxacin for 7 d. Next, preactivated ciprofloxacin-treated T cells were activated via stimulation with plate-bound anti-CD3 Ab for 1 h. IL-2 and IL-4 gene expression was analyzed using real-time PCR. Ciprofloxacin treatment led to a moderate increase in basal IL-2 and -4 expression levels in PHA-preactivated T cells (Fig. 1A). However, prolonged ciprofloxacin treatment clearly inhibited anti-CD3-induced IL-2 and -4 expression in a dose-dependent manner (Fig. 1B).

Ciprofloxacin treatment induces mtDNA loss, impairs mitochondrial function, and inhibits cellular growth in cultured preactivated human T cells

The addition of ciprofloxacin to in vitro cultures of preactivated human T cells exhibited a strong cytostatic effect together with low

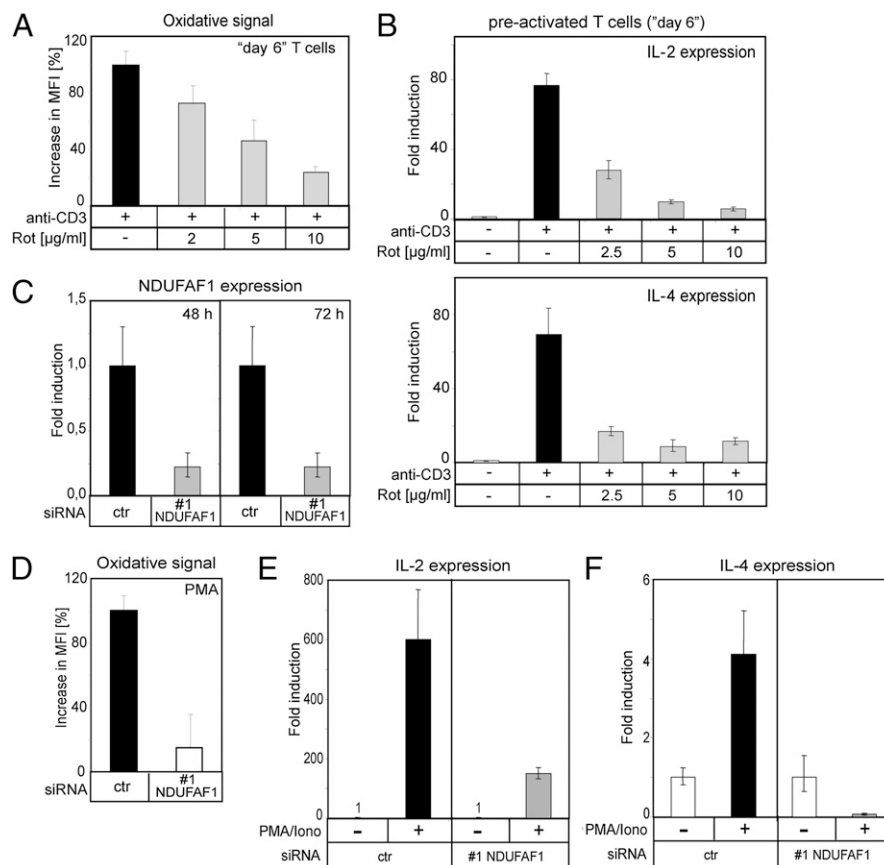


FIGURE 3. Mitochondrial respiratory complex I-generated oxidative signal drives T cell activation-induced IL-2 and -4 expression. *A* and *B*, Inhibition of complex I blocks TCR-induced oxidative signal generation and IL-2/IL-4 expression in preactivated human T cells. *A*, Preactivated T cells were stained with H₂DCF-DA, 5 min pretreated with increasing amounts of Rot, and stimulated via plate-bound anti-CD3 Abs (30 μ g/ml) for 1 h. ROS generation was measured by FACS and calculated as the percentage increase in MFI (untreated control set to 100%). *B*, "Day 6" T cells were pretreated with increasing amounts of Rot (5 min) and stimulated via plate-bound anti-CD3 Abs for 1 h. IL-2 and -4 expression levels were analyzed by quantitative real-time PCR. *C–F*, Downregulation of NDUFAF1 expression inhibits activation-induced ROS generation and IL-2 and -4 expression. *C*, Jurkat T cells were transfected with 250 nM of nonsilencing (ctr) or anti-NDUFAF1-directed siRNA oligonucleotides (#1 anti-NDUFAF1) by nucleofection. Forty-eight or 72 h post-transfection, RNA was isolated, and expression levels of NDUFAF1 were analyzed by quantitative real-time PCR. *D*, After nucleofection (72 h), cells were stained with H₂DCF-DA, treated with PMA (10 ng/ml) for 30 min, and the level of oxidative signal was measured by FACS. Results are shown as the percentage increase in MFI. *E* and *F*, Seventy-two hours after nucleofection, Jurkat T cells were activated by treatment with PMA (10 ng/ml) and Iono (1 μ M) for 1 h. Next, RNA was isolated, reverse transcribed, and IL-2 or -4 expression was assessed by quantitative real-time PCR.

toxicity (Fig. 1C, 1D). In addition, ciprofloxacin induced mtDNA depletion in cultured PHA-preactivated T cells by up to 50%, as estimated by real-time PCR analysis (Fig. 1E). Moreover, mtDNA loss resulted in an impairment of mitochondrial function. This is reflected by significantly decreased activity of the mtDNA-encoded respiratory complex I (Fig. 2A). Activities of non-mtDNA-encoded mitochondrial enzymes, such as citrate synthase and complex II, were not significantly affected by prolonged ciprofloxacin treatment (Fig. 2B). This is in line with previous reports demonstrating a delayed cellular proliferation upon prolonged ciprofloxacin treatment via a mechanism associated with the progressive loss of mtDNA and energy shortage (25).

Ciprofloxacin-induced loss of mtDNA abrogates the activation-induced oxidative signal

A mitochondria-generated activation-induced oxidative signal plays an important regulatory role for CD95L expression in AICD of preactivated T cells (9). Thus, it was interesting to analyze whether mitochondrial oxidative signals are involved in transcriptional regulation of the TCR-induced cytokines IL-2 and -4 in preactivated T cells. Peripheral human T cells, expanded for 7 d in the presence or absence of ciprofloxacin, were stained with oxidation-dependent fluorescent dye H₂DCF-DA and stimulated via plate-bound anti-CD3 Ab for 1 h. As shown in Fig. 2C, ciprofloxacin dose-dependently blocked activation-induced ROS generation, suggesting a causative role for ciprofloxacin-induced mtDNA depletion in inhibiting the expression of IL-2 and -4. To verify this assumption, Jurkat T cells transiently depleted of mtDNA (pseudo-p⁰ phenotype [ps-p⁰]) were generated as described previously (Fig. 2D) (9). Subsequently, ps-p⁰ cells were stimulated with PMA/Iono for 1 h, and gene expression of IL-2 and -4 was analyzed. In addition, the extent of the oxidative signal induced by PMA treatment was assessed (Fig. 2E, 2F). Consistent with a reduced oxidative signal (Fig. 2E), ps-p⁰ cells displayed an abrogated expression of IL-2 and -4 (Fig. 2F). Thus, transcriptional downregulation of these cytokines parallels the inhibition of the mitochondria-generated activation-induced oxidative signal.

The respiratory complex I-generated ROS control activation-induced expression of IL-2 and -4 in preactivated, ciprofloxacin-treated human T cells

Genes located on mtDNA encode crucial components of the mitochondrial ETC, such as complex I, III, and IV and ATP synthase. Thus, the loss of mtDNA results in a decreased activity of the ETC (25). Because activity of the respiratory complex I is necessary for activation-induced ROS generation and CD95L expression in AICD of preactivated T cells (9), we investigated whether the same mechanism accounts for the regulation of IL-2 and -4 expression. The presence of ciprofloxacin during T cell expansion induced a loss of complex I activity (Fig. 2A). To further analyze the participation of complex I in the regulation of IL-2 and -4 expression, we assayed the effects of complex I inhibition by Rot. Human T cells were preactivated by PHA addition and expanded for 6 d in the presence of IL-2 ("day 6" T cells). Next, the cells were stimulated by plate-bound anti-CD3 Abs for 1 h in the presence or absence of subtoxic doses of Rot (Supplemental Fig. 1A); the expression of IL-2 and -4, as well as ROS production, was measured (Fig. 3A, 3B, Supplemental Fig. 1B). T cell activation resulted in upregulation of IL-2 and -4 expression. The induction was almost completely blocked by Rot (Fig. 3B, Supplemental Fig. 1B). Correspondingly, Rot inhibited generation of the TCR-induced oxidative signal (Fig. 3A). To verify the results obtained by inhibition of mitochondrial respiration, we analyzed the activation-induced IL-2 and -4 expression in cells with a decreased amount of complex I. Recently, it was demonstrated that

NDUFAF1 is an essential chaperone for complex I assembly (19). Using an siRNA-mediated approach and two transfection methods, we downregulated NDUFAF1 expression in Jurkat T cells (Fig. 3C, Supplemental Fig. 1C). The knockdown of NDUFAF1 inhibited PMA-induced ROS production (Fig. 3D, Supplemental Fig. 1D) and, subsequently, activation-induced IL-2 and -4 expression (Fig. 3E, 3F, Supplemental Fig. 1E). The obtained results indicate that activation-induced IL-2 and -4 expression in preactivated T cells depends on complex I-generated ROS. Moreover, they suggest that ciprofloxacin-dependent mtDNA loss (Fig. 1E), resulting in inhibition of complex I activity (Fig. 2A), is responsible for the ciprofloxacin-mediated abrogation of the activation-induced oxidative signal (Fig. 2C) and blocking of activation-induced IL-2 and -4 expression in preactivated T cells (Fig. 1B).

The H₂O₂-mediated oxidative signal regulates IL-2 and IL-4 gene transcription

The data obtained thus far implicated a similar regulatory principle for the regulation of IL-2/IL-4 and CD95L transcription. CD95L expression in TCR-stimulated, preactivated T cells strictly depends on the simultaneous presence of two signals: an increase in the intracellular concentration of H₂O₂ and an influx of Ca²⁺ (9). To verify this assumption, Jurkat T cells were transiently transfected with luciferase reporter constructs for IL-2 and -4 promoters. Transcriptional upregulation of IL-2 and -4 was strongly induced upon PMA/Iono treatment (Fig. 4). Nevertheless, treatment with PMA or Iono only failed to induce either of the two promoters (Fig. 4B). This indicates that the IL-2 and -4 promoters depend on the simultaneous presence of the increased cytosolic Ca²⁺ concentration and the PMA-induced oxidative signal. Selective blocking of ROS (with the antioxidant NAC) and the Ca²⁺ influx (with the intracellular Ca²⁺ chelator BAPTA-AM) (Fig. 4A, 4C) led to a significant inhibition of IL-2 and -4 promoter activities (Fig. 4B, 4D).

In addition, the transcriptional upregulation of IL-2 and -4 could also be induced by supplementation of the Iono-derived Ca²⁺ signal with a H₂O₂ signal produced by low amounts of GOX present in the culture media. The GOX-catalyzed reaction transforms glucose into gluconic acid (C₆H₁₂O₆ + O₂ + H₂O → C₆H₁₂O₇ + H₂O₂). H₂O₂ is generated at a consistently low rate as a by-product of this reaction. Because H₂O₂ is an uncharged molecule, it can diffuse through plasma membranes. The extent of intracellular H₂DCF-DA oxidation by GOX-derived H₂O₂ after 1 h of treatment was comparable to the amount induced by PMA treatment (Fig. 4E). Jurkat T cells were treated with PMA and/or Iono or with GOX and/or Iono for 1 h, and IL-2 or IL-4 gene expression was analyzed. IL-2 and -4 expression was detected exclusively after GOX/Iono or PMA/Iono treatment, demonstrating that IL-2 and -4 expression was only possible when the Ca²⁺ and H₂O₂ signals act synergistically (Fig. 4E, 4F).

The oxidative signal controls activation of NF-κB and AP-1 transcription factors

Transcriptional regulation of IL-2 and -4 promoters is known to be controlled by three major transcription factors: NF-κB, AP-1, and NF-AT (1, 2). Therefore, PMA/Iono-induced transcriptional activation of IL-2 and -4 promoters could be blocked by specific inhibition of NF-κB (overexpression of IκBα) (Fig. 5A), AP-1 (overexpression of DN-JNKK) (Fig. 5B), or NF-AT (treatment with CsA) (Fig. 5C). To investigate which of these transcription factors is activated by the oxidative signal, Jurkat T cells were transiently transfected with plasmids carrying luciferase reporter constructs for the respective transcription factors. NAC blocked the PMA-induced oxidative signal (Fig. 4A). Fig. 5D shows that

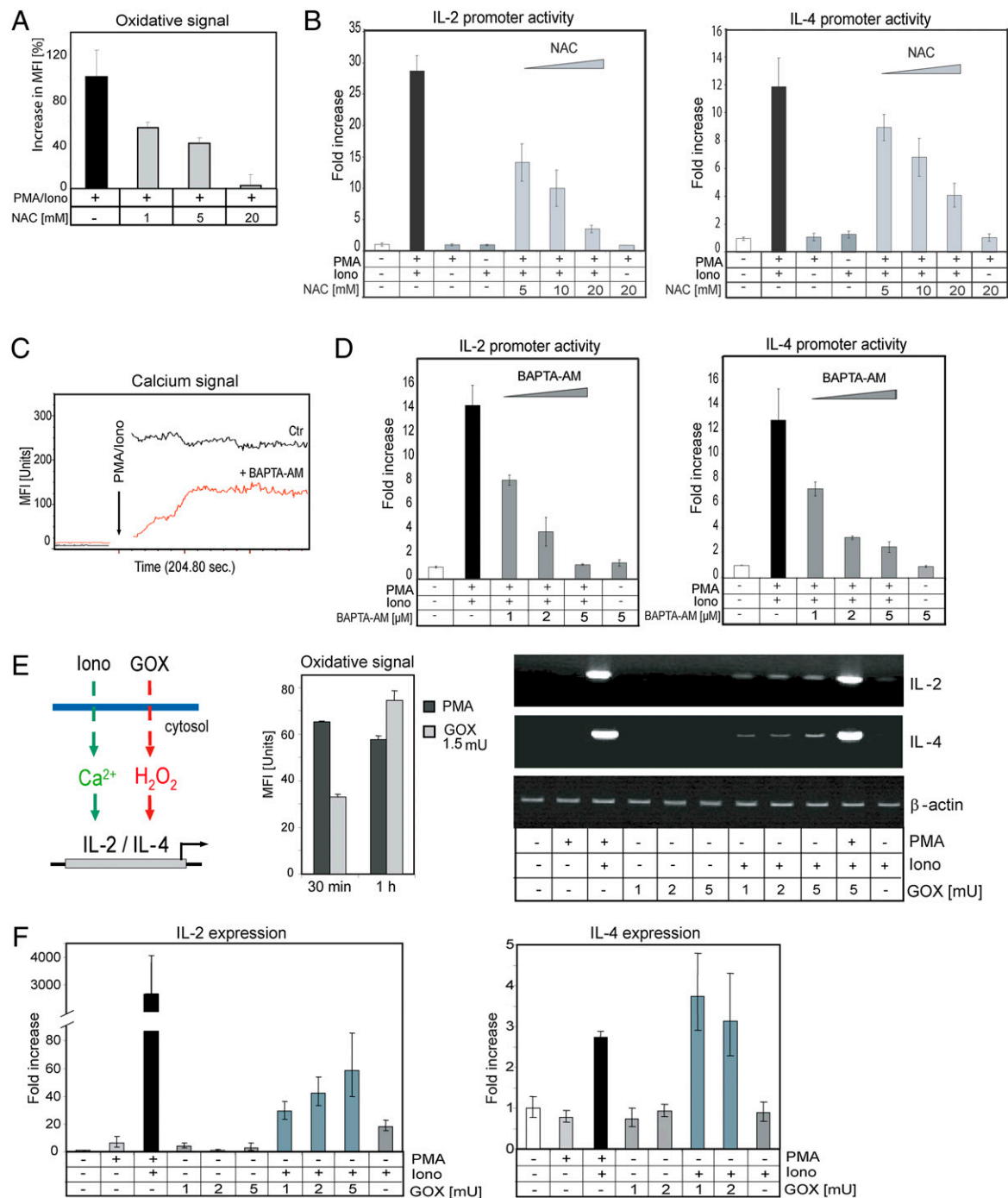


FIGURE 4. Simultaneous presence of the oxidative signal and the Ca^{2+} signal is necessary for T cell activation-induced expression of IL-2 and -4. **A**, Pretreatment of Jurkat T cells with the antioxidant NAC blocks the PMA/Iono-induced oxidative signal. NAC-pretreated (30 min) and H₂DCF-DA-stained Jurkat T cells were activated by PMA (10 ng/ml)/Iono (1 μM) treatment, and the oxidative (30 min) signal was measured by FACS. **B**, Jurkat T cells were transfected with plasmids carrying firefly luciferase reporter constructs (5 μg DNA/transfection) under the control of IL-2 (left panel) and IL-4 (right panel) promoters and cotransfected with a pRL-TK plasmid (2 μg DNA/transfection) harboring the *Renilla luc* gene. After overnight recovery (18 h), cells were treated with PMA (10 ng/ml) and/or Iono (1 μM) with or without 30 min of preincubation with 5–20 mM NAC for 7 h. Thereafter, cells were lysed, and luciferase activity was measured and normalized to *Renilla luc* expression. Data presented are average values $\pm$ SD of the mean chemiluminescence for representative experiments performed in triplicate. **C**, Pretreatment of Jurkat T cells with the intracellular Ca^{2+} chelator BAPTA-AM blocks the PMA/Iono-induced Ca^{2+} signal. BAPTA-AM-pretreated (30 min) and Fluo-4-AM-stained Jurkat T cells were activated by PMA (10 ng/ml)/Iono (1 μM) treatment, and the Ca^{2+} signal was measured by real-time FACS. **D**, Jurkat T cells were transfected with plasmids carrying firefly luciferase reporter constructs under the control of IL-2 (left panel) and IL-4 (right panel) promoters and cotransfected with a pRL-TK plasmid harboring the *Renilla luc* gene as described for **B**. Subsequently, cells were treated with PMA (10 ng/ml) and/or Iono (1 μM) with or without 30 min preincubation with 1–5 μM of BAPTA-AM for 7 h. Promoter activation was determined according to luciferase activity (as in **B**). **E** and **F**, Simultaneous presence of the oxidative signal and the Ca^{2+} signal induces the expression of IL-2 and -4 (**E**, left panel). **E**, middle panel, Jurkat T cells were incubated with GOX (1.5 mU/ml) or PMA (10 ng/ml) for the indicated times. Thereafter, cells were stained with H₂DCF-DA, and the oxidative signal was measured by FACS. Results are shown as an increase in MFI. **E**, right panel and **F**, Jurkat T cells were incubated with different concentrations of GOX (1–5 mU/ml) or PMA (10 ng/ml) with or without Iono (1 μM) for 1 h. mRNA was reverse-transcribed and amplified using IL-2- and -4- and actin- or GAPDH-specific primers. IL-2 and -4 expression was analyzed by semiquantitative (**E**) or quantitative real-time (**F**) RT-PCR.

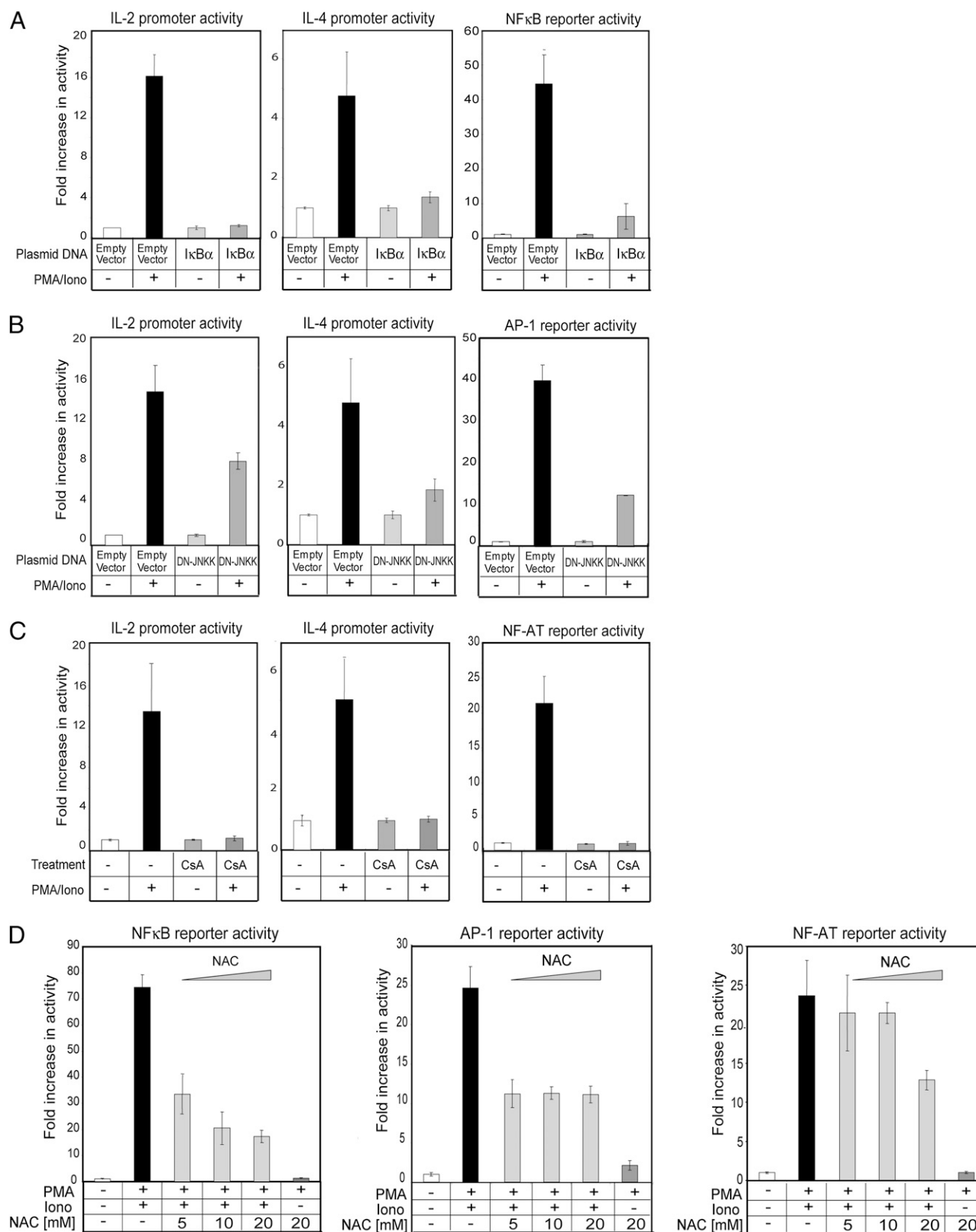


FIGURE 5. The activation-induced oxidative signal controls IL-2 and IL-4 gene expression via triggering of NF-κB and AP-1 transcription factors. A–C, Jurkat T cells were transiently transfected with firefly luciferase reporter constructs controlled by IL-2 and -4 promoters or NF-κB (A), AP-1 (B), and NF-AT (C) based promoters. Cells were cotransfected with a pRL-TK plasmid harboring the *Renilla luc* gene and with expression vectors (5 μg/transfection) for IκBα (wild type) (A) or DN-JNKK (B) or were pretreated for 30 min with 60 ng/ml CsA (C) prior to induction. After overnight recovery (18 h), cells were treated with PMA (10 ng/ml) and Iono (1 μM) for 7 h. Thereafter, cells were lysed, and luciferase activity was measured and normalized to *Renilla luc* expression. Data presented are average values ± SD of the mean chemiluminescence for representative experiments performed in triplicate. D, Jurkat T cells were transfected with firefly luciferase reporter constructs controlled by NF-κB (left panel), AP-1 (middle panel) and NF-AT (right panel) based promoters (5 μg/ml DNA/transfection) and cotransfected with a pRL-TK plasmid harboring the *Renilla luc* gene (2 μg DNA/transfection). After overnight recovery (18 h), cells were treated with PMA (10 ng/ml) and Iono (1 μM) with or without the addition of 5–20 mM NAC (30 min preincubation) for 7 h. Luciferase activity was analyzed as described previously.

blocking of the oxidative signal by NAC differentially inhibited PMA/Iono-induced activation of the NF- κ B, AP-1, and NF-AT luciferase reporter constructs. The oxidative signal positively regulates activation of NF- κ B and AP-1, which corresponds with their known redox dependency (26). However, Ca²⁺ influx-induced activation of NF-AT is largely oxidative signal independent. Moderate effects of high NAC concentrations on the activation of the NF-AT luciferase reporter construct can result from AP-1/NF-AT binding cooperativity, which is characteristic of the IL-2-originated sequence used in the reporter construct (1, 5). Thus, the activation-induced oxidative signal facilitates NF- κ B- and AP-1-mediated transcription.

Resting human T cells regulate activation-induced IL-2 and -4 expression via a mitochondrial complex I-generated oxidative signal

Knowing that mitochondrial complex I-produced ROS positively regulate IL-2 and -4 expression in preactivated human T cells (Fig. 3, Supplemental Fig. 1B–E), we investigated whether the same reg-

ulatory principle applies to resting T cells. Freshly isolated peripheral blood T cells ("day 0" T cells) were stained with H₂DCF-DA. After 5 min of preincubation with different amounts of Rot, cells were stimulated for 1 h with anti-CD3 Abs, and ROS generation was measured. The addition of Rot efficiently blocked the activation-induced oxidative signal (Fig. 6A), suggesting that mitochondrial complex I is the source of the oxidative signal in resting T cells. Consequently, Rot treatment strongly inhibited CD3-induced IL-2 and -4 expression (Fig. 6B). Interestingly, much lower amounts of Rot were sufficient for the inhibition of IL-2 and -4 expression in resting T cells compared with preactivated T cells (Figs. 3B, 6B, Supplemental Fig. 1B). This was paralleled by a stronger Rot-mediated inhibition of activation-induced ROS production in resting T cells compared with preactivated T cells (Figs. 3A, 6A). The differential effects of Rot could be explained, in part, by the significantly lower level of the activation-induced ROS in resting T cells ("day 0") compared with preactivated T cells ("day 6") (Fig. 6C). Furthermore, the inhibition of complex I effectively blocked CD3/CD28- and PMA/Iono- induced secretion of

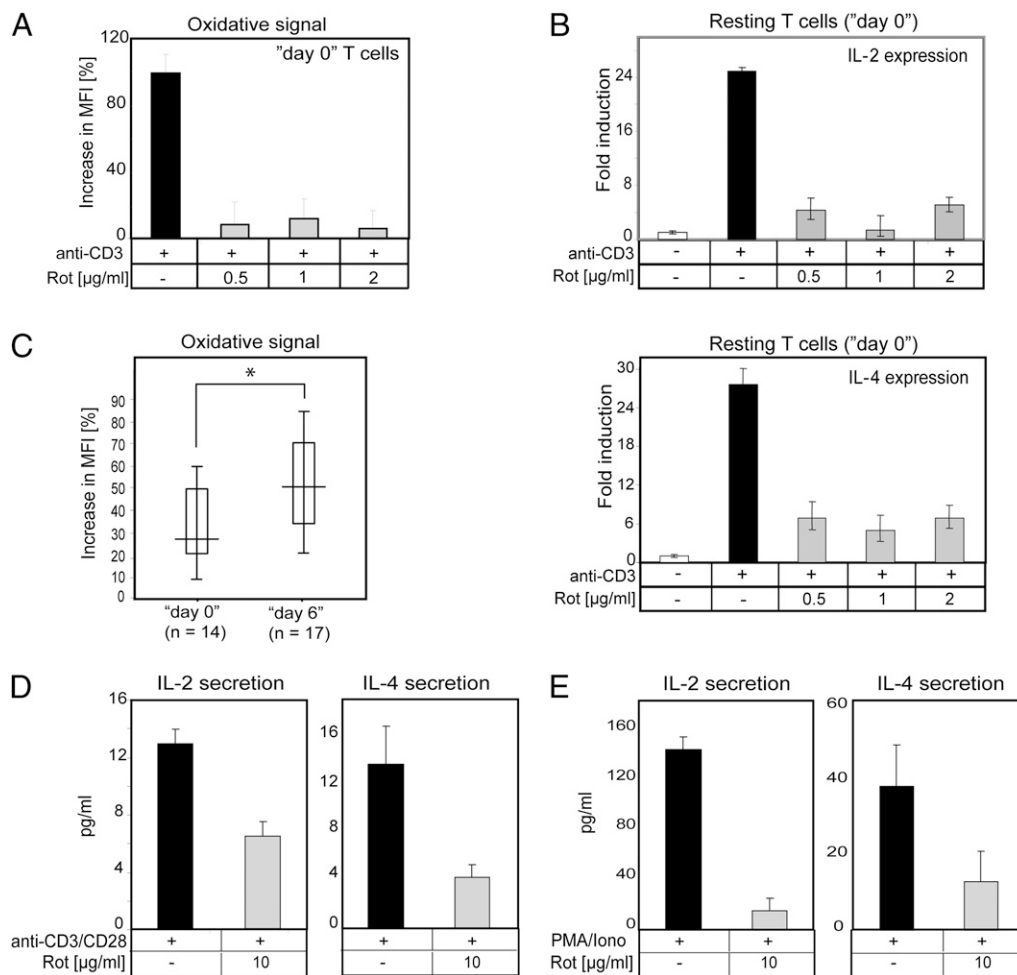


FIGURE 6. Mitochondrial respiratory complex I-generated oxidative signal positively regulates IL-2 and -4 expression in resting ("day 0") T cells. **A** and **B**, Freshly isolated human peripheral T cells were stained with H₂DCF-DA, 5 min-pretreated with different concentrations of Rot and stimulated with plate-bound anti-CD3 Abs (30 μg/ml) for 1 h. **A**, The oxidative signal was analyzed by FACS and calculated as the percentage increase in MFI (untreated control set to 100%). **B**, Total cellular RNA was isolated, and IL-2 (upper panel) or IL-4 expression (lower panel) was analyzed by quantitative real-time PCR. **C**, Resting ("day 0") and preactivated ("day 6") T cells generate the activation-induced oxidative signal to different extents. Resting or preactivated T cells from 14 or 17 healthy donors, respectively, were stained with H₂DCF-DA and stimulated with plate-bound anti-CD3 Ab (30 μg/ml) for 1 h. The increase in MFI was measured and calculated as described previously. Data presented are median values ± range. **p* = 0.0144, Student *t* test; **p* = 0.01035, Wilcoxon rank-sum test. **D** and **E**, Inhibition of the mitochondrial respiratory complex I blocks activation-induced secretion of IL-2 and -4. Resting peripheral human T cells ("day 0" T cells) were pretreated with Rot for 15 min. Next, cells were activated by simultaneous treatment with plate-bound anti-CD3 Ab (30 μg/ml) and soluble anti-CD28 Ab (1 μg/ml) (**C**) or PMA (10 ng/ml) and Iono (1 μM) (**D**) for 4 h. Cell culture medium was collected, and IL-2/IL-4 levels were determined via ELISA.

IL-2 and -4 by resting T cells (Fig. 6D, 6E). Thus, the mitochondrial respiratory complex I actively participates in T cell activation via control over IL-2 and -4 expression.

Blocking mitochondrial complex I abrogates constitutive hyperexpression and TCR-induced upregulation of IL-4 in peripheral blood T lymphocytes of patients with atopic dermatitis

Atopic dermatitis is a heterogeneous allergic skin disease often characterized by elevated blood IL-4 levels (3, 4). Thus, the abrogation of IL-4 expression via inhibition of complex I-mediated ROS generation or ciprofloxacin treatment might have therapeutic effects in these patients. Peripheral blood T cells were isolated from healthy donors or patients with acute atopic dermatitis. Next, freshly isolated T cells were pretreated with Rot and/or activated by CD3 induction, and IL-4 expression levels were analyzed by quantitative PCR. In four of nine cases, T cells from patients with atopic dermatitis exhibited significantly higher basal IL-4 expression compared with resting T cells isolated from healthy donors (Fig. 7A). In addition, CD3 stimulation further increased IL-4 expression in the isolated T cells. Rot efficiently downregulated basal hyperexpression and CD3-induced expression of IL-4 in T cells from patients with atopic dermatitis (Fig. 7). However, in the case of basal IL-4 expression, the inhibitory effect of Rot was only detectable in samples in which the transcript level exceeded the average value for healthy control T cells (relative expression level >0.043).

In addition, we investigated whether prolonged treatment with ciprofloxacin could alleviate basal hyperexpression or TCR-induced expression of IL-4 in T cells from patients with atopic dermatitis. After overnight PHA activation, T cells from patients and healthy controls were treated with 50 $\mu\text{g/ml}$ ciprofloxacin and expanded for 7 d. Subsequently, basal IL-4 expression in preactivated ciprofloxacin-treated T cells was analyzed and compared with transcript levels of resting T cells before expansion (Supplemental Fig. 2). Interestingly, basal expression levels for IL-4 transcripts generally decreased during 7 d of expansion (Supplemental Fig. 2A), indicating a change in the cellular phenotype due to the expansion procedure in cell culture. Thus, no significant downregulation of basal IL-4 expression could be detected when PHA-preactivated

T cells from patients were treated with ciprofloxacin for a prolonged time (Supplemental Fig. 2B). However, ciprofloxacin treatment clearly blocked TCR-induced IL-4 expression in preactivated T cells from patients with atopic dermatitis (Fig. 8A) in a similar fashion as observed for T cells from healthy donors (Figs. 1B, 8A). Furthermore, prolonged ciprofloxacin treatment significantly impaired TCR-induced expression of IL-2, as well as CD95L (Fig. 9). T cell activation-induced CD95L expression is known to be regulated by a mitochondria-originated oxidative signal, as well as by NF- κB and AP-1 (9, 27). Thus, our results support the postulated mechanism of immunosuppressive activity of ciprofloxacin. Prolonged ciprofloxacin treatment leads to mtDNA depletion-mediated impairment of ROS generation and impaired NF- κB /AP-1 activation.

To investigate whether ciprofloxacin also exerts an inhibitory effect on IL-4 secretion, preactivated T cells from patients with atopic dermatitis or healthy donors were expanded in the presence or absence of ciprofloxacin for 7 d and subsequently activated via CD3/CD28 stimulation for 16 h. The concentration of IL-4 in cell culture media was assessed by ELISA. Prolonged ciprofloxacin treatment clearly abrogated activation-induced IL-4 secretion in T cells from healthy controls (Fig. 8B). The same inhibitory effect could be observed in T cells from patients with atopic dermatitis, albeit to a much lower extent (Fig. 8B). Despite comparable efficiency of CD3-mediated gene transcription (Fig. 8A), the amounts of secreted IL-4 were significantly lower compared with those from T cells of healthy donors (Fig. 8B). Therefore, it can be assumed that expanded, preactivated T cells from patients with atopic dermatitis represent a refractory phenotype with regard to CD28-mediated stimulation.

In conclusion, inhibition of the RC complex I or prolonged ciprofloxacin treatment represents a novel therapeutic tool to alleviate deleterious effects of elevated IL-4 levels in patients with atopic dermatitis.

Discussion

The immunomodulatory properties of ciprofloxacin and other drugs of the fluoroquinolone group are well documented (10). Most of the in vitro studies showed stimulatory effects of immediate or

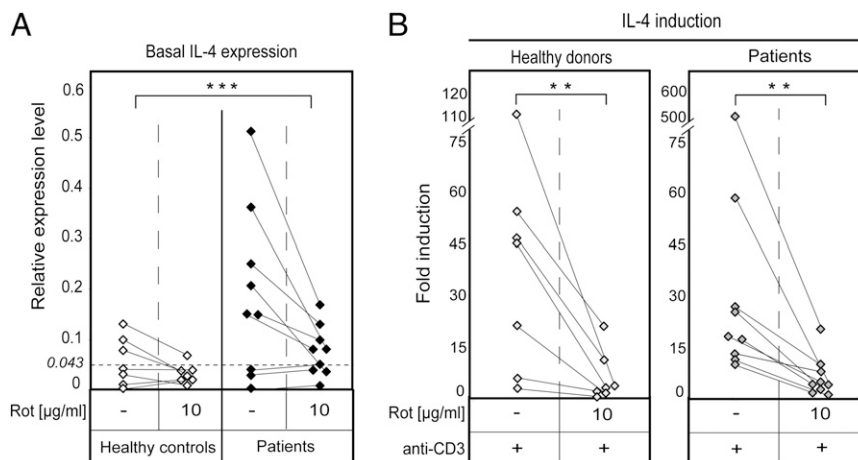
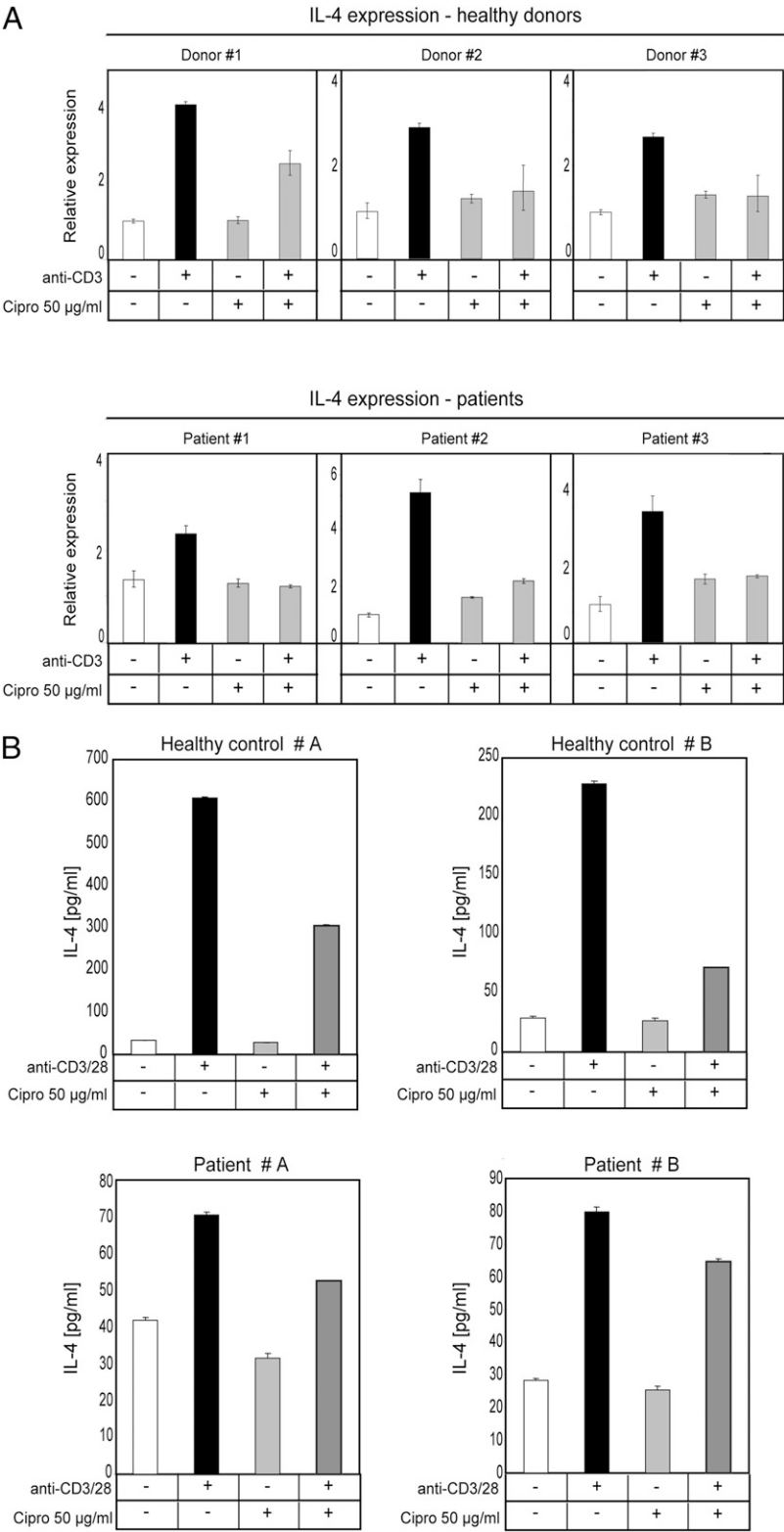


FIGURE 7. Inhibition of complex I activity downregulates basal and activation-induced IL-4 hyperexpression in peripheral blood T cells of patients with atopic dermatitis. Freshly isolated peripheral blood T cells from seven healthy control donors and nine patients with atopic dermatitis were pretreated with Rot (10 $\mu\text{g/ml}$, 15 min) and activated via anti-CD3 Ab for 2 h (10 $\mu\text{g/ml}$; soluble Ab cross-linked by goat anti-mouse polyclonal Ab). Next, RNA was isolated and reverse-transcribed and relative IL-4 gene expression level was analyzed by quantitative real-time PCR. An effect of Rot treatment on basal (A) and TCR-induced (B) IL-4 expression levels is presented. A, the statistical difference for the Rot-induced reduction in basal IL-4 expression of healthy control T cells versus those from atopic dermatitis patients was calculated for values exceeding the average value of expression for control cells (>0.043) using Fortran-subroutine FYTEST, *** $p < 0.0001$ (exact p value 0.0000874). B, the statistical significance of the Rot-induced downregulation of IL-4 expression was calculated using the Wilcoxon signed-rank test. ** $p < 0.01$ (exact p value 0.00781, healthy donors; exact p value 0.00195, patients).

FIGURE 8. Prolonged ciprofloxacin treatment downregulates activation-induced IL-4 expression and secretion in peripheral blood T cells of healthy donors or patients with atopic dermatitis. Human peripheral blood T cells from healthy donors or patients with atopic dermatitis were preactivated by PHA treatment and subsequently cultured for 7 d in the presence or absence of 50 $\mu\text{g/ml}$ Cipro. **A**, T cells were activated via plate-bound anti-CD3 Ab (30 $\mu\text{g/ml}$) for 1 h, and the gene expression levels for IL-4 were assayed by quantitative real-time PCR. Results obtained for samples isolated from T cells of three healthy donors (*upper panel*) and patients with atopic dermatitis (*lower panel*) are presented. **B**, T cells were activated via plate-bound anti-CD3 Ab (30 $\mu\text{g/ml}$) and soluble anti-CD28 Ab (1 $\mu\text{g/ml}$) for 16 h. Next, the secreted amounts of IL-4 were measured in culture media by ELISA. The results obtained for T cells of two healthy donors (*upper panel*) and two patients (*lower panel*) are depicted.

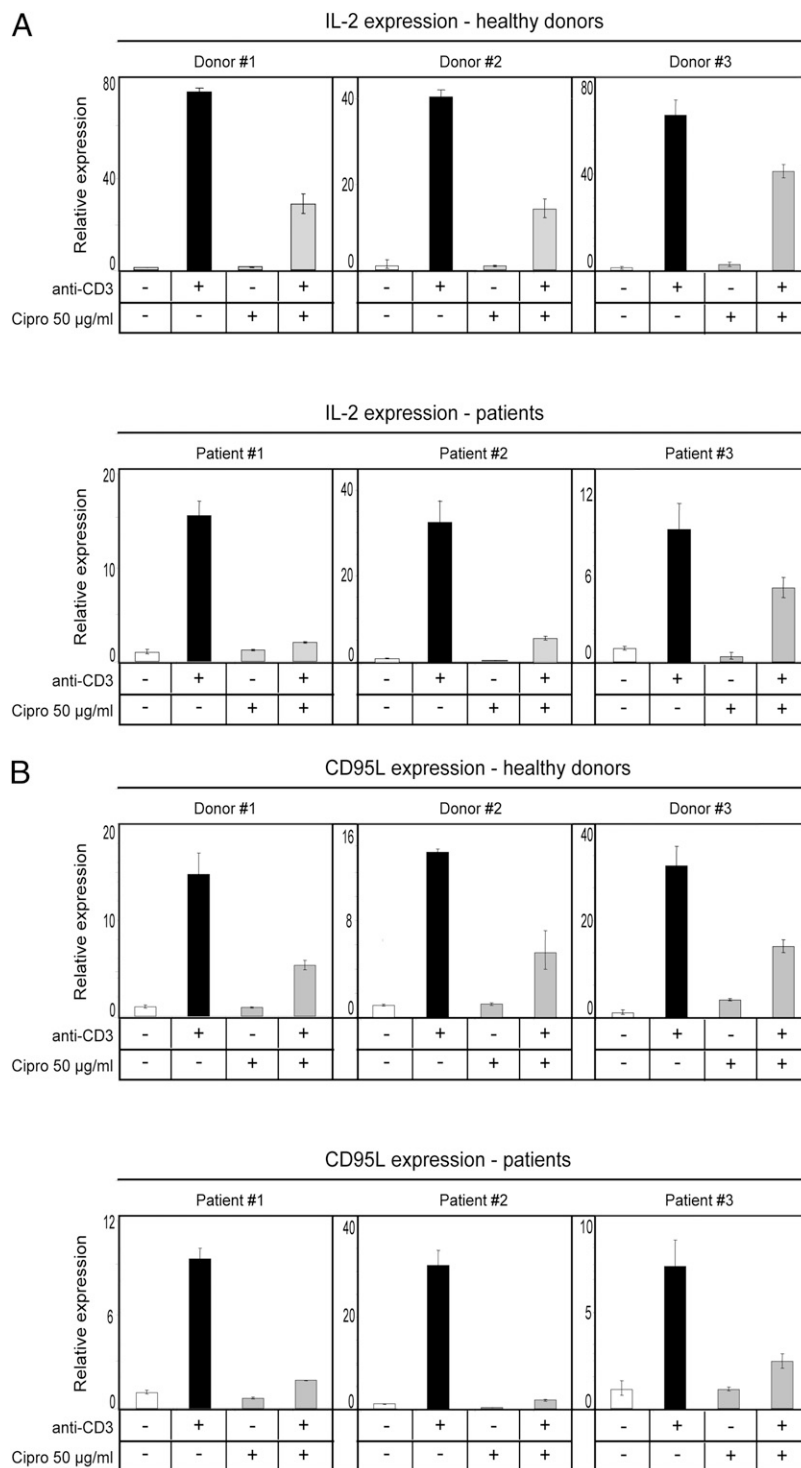


short-term (up to 72 h) ciprofloxacin treatment on basal gene expression in peripheral mitogen-preactivated human T cells (11, 12, 24). However, several in vitro and in vivo studies suggested that ciprofloxacin has inhibitory properties toward T cell activation (10, 13, 14, 28). In addition, in vitro experiments demonstrated that prolonged ciprofloxacin treatment retards cellular growth (25). This cytostatic effect is mediated by inhibition of the putative mitochondrial topoisomerase II in proliferating cells, resulting in a gradual mtDNA loss and energy shortage (16, 25). Our

previous work showed that the mitochondria-generated oxidative signal, in the form of H_2O_2 , is indispensable for T cell activation-induced expression of CD95L, a crucial AICD mediator (9). Thus, it is important to clarify whether ciprofloxacin-induced mitochondrial dysfunction could account for differential effects of ciprofloxacin on activation-induced gene expression in T cells.

Supporting previously published data, we show that long-term ciprofloxacin treatment (7 d) of mitogen-activated proliferating peripheral human T lymphocytes led to a decreased mtDNA

FIGURE 9. Prolonged ciprofloxacin treatment downregulates activation-induced IL-2 and CD95L expression in peripheral blood T cells of healthy donors or patients with atopic dermatitis. Human peripheral blood T cells from healthy donors or patients with atopic dermatitis were preactivated by PHA treatment and subsequently cultured for 7 d in the presence or absence of 50 $\mu\text{g/ml}$ Cipro. T cells were activated via plate-bound anti-CD3 Ab (30 $\mu\text{g/ml}$) for 1 h, and the gene expression levels for IL-2 (A) and CD95L (B) were assayed by quantitative real-time PCR. Results obtained for T cells of three healthy donors (upper panels) and patients with atopic dermatitis (lower panels) are presented.



content (Fig. 1E). Interestingly, prolonged ciprofloxacin treatment clearly blocked TCR-induced expression of IL-2 and IL-4 genes (Fig. 1B). Of note, ciprofloxacin moderately increased basal IL-2 and -4 expression (Fig. 1A), which corresponds with previously reported data (11, 12, 24). Parallel to the inhibitory effect on activation-induced IL-2 and IL-4 gene expression, ciprofloxacin reduced TCR-triggered ROS levels in a dose-dependent fashion (Fig. 2C). Experimental results obtained using Jurkat T cells transiently depleted of mtDNA (ps- ρ^0 phenotype) clearly attributed mtDNA loss to observed effects of ciprofloxacin treatment (Fig. 2D–F). The activation-induced IL-2 and -4 expression levels, as well as the level of activation-induced

ROS, were lower in ps- ρ^0 Jurkat T cells compared with the parental cell line.

Mitochondrial ETC complex I functions as a generator of the activation-induced oxidative signal in preactivated human T cells (“day 6” T cells) (9). As a result of mtDNA depletion (Fig. 1E), long-term ciprofloxacin treatment reduced the activity of mtDNA-encoded complex I (Fig. 2A). Therefore, we investigated whether mitochondrial complex I-generated ROS influences IL-2 and -4 expression in preactivated human T cells. Pretreatment with sub-toxic doses of Rot (9) (Supplemental Fig. 1A), an inhibitor of the mitochondrial respiratory complex I, efficiently blocked the generation of the activation-induced oxidative signal (Fig. 3A), as well

as the TCR-induced expression of IL-2 and IL-4 genes (Fig. 3*B*, Supplemental Fig. 1*B*), in “day 6” T cells. Furthermore, Rot proved to be an even more potent inhibitor of activation-induced IL-2 and -4 expression and ROS production in resting T cells (“day 0” T cells) (Fig. 6*A*, 6*B*). In addition, CD3/CD28- or PMA/Iono-induced secretion of IL-2 and -4 in resting T cells was inhibited by Rot treatment (Fig. 6*D*, 6*E*). CD28-mediated stimulation is necessary for the stabilization of CD3-triggered transcripts and efficient translation (29, 30). The results obtained strongly suggest that in preactivated or in resting T cells, the TCR-induced oxidative signal depends on the activity of mitochondrial complex I. Furthermore, the oxidative signal seems to be crucial for IL-2 and -4 expression. Moreover, the efficiency of Rot is greater in “day 0” T cells compared with “day 6” T cells (Figs. 3*A*, 3*B*, 6*A*, 6*B*). This could be explained, in part, by different levels of activation-induced ROS. Resting T cells (“day 0”) tend to produce lower amounts of ROS compared with preactivated T cells (“day 6”) (Fig. 6*C*).

Previously published studies on the inhibitory effects of Rot on CD8⁺ T cell function implied a regulatory role for the respiratory complex I (31). Nevertheless, Rot was also described to lead to an arrest of the cell cycle due to the inhibition of microtubular spindle formation, centrosome disorganization, and tubulin assembly (32–35). Rot is also known to induce the disassembly of the Golgi apparatus and to disturb tubulin-dependent signaling events (36–38). Thus, it is likely that Rot interferes with T cell activation-triggered microtubule-dependent processes, such as the proliferation and degranulation of CD8⁺ T cells (39). Nevertheless, downregulating the expression of the crucial complex I chaperone NDUFAF1 (19) effectively blocked the generation of the activation-induced oxidative signal and led to decreased expression levels of IL-2 and -4 (Fig. 3*C–F*, Supplemental Fig. 1*C–E*). Thus, we could clearly demonstrate that the activity of mitochondrial respiratory complex I is necessary for T cell activation-induced IL-2 and -4 transcription.

Early investigations showed that T cell mitogenesis requires ROS production (40, 41). Devadas et al. (42) demonstrated that TCR triggering induces the generation of two kinds of ROS: H₂O₂ and superoxide anion (O₂^{•−}). Nevertheless, the unique chemical properties of H₂O₂, such as selective, reversible oxidation of cysteines, membrane permeability, and relatively long half-life, favor it over other ROS as a possible second messenger (26). The postulated positive regulatory role for H₂O₂ includes inactivation of protein tyrosine phosphatases (43) and/or activation of transcription factors, such as NF-κB and AP-1 (26). Our previous work demonstrated that in the case of CD95L expression, the IP₃/Iono-induced Ca²⁺ signal is complemented by a DAG/PMA-induced H₂O₂ signal. The combination of a mitochondria-generated H₂O₂ signal with a simultaneous Ca²⁺ influx into the cytosol constitutes the minimal requirement for induction of CD95L expression (8).

The application of antioxidants blocks TCR-induced IL-2 expression (44–46). Likewise, the treatment of primary human T cells with NAC efficiently attenuates IL-4 expression and secretion (47, 48). Our work confirms these studies and extends the regulatory principle previously identified for CD95L to the transcriptional regulation of IL-2 and IL-4 genes. Selective interference with the H₂O₂ signal by NAC or with the Ca²⁺ influx by BAPTA-AM blocked PMA/Iono-induced activation of IL-2 and IL-4 gene promoters (Fig. 4*A–D*). Either signal alone is insufficient for IL-2 and -4 expression. The present study also clearly demonstrates that a low, physiologically relevant H₂O₂-mediated oxidative signal is indispensable for the activation-induced gene expression in T cells. A full transcriptional induction of IL-2 and -4 was only observed when a GOX-derived H₂O₂ signal was complemented by an Iono-mediated Ca²⁺ signal (Fig. 4*E*, 4*F*).

Gene promoter sequences of IL-2 and -4 largely show binding sites for three major transcription factors: NF-κB, AP-1, and NF-AT (2, 5). Concerted induction of these transcription factors drives T cell activation-triggered IL-2 and IL-4 gene transcription (Fig. 5*A–C*). Upon T cell activation, mitochondrial complex I-generated O₂^{•−} dismutates to H₂O₂ and diffuses to the cytosol where it serves as an oxidative signal (9). This oxidative signal controls the activation of NF-κB and AP-1, whereas NF-AT activation is largely H₂O₂ independent (Fig. 5*D*). Our findings support previous reports on redox-dependent regulation of NF-κB and AP-1 activation in T cells (46, 49, 50).

Overproduction of IL-4 by lesional and peripheral T cells from patients with atopic dermatitis often constitutes a major hallmark of the disease (3, 4). Atopic dermatitis is a chronic, heterogeneous, inflammatory skin disease of increasing prevalence. Currently, it affects 15–30% of children and 2–10% of adults in industrialized countries (4). Thus, we decided to test whether blocking the activity of mitochondrial respiratory complex I would alleviate IL-4 hyperexpression in patients’ T cells. Freshly isolated peripheral T cells from four of nine patients with atopic dermatitis showed significantly enhanced basal levels of IL-4 transcripts. Pretreatment of these samples with Rot efficiently reduced IL-4 expression to levels corresponding with those of T cells from healthy donors (Fig. 7*A*). Furthermore, Rot totally abrogated the TCR-induced increase in IL-4 expression in patients’ T cells, as well as in T cells from healthy donors (Fig. 7*B*). In addition, prolonged ciprofloxacin treatment proved to be efficient in blocking TCR-induced IL-4, IL-2, and CD95L gene expression in PHA-preactivated T cells (Figs. 8*A*, 9). Moreover, long-term culture in the presence of ciprofloxacin abrogated CD3/CD28-induced IL-4 secretion in T cells from healthy donors and patients (Fig. 8*B*). In patients’ T cells, the inhibitory effects of ciprofloxacin on IL-4 secretion were mild, probably because of the diminished ability to secrete IL-4 after 7 d of PHA-mediated expansion compared with T cells of healthy donors (Fig. 8*B*). This could be explained, in part, by disturbed CD28-induced signaling, because PHA-preactivated T cells isolated from patients with atopic dermatitis showed a normal CD3-induced IL-4 gene expression compared with T cells from healthy donors (Fig. 8*A*). In general, our results are in line with the proposed mechanism for ciprofloxacin-mediated immunosuppression (i.e., immunosuppression by ciprofloxacin treatment is based on mtDNA depletion during PHA-induced expansion and consequent impairment of mitochondrial ROS generation and NF-κB/AP-1 activation).

To our knowledge, these observations strongly suggest that the inhibition of ROS production by mitochondrial respiratory complex I might have a therapeutic potential for the treatment of T cell-mediated inflammatory diseases. Moreover, we found that ciprofloxacin-treated preactivated T cells displayed an immunosuppressed phenotype as the result of lower activation-induced ROS production and, consequently, lower IL-2 and -4 expression. In this respect, it cannot be excluded that T cell activation-induced expression of other NF-κB/AP-1-dependent cytokines is influenced by inhibition of the ROS signal, as is seen for CD95L expression. In conclusion, these findings open new possibilities for use of this drug. However, the ability of ciprofloxacin to induce delayed-type hypersensitivity via direct TCR triggering (51) may pose difficulties to the topical application of ciprofloxacin to alleviate skin inflammation.

Thus, we postulate that our findings may have a profound impact on the treatment of inflammatory diseases, such as atopic dermatitis, in which pathologic conditions develop from increased IL-4 production by hyperactivated Th2 cells. Moreover, our data shed new light on the etiology of immunological phenotypes associated

with mitochondrial disorders, such as mtDNA deletions or complex I deficiencies. Patients with mitochondrial dysfunctions often present with recurrent infections (52). In addition, fatal neonatal-onset mitochondrial RC disease with the manifestation of T cell immunodeficiency has been described (53). Furthermore, it was demonstrated that the malfunctioning of complex I leads to excessive generation of ROS (54). Thus, it seems interesting that Leber hereditary optic neuropathy, caused by deficient function of mitochondrial respiratory complex I, is often associated with T cell-mediated autoimmune multiple sclerosis-like syndrome (55). In addition, recent epidemiologic studies on a cohort of patients with mitochondrial disorders showed a high statistical association between these pathologies and lymphoid malignancies (56).

The present study demonstrates, for the first time, that mitochondrial complex I-derived H_2O_2 controls T cell activation due to regulation of IL-2 and -4 expression. Therefore, the experimental results of our work call for careful analysis of T cell profiling and activation defects in the case of mitochondria-associated disorders.

Acknowledgments

We thank the patients with atopic dermatitis for blood donations, S. Sass, D. Röth, A.N. Tan, and W.A. Grandy for critical reading of the manuscript, Dr. W. Rittgen for help with the statistical analysis, and Prof. Dr. S. Goerdit for support.

Disclosures

The authors have no financial conflicts of interest.

References

- Malek, T. R. 2008. The biology of interleukin-2. *Annu. Rev. Immunol.* 26: 453–479.
- Li-Weber, M., and P. H. Krammer. 2003. Regulation of IL4 gene expression by T cells and therapeutic perspectives. *Nat. Rev. Immunol.* 3: 534–543.
- Avgerinou, G., A. V. Goules, P. G. Stavropoulos, and A. D. Katsambas. 2008. Atopic dermatitis: new immunologic aspects. *Int. J. Dermatol.* 47: 219–224.
- Bieber, T. 2008. Atopic dermatitis. *N. Engl. J. Med.* 358: 1483–1494.
- Katsiari, C. G., and G. C. Tsokos. 2006. Transcriptional repression of interleukin-2 in human systemic lupus erythematosus. *Autoimmun. Rev.* 5: 118–121.
- Yuang, Y., and R. L. Wange. 2004. T cell receptor signaling: beyond complex complexes. *J. Biol. Chem.* 279: 28827–28830.
- Truneh, A., F. Albert, P. Golstein, and A. M. Schmitt-Verhulst. 1985. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. *Nature* 313: 318–320.
- Gülöw, K., M. Kaminski, K. Darvas, D. Süß, M. Li-Weber, and P. H. Krammer. 2005. HIV-1 trans-activator of transcription substitutes for oxidative signaling in activation-induced T cell death. *J. Immunol.* 174: 5249–5260.
- Kaminski, M., M. Kiessling, D. Süß, P. H. Krammer, and K. Gülöw. 2007. Novel role for mitochondria: protein kinase C θ -dependent oxidative signaling organelles in activation-induced T-cell death. *Mol. Cell. Biol.* 27: 3625–3639.
- Dalhoff, A. 2005. Immunomodulatory activities of fluoroquinolones. *Infection* 33(Suppl. 2): 55–70.
- Riesbeck, K., M. Sigvardsson, T. Leanderson, and A. Forsgren. 1994. Superinduction of cytokine gene transcription by ciprofloxacin. *J. Immunol.* 153: 343–352.
- Stünkel, K. G., G. Hewlett, and H. J. Zeiler. 1991. Ciprofloxacin enhances T cell function by modulating interleukin activities. *Clin. Exp. Immunol.* 86: 525–531.
- Williams, A. C., H. F. Galley, A. M. Watt, and N. R. Webster. 2005. Differential effects of three antibiotics on T helper cell cytokine expression. *J. Antimicrob. Chemother.* 56: 502–506.
- Katsuno, G., H. K. Takahashi, H. Iwagaki, K. Mizuno, T. Yagi, S. Mori, S. Saito, T. Yoshino, M. Nishibori, and N. Tanaka. 2006. The immunosuppressive effects of ciprofloxacin during human mixed lymphocyte reaction. *Clin. Immunol.* 119: 110–119.
- Castora, F. J., F. F. Vissering, and M. V. Simpson. 1983. The effect of bacterial DNA gyrase inhibitors on DNA synthesis in mammalian mitochondria. *Biochim. Biophys. Acta* 740: 417–427.
- Lawrence, J. W., S. Darkin-Rattray, F. Xie, A. H. Neims, and T. C. Rowe. 1993. 4-Quinolones cause a selective loss of mitochondrial DNA from mouse L1210 leukemia cells. *J. Cell. Biochem.* 51: 165–174.
- Koziele, R., K. Zablocki, and J. Duszyński. 2006. Calcium signals are affected by ciprofloxacin as a consequence of reduction of mitochondrial DNA content in Jurkat cells. *Antimicrob. Agents Chemother.* 50: 1664–1671.
- Li-Weber, M., O. Laur, K. Dern, and P. H. Krammer. 2000. T cell activation-induced and HIV tat-enhanced CD95(APO-1/Fas) ligand transcription involves NF-kappaB. *Eur. J. Immunol.* 30: 661–670.
- Vogel, R. O., R. J. Janssen, C. Ugalde, M. Grovenstein, R. J. Huijbens, H. J. Visch, L. P. van den Heuvel, P. H. Willems, M. Zeviani, J. A. Smeitink, and L. G. Nijtmans. 2005. Human mitochondrial complex I assembly is mediated by NDUFAF1. *FEBS J.* 272: 5317–5326.
- Novak, H., A. Müller, N. Harter, C. Günther, J. M. Carballido, and M. Woisetschlager. 2007. CCL23 expression is induced by IL-4 in a STAT6-dependent fashion. *J. Immunol.* 178: 4335–4341.
- Okun, J. G., P. Lümmer, and U. Brandt. 1999. Three classes of inhibitors share a common binding domain in mitochondrial complex I (NADH:ubiquinone oxidoreductase). *J. Biol. Chem.* 274: 2625–2630.
- Sauer, S. W., J. G. Okun, M. A. Schwab, L. R. Crnic, G. F. Hoffmann, S. I. Goodman, D. M. Koeller, and S. Köler. 2005. Bioenergetics in glutaryl-coenzyme A dehydrogenase deficiency: a role for glutaryl-coenzyme A. *J. Biol. Chem.* 280: 21830–21836.
- Chretien, D., P. Bénil, M. Chol, S. Lebon, A. Rötig, A. Munnich, and P. Rustin. 2003. Assay of mitochondrial respiratory chain complex I in human lymphocytes and cultured skin fibroblasts. *Biochem. Biophys. Res. Commun.* 301: 222–224.
- Riesbeck, K., A. Forsgren, A. Henriksson, and A. Bredberg. 1998. Ciprofloxacin induces an immunomodulatory stress response in human T lymphocytes. *Antimicrob. Agents Chemother.* 42: 1923–1930.
- Rowe, T. C., V. Weissig, and J. W. Lawrence. 2001. Mitochondrial DNA metabolism targeting drugs. *Adv. Drug Deliv. Rev.* 49: 175–187.
- Dröge, W. 2002. Free radicals in the physiological control of cell function. *Physiol. Rev.* 82: 47–95.
- Li-Weber, M., and P. H. Krammer. 2003. Function and regulation of the CD95 (APO-1/Fas) ligand in the immune system. *Semin. Immunol.* 15: 145–157.
- Riesbeck, K., H. Schatz, O. Ostraat, G. Tufveson, A. Forsgren, and H. Ekberg. 1995. Enhancement of the immunosuppressive effect of cyclosporin A by ciprofloxacin in a rat cardiac allograft transplantation model. *Transpl. Int.* 8: 96–102.
- Powell, J. D., J. A. Ragheb, S. Kitagawa-Sakakida, and R. H. Schwartz. 1998. Molecular regulation of interleukin-2 expression by CD28 co-stimulation and anergy. *Immunol. Rev.* 165: 287–300.
- June, C. H., K. M. Jackson, J. A. Ledbetter, J. M. Leiden, T. Lindsten, and C. B. Thompson. 1989. Two distinct mechanisms of interleukin-2 gene expression in human T lymphocytes. *J. Autoimmun.* 2(Suppl): 55–65.
- Yi, J. S., B. C. Holbrook, R. D. Michalek, N. G. Laniewski, and J. M. Grayson. 2006. Electron transport complex I is required for CD8+ T cell function. *J. Immunol.* 177: 852–862.
- Brinkley, B. R., S. S. Barham, S. C. Barranco, and G. M. Fuller. 1974. Rotenone inhibition of spindle microtubule assembly in mammalian cells. *Exp. Cell Res.* 85: 41–46.
- Barham, S. S., and B. R. Brinkley. 1976. Action of rotenone and related respiratory inhibitors on mammalian cell division. 2 Ultrastructural studies. *Cytobios* 15: 97–109.
- Barham, S. S., and B. R. Brinkley. 1976. Action of rotenone and related respiratory inhibitors on mammalian cell division. 1 Cell kinetics and biochemical aspects. *Cytobios* 15: 85–96.
- Diaz-Corrales, F. J., M. Asanuma, I. Miyazaki, K. Miyoshi, and N. Ogawa. 2005. Rotenone induces aggregation of gamma-tubulin protein and subsequent disorganization of the centrosome: relevance to formation of inclusion bodies and neurodegeneration. *Neuroscience* 133: 117–135.
- Marshall, L. E., and R. H. Himes. 1978. Rotenone inhibition of tubulin self-assembly. *Biochim. Biophys. Acta* 543: 590–594.
- Ren, Y., W. Liu, H. Jiang, Q. Jiang, and J. Feng. 2005. Selective vulnerability of dopaminergic neurons to microtubule depolymerization. *J. Biol. Chem.* 280: 34105–34112.
- Jiang, Q., Z. Yan, and J. Feng. 2006. Activation of group III metabotropic glutamate receptors attenuates rotenone toxicity on dopaminergic neurons through a microtubule-dependent mechanism. *J. Neurosci.* 26: 4318–4328.
- Grayson, J. M., K. Murali-Krishna, J. D. Altman, and R. Ahmed. 2001. Gene expression in antigen-specific CD8+ T cells during viral infection. *J. Immunol.* 166: 795–799.
- Novogrodsky, A., A. Ravid, A. L. Rubin, and K. H. Stenzel. 1982. Hydroxyl radical scavengers inhibit lymphocyte mitogenesis. *Proc. Natl. Acad. Sci. USA* 79: 1171–1174.
- Chaudhri, G., N. H. Hunt, I. A. Clark, and R. Ceredig. 1988. Antioxidants inhibit proliferation and cell surface expression of receptors for interleukin-2 and transferrin in T lymphocytes stimulated with phorbol myristate acetate and ionomycin. *Cell. Immunol.* 115: 204–213.
- Devadas, S., L. Zaritskaya, S. G. Rhee, L. Oberley, and M. S. Williams. 2002. Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: selective regulation of mitogen-activated protein kinase activation and fas ligand expression. *J. Exp. Med.* 195: 59–70.
- Xu, D., I. I. Rovira, and T. Finkel. 2002. Oxidants painting the cysteine chapel: redox regulation of PTPs. *Dev. Cell* 2: 251–252.
- Tatla, S., V. Woodhead, J. C. Foreman, and B. M. Chain. 1999. The role of reactive oxygen species in triggering proliferation and IL-2 secretion in T cells. *Free Radic. Biol. Med.* 26: 14–24.
- Goldstone, S. D., A. D. Milligan, and N. H. Hunt. 1996. Oxidative signalling and gene expression during lymphocyte activation. *Biochim. Biophys. Acta* 1314: 175–182.
- Goldstone, S. D., J. C. Fragonas, T. M. Jeitner, and N. H. Hunt. 1995. Transcription factors as targets for oxidative signalling during lymphocyte activation. *Biochim. Biophys. Acta* 1263: 114–122.
- Jeannin, P., Y. Delneste, S. Lecoanet-Henchoz, J. F. Gauchat, P. Life, D. Holmes, and J. Y. Bonnefoy. 1995. Thiols decrease human interleukin (IL) 4 production and IL-4-induced immunoglobulin synthesis. *J. Exp. Med.* 182: 1785–1792.

48. Bengtsson, A., M. Lundberg, J. Avila-Cariño, G. Jacobsson, A. Holmgren, and A. Scheynius. 2001. Thiols decrease cytokine levels and down-regulate the expression of CD30 on human allergen-specific T helper (Th) 0 and Th2 cells. *Clin. Exp. Immunol.* 123: 350–360.
49. Goldstone, S. D., and N. H. Hunt. 1997. Redox regulation of the mitogen-activated protein kinase pathway during lymphocyte activation. *Biochim. Biophys. Acta* 1355: 353–360.
50. Los, M., W. Dröge, K. Stricker, P. A. Baeuerle, and K. Schulze-Osthoff. 1995. Hydrogen peroxide as a potent activator of T lymphocyte functions. *Eur. J. Immunol.* 25: 159–165.
51. Schmid, D. A., J. P. Depta, and W. J. Pichler. 2006. T cell-mediated hypersensitivity to quinolones: mechanisms and cross-reactivity. *Clin. Exp. Allergy* 36: 59–69.
52. Finsterer, J. 2007. Hematological manifestations of primary mitochondrial disorders. *Acta Haematol.* 118: 88–98.
53. Reichenbach, J., R. Schubert, R. Horváth, J. Petersen, N. Fütterer, E. Malle, A. Stumpf, B. R. Gebhardt, U. Koehl, B. Schraven, and S. Zielen. 2006. Fatal neonatal-onset mitochondrial respiratory chain disease with T cell immunodeficiency. *Pediatr. Res.* 60: 321–326.
54. Distelmaier, F., W. J. Koopman, L. P. van den Heuvel, R. J. Rodenburg, E. Mayatepek, P. H. Willems, and J. A. Smeitink. 2009. Mitochondrial complex I deficiency: from organelle dysfunction to clinical disease. *Brain* 132: 833–842.
55. Vyshkina, T., I. Banisor, Y. Y. Shugart, T. P. Leist, and B. Kalman. 2005. Genetic variants of Complex I in multiple sclerosis. *J. Neurol. Sci.* 228: 55–64.
56. Mende, S., R. Koch, J. Schmiedel, H. Reichmann, and A. Storch. 2007. Association of mitochondrial disorders with lymphoid malignancies. *Mitochondrion* 7: 401–403.

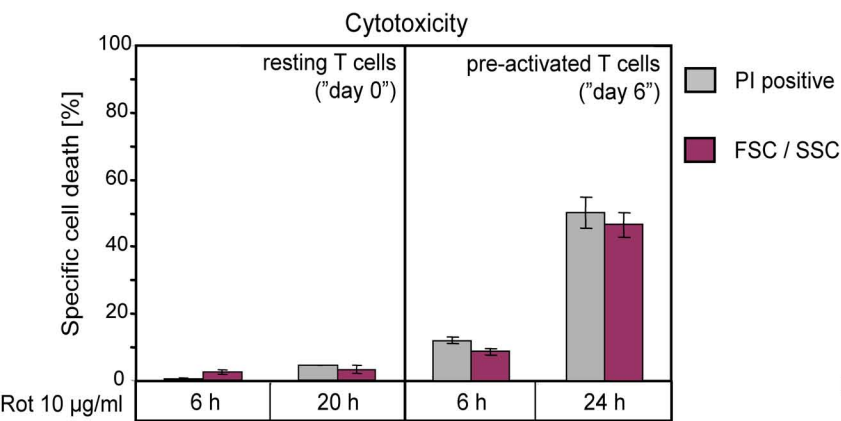
Supplementary figure legends

Supplementary Figure 1. ROS released from mitochondrial respiratory complex I are indispensable for activation-induced IL-2 and IL-4 expression. **A** and **B**, Sub-toxic doses of rotenone block TCR-induced IL-2 and IL-4 expression in pre-activated human T cells. **A**, Resting (“day 0”) or pre-activated (“day 6”) human peripheral blood T cells were treated with 10 µg/ml rotenone for indicated time periods. Cell death was determined by a drop in the FSC/SSC profile or by propidium iodide (PI) uptake in comparison to living cells. The data were re-calculated to “specific cell death” as described before (8). **B**, Pre-activated T cells (“day 6” T cells) were 5 min pre-treated with increasing amounts of rotenone (Rot) and stimulated *via* plate-bound anti-CD3 antibodies (30 µg/ml) for 1 h. Total cellular RNA was isolated and IL-2/IL-4 expression was analyzed by semi-quantitative RT-PCR. **C-E**, siRNA-mediated knock-down of NDUFAF1 expression inhibits activation-induced ROS generation as well as IL-2 and IL-4 gene induction. Jurkat T cells were transfected with 150 nM scrambled (ctr) or two different NDUFAF1-specific siRNA oligonucleotides (#1, #2) by lipofection. After 48 h NDUFAF1 expression was assessed by semi-quantitative RT-PCR. **D**, Quantification of PMA-induced oxidative signals in lipofection-transfected Jurkat cells after 72 h. **E**, Jurkat T cells were transfected by lipofection with 150 nM of scrambled (ctr) or NDUFAF1-specific siRNA oligonucleotides (#1, #2). After 72 h, cells were treated with PMA/ionomycin for 1 h. Levels of IL-2 and IL-4 transcripts were analyzed by semi-quantitative PCR.

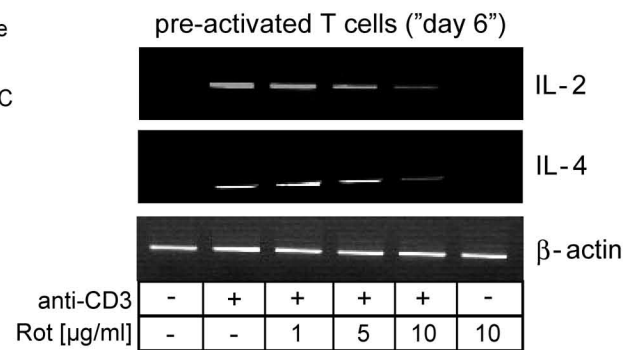
Supplementary Figure 2. Changes of basal expression of IL-4 after 7 days of PHA-mediated T cell expansion in the presence or absence of ciprofloxacin. Freshly isolated peripheral

blood T cells (day 0") from 5 healthy donors and 7 patients with atopic dermatitis were pre-activated with PHA and expanded for 7 days *in vitro* in the presence or absence of 50 µg/ml ciprofloxacin (Cipro). Next, RNA was isolated, reverse-transcribed and the relative IL-4 gene expression level was analyzed by quantitative real-time PCR. Changes of basal expression of IL-4 upon T cell expansion (**A**) and ciprofloxacin treatment (**B**) on IL-4 expression levels are presented. Statistical significance of culture conditions-induced down-regulation of IL-4 expression was calculated using Wilcoxon signed-rank test, for (**A**) $P < 0.05$ (0.03125 – healthy donors; 0.01563 - patients), (*), for (**B**) no statistical significance, (n. s.).

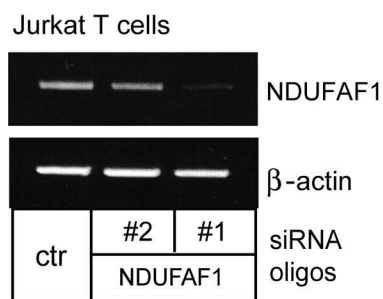
A



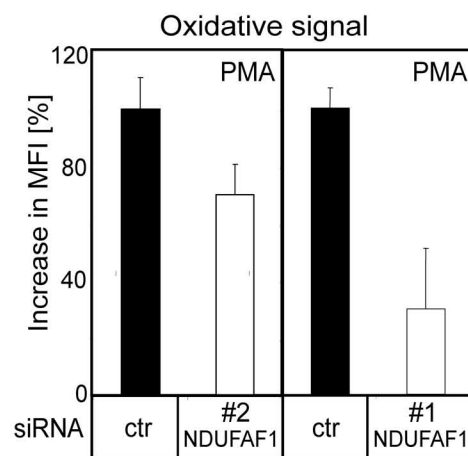
B



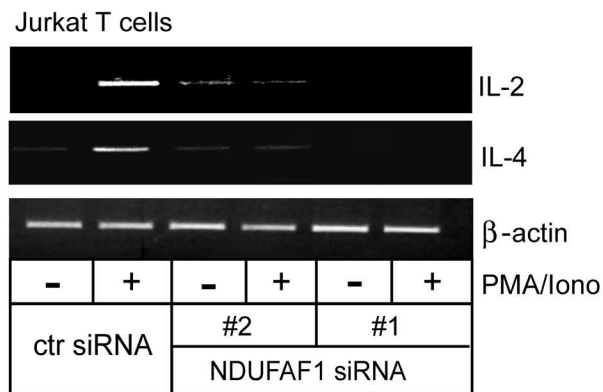
C



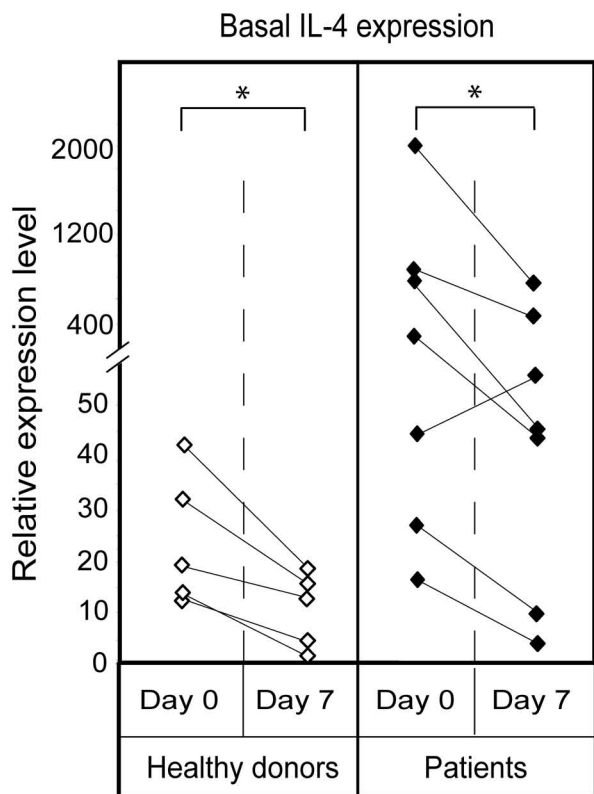
D



E



A



B

