The Journal of Immunology

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J Immunol 2006; 176:2765-2772; ;
doi: 10.4049/jimmunol.176.5.2765
http://www.jimmunol.org/content/176/5/2765

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Oxidative Stress Promotes Polarization of Human T Cell Differentiation Toward a T Helper 2 Phenotype[1]

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These studies were conducted to determine the effects of oxidative stress on human T cell differentiation and polarization into Th1 or Th2 phenotypes. Highly purified naive CD4$^+$ T cells were isolated from PBMC of healthy, nonatopic donors. CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAb in the presence or absence of oxidative stress as supplied by 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), which generates a low level of superoxide anion. Increases in cellular superoxide were observed by exposure to DMNQ. Exposure of unpolarized CD4$^+$ T cells to IL-12 or IL-4 resulted in a Th1 or Th2 phenotype, respectively. T cells stimulated in the absence of polarizing cytokines secreted modest amounts of IFN-γ and TNF-α. Cells stimulated in the continuous presence of 5 μM DMNQ, displayed a marked up-regulation in Th2 cytokines, including IL-4, IL-5, and IL-13, but not the Th1 cytokine IFN-γ. Th2 responses were blunted by concomitant exposure to thiol antioxidants. Long-term exposure of T cells to DMNQ resulted in growth of cells expressing CCR4, and a decrease in cells expressing CXCR3, indicating phenotypic conversion to Th2 cells. These results suggest that oxidative stress favors a Th2-polarizing condition. ***The Journal of Immunology,*** **2006, 176: 2765–2772.**

Upon activation, naive Th cells differentiate into at least two types of polarized responses (1, 2). Th1 cells secrete IFN-γ, TNF, and lymphotoxin (1, 3). They are associated with cell-mediated immunity and pathological autoimmune states characterized by organ-specific inflammation (4, 5). Th2 cells secrete IL-4, IL-5, and IL-13 (1, 2, 6). They are important for Ab-mediated immunity and resistance to parasitic infection, and are associated with pathologic states such as allergy and asthma (7–9).

There are a number of factors that influence the decision of naive CD4$^+$ T cells to differentiate into Th1 or Th2 effectors. The primary factor is the presence of key cytokines at the time of T cell activation (10). Cells exposed to IL-12, a product of APCs, produce IFN-γ and become Th1 cells, whereas T cells exposed to IL-4, a product of other CD4$^+$ T cells as well as mast cells, tend to become Th2 cells (1, 2, 10, 11). The process of T cell polarization involves both transcriptional regulation as well as remodeling of chromatin (12, 13). IL-12 signals through the IL-12R, activating STAT4 to induce IFN-γ, which then induces macrophages to produce more IL-12 (11). IFN-γ also induces the expression of the Th1-specific transcription factor, T-bet (14, 15). T-bet up-regulates IFN-γ expression creating a positive feedback loop favoring Th1 responses and attenuating Th2 responses through the active suppression of the *IL-4* gene (16, 17). In a similar manner, IL-4 activates STAT6 leading to the induction of GATA3, a zinc finger transcription factor essential for the expression of Th2-specific cytokines (18–21). GATA3 also represses IL-12 signaling, leading to an inhibition of Th1 responses (22).

In addition to cytokines, other factors influence the decision of T cells to become polarized to Th1 or Th2 phenotypes. Signals from costimulatory molecules such as CD28 or ICOS have been suggested to skew responses to Th2 types, in part through a cytokine-independent pathway to GATA3 expression (23–25). Signaling through the adhesion molecule LFA-1 favors Th1 responses (26–28). It has been suggested that APC modulation of ICAM-1, the LFA-1 ligand, could thus alter the Th1/Th2 balance in a given response.

Regulation of nonspecific intracellular activation pathways also influences Th cell differentiation, specifically with regard to the expression of the key Th2-specific cytokine IL-4. The promoter for IL-4 contains binding sites for NF-κB, NFAT family members, and STAT6 (2). Up-regulation of NF-κB activity through genetic manipulation leads to increased IL-4 production (29), whereas inhibition of NF-κB activation through the expression of a dominant-negative form of IκB or through chemical inhibition, suppresses IL-4 production (30, 31). Similarly, Th2 responses are reduced in genetically modified mice lacking NFAT1 and or NFAT2 as well as when NFAT activation is inhibited by cyclosporine or FK506 (32, 33).

Modulation of intracellular signaling pathways also occurs when cells are exposed to reactive oxygen species (ROS)[3] such as peroxide or superoxide. It is well documented that "oxidative stress" activates NF-κB, although the exact mechanism is unclear. Exposure of freshly isolated peripheral blood T cells to the antioxidant vitamin α tocopherol (vitamin E) results in a reduction in IL-4 production (34). Possible mechanisms for this effect include the inhibition of NF-κB binding to chromatin, or the activation of NF-κB in a protein kinase C-dependent manner (33). There is also epidemiological evidence linking allergic asthma to low intake of antioxidant vitamins, as well as evidence that high intake of vitamin E is associated with decreased serum levels of IgE (35, 36). ROS are produced at sites of inflammation by myelophagocytic cells as well as in response to exogenous factors such as aryl hydrocarbons contained in environmental tobacco smoke and diesel

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Received for publication July 1, 2005. Accepted for publication December 29, 2005.

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[1] This work was supported by National Institutes of Health Grant R01-AI50717 (to D.R.K.) and National Institutes of Health Grant R01-AR45293 (to L.S.D.).

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[3] Abbreviations used in this paper: ROS, reactive oxygen species; DHE, dihydroethidium; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone.

0022-1767/06/$02.00

exhaust particles. Therefore, oxidative stress and its modulation may be important influences on the development of chronic Th2 responses such as allergic asthma. This study was undertaken to investigate the role of chronic oxidative stress in the polarization of Th2 responses. We demonstrate that exposure of $CD4^+$ T cells to low levels of superoxide anion leads to up-regulation of the entire family of Th2-specific cytokines, as well as modulation of chemokine receptors associated with T cell polarization.

Materials and Methods

Cellular ATP measurement

This study was approved by the University of Texas Southwestern Institutional Review Board. Jurkat T lymphoma cells were incubated in the presence or absence of 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) for 16 h. Cells were tested for ATP release using the Bioluminescent Somatic Cell Assay kit (Sigma-Aldrich) according to the manufacturer's protocol. Briefly, 1×10^5 cells were incubated with ATP assay mix for 3 min at room temperature in a white 96-well plate. Somatic cell releasing agent was added to each well and light emission was measured using a plate luminometer. Purified $CD4^+$ T cells were activated as described below in the presence or absence of 5 μM DMNQ for 5 days and rested for 48 h. Cells were stimulated with PMA (10 ng/ml) and ionomycin (0.5 μM) for 5 h. ATP was measured as above.

Cellular ROS measurements

Freshly isolated $CD4^+$ T cells were suspended in Kreb's Ringer phosphate buffer (145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM glucose (pH 7.35)) and incubated in the presence or absence of 5 μM DMNQ. A portion of the cells was activated using soluble anti-CD3 (5 μg/ml) and anti-CD28 (0.5 μg/ml). Cells were incubated for 2 h at 37°C. Hydrogen peroxide production was detected using the Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Invitrogen Life Technologies). Supernatants were added to a mixture containing 50 μM Amplex Red and 0.1 U/ml HRP in a black-side, clear-bottom, 96-well plate. Fluorescence was measured immediately using a fluorescence plate reader.

Purified $CD4^+$ T cells were incubated in the presence or absence of 5 μM DMNQ for 24 h. The oxidative sensitive dye dihydroethidium (DHE; 10 μM final concentration) was added to the cultures 30 min before harvest. Incubation was terminated by the addition of a 10-fold dilution of ice-cold PBS, and the cells were washed before analysis. Live cells were gated based on forward and side scatter properties and analysis was performed using FlowJo software (Tree Star).

T cell stimulation

Mononuclear cells were prepared from the peripheral blood of healthy, nonatopic (by history) volunteers. $CD4^+$ T cells were isolated by immunomagnetic depletion of cells expressing CD8, CD19, CD14, CD16, and HLA-DR. The resulting population was >95% $CD3^+$ and $CD4^+$ (37). These cells were then stimulated using anti-CD3 mAb (UCHT1; R&D Systems) and anti-CD28 mAb (eBioscience) in the presence or absence of polarizing cytokines or DMNQ (38). Flat-bottom 24-well plates were coated with 5 μg/ml anti-CD3 mAb overnight at room temperature. After a washing to remove unbound anti-CD3 mAb, T cells were added along with soluble anti-CD28 mAb (0.5 μg/ml) and rIL-2 (10 U/ml) plus the following cytokine and oxidant combinations: human rIL-12 (R&D Systems; 12.5 ng/ml) and neutralizing anti-IL-4 (R&D Systems; 5 μg/ml), human rIL-4 (R&D Systems; 12.5 ng/ml), and neutralizing anti-IFN-γ (BioSource International; 5 μg/ml), or 5 μM DMNQ (Alexis Biochemicals). Cells were removed from activation signaling after 5 days and expanded in rIL-2-containing medium for 48 h (including DMNQ, if indicated). For immediate analysis, the cells were then restimulated with 10 ng/ml PMA and 0.5 μM ionomycin (38). To generate short-term T cell lines, the activation/resting cycle was repeated two times, with the final cycle not containing cytokines. The culture medium used in all experiments was RPMI 1640 supplemented with 10% normal human serum.

Analysis of cytokine production

Supernatants were harvested from T cell cultures at the end of the first and third activation and resting period. They were tested for presence of cytokines (IL-2, IFN-γ, TNF-α, IL-10, IL-4, and IL-5) using the Human TH1/TH2 Cytokine Bead Array kit (BD Biosciences) and were analyzed with a FACSCalibur four-color cytometer according to the manufacturer's instructions. Supernatants were tested for the presence of IL-13 using an IL-13 ELISA kit (BD Biosciences).

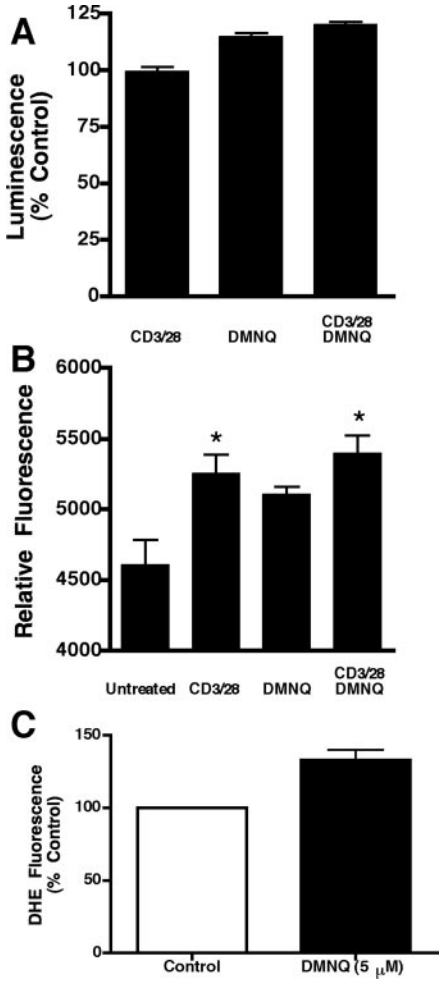

FIGURE 1. Effect of DMNQ exposure on T cell oxidative stress. *A*, $CD4^+$ peripheral blood T cells were stimulated with anti-CD3/28, exposed to 5 μM DMNQ for 7 days, or stimulated with anti-CD3/CD28 in addition to DMNQ exposure. Intracellular ATP was determined and compared with that in cells maintained in exogenous IL-2 alone. *B*, $CD4^+$ peripheral blood T cells were stimulated with anti-CD3/28, treated with DMNQ, or both for 2 h, and H_2O_2 production was determined spectrofluorimetrically. *, $p < 0.05$ compared with untreated Student's *t* test. *C*, $CD4^+$ peripheral blood T cells were treated with 5 μM DMNQ for 24 h, then stained with DHE and analyzed by flow cytometry. The mean fluorescence intensity of the DHE staining was normalized to control. Mean and SEM of three independent experiments.

RNase protection assay

Purified $CD4^+$ T cells were activated with anti-CD3 mAb and anti-CD28 mAb as described in the presence or absence of 5 μM DMNQ for 5 days and then rested for 48 h. Cells were restimulated with PMA (10 ng/ml) and ionomycin (0.5 μg/ml) for 24 h as indicated. Cells were harvested 1, 3, and 5 days after original activation and then after restimulation. Total RNA was extracted from cells using TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions and the RNeasy Mini kit (Qiagen). The RNA was suspended in DEPC-treated H_2O and quantified by spectrophotometry. Multiplex RNase protection assay was performed using reagents from BD Pharmingen according to the manufacturer's directions (39). The multiprobe template set hCK-1 consisting of IL-5, IL-4, IL-10, IL-15, IL-14, IL-9, IL-2, IL-13, IFN-γ, L32, and GAPDH was used to generate antisense riboprobes labeled with [^{32}P]UTP using the RiboQuant In Vitro Transcription kit. Five micrograms of sample RNA was hybridized for 16 h at 56°C with the ^{32}P-labeled riboprobes. The samples were treated with RNase A/T1 mixture and then with proteinase K. After extraction and

precipitation, the samples were run on a denaturing 4.75% polyacrylamide gel. The gel was transferred to filter paper, dried, and subjected to autoradiography.

Analysis of STAT phosphorylation

Purified $CD4^+$ T cells were activated in the presence or absence of 5 μM DMNQ. At the indicated time points, 2×10^6 cells were lysed in Nonidet P-40 buffer containing protease and phosphatase inhibitors. Fifteen micrograms of cytoplasmic protein were fractionated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore) as previously described (37). Blots were probed with anti-phospho-STAT6, anti-STAT4, anti-STAT6 (all from Santa Cruz Biotechnology), or anti-phospho-STAT4 (Zymed Laboratories) Abs followed by HRP-conjugated anti-rabbit IgG (Cell Signaling Technologies) and chemiluminescence detection. To normalize for protein content, the membranes were stripped using 0.2 M NaOH for 5 min at room temperature. Stripped membranes were then probed with HRP-conjugated anti-actin (Santa Cruz Biotechnology).

Results

Quinone-induced oxidative stress in T cells

The effect of low-level oxidative stress on T cell polarization was tested by exposing proliferating cells to the recycling quinone, DMNQ. In tissue culture, DMNQ generates superoxide anion and hydrogen peroxide (40, 41). At a concentration of 25 μM, DMNQ has been shown to be cytotoxic to respiratory epithelial cells. Preliminary experiments suggested that short-term exposure of T cells to DMNQ does cause a decrease in cellular ATP (data not shown). However, long-term exposure of peripheral blood T cells to DMNQ did not result in cell death. In fact, a small increase in cellular ATP was seen after 7 days in culture with 5 μM DMNQ (Fig. 1*A*). The immediate production of hydrogen peroxide in tissue culture was verified by measuring the oxidation of Resorufin (Amplex Red) in the presence of HRP. After 2 h of exposure to DMNQ, there was a 14% increase in extracellular H_2O_2 (Fig. 1*B*). Stimulation of the cells with anti-CD3 mAb and anti-CD28 mAb (anti-CD3/28 mAb) also led to an increase in peroxide production, but did not significantly augment the level seen with DMNQ alone. Finally, intracellular superoxide levels in $CD4^+$ T cells were measured by flow cytometry after staining the cells with DHE (Fig. 1*C*). Overall, there was an increase of 33 $\pm$ 7% in the level of superoxide in the DMNQ-treated cells compared with untreated cells ($p < 0.05$, Student's *t* test). Together, these data show that 5 μM DMNQ treatment exposes cultured cells to chronic, nontoxic generation of ROS.

DMNQ influences polarization of T cells

The typical method to generate Th1 or Th2 cells is to stimulate nonpolarized cells in the presence of IL-12 and anti-IL-4 for Th1 and IL-4 and anti-IFN-γ for Th2 cells, respectively (9–12). To study the effect of oxidative stress on T cell polarization, peripheral blood $CD4^+$ T cells were stimulated with a combination of anti-CD3/28 mAb without polarizing cytokines, but with 5 μM DMNQ present. Cytokine production in the cell supernatants was analyzed after restimulation with PMA and ionomycin. The results from five to six separate nonatopic donors are shown in Fig. 2. Although the production of IFN-γ, TNF-α, and IL-10 were not significantly altered by DMNQ treatment, the levels of type 2 cytokines, in particular IL-5 and IL-13, were significantly increased by exposure to low-level oxidative stress. A similar effect was seen in cultures of T cells polarized to either Th1 or Th2 phenotypes (Fig. 3). In cell cultures polarized to Th1 by the addition of IL-12 and anti-IL-4, the inclusion of DMNQ did not change the amount of IFN-γ produced, yet caused significant increases in IL-5 and IL-13. In cultures containing IL-4 and anti-IFN-γ, there was an augmented production of IL-4, IL-5, and IL-13, suggesting an ability of oxidative stress to amplify cytokine signals. The Th2 promoting effect was also seen in cultures containing low levels of xanthine oxidase, which also generates superoxide anion (Fig. 4, *A* and *B*). In addition, the effect of DMNQ could be reversed by anti-oxidants, as shown in Fig. 4*C*. α Lipoic acid (thioctic acid) is a cell permeable dithiol compound that causes increased intracellular glutathione content (42, 43). Inclusion of 25–50 μM lipoic acid in cultures containing DMNQ led to a decrease in IL-4, IL-5, and IL-13 production, while having little or no effect on IFN-γ

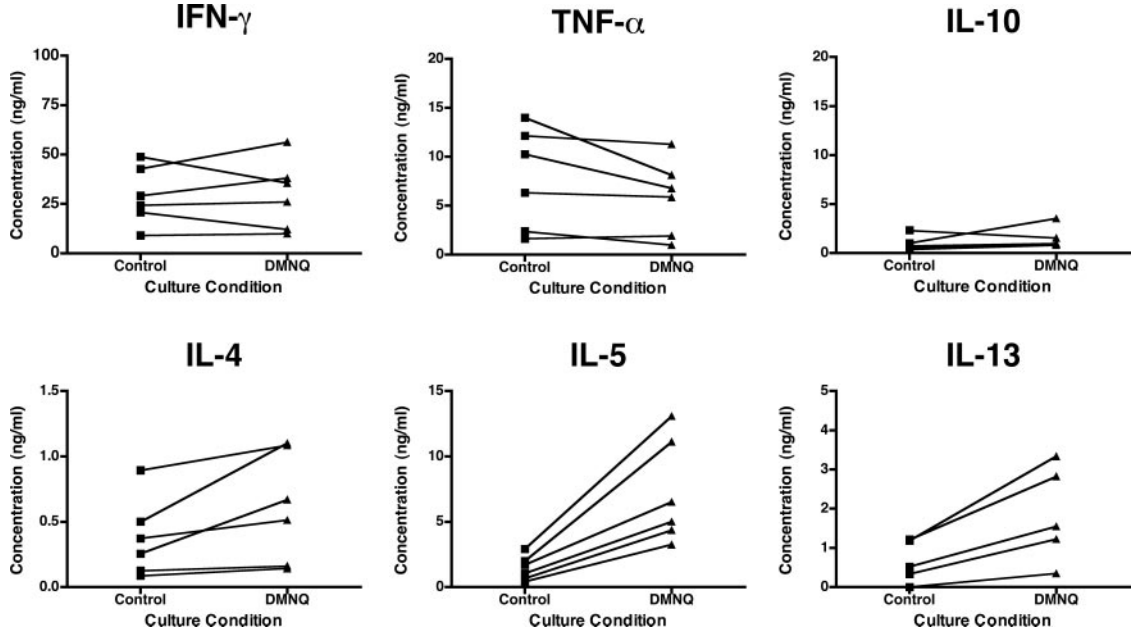

FIGURE 2. Effect of DMNQ on T cell polarization. $CD4^+$ peripheral blood T cells were stimulated with anti-CD3/28 in the absence or presence of 5 μM DMNQ for 5 days, then rested for 2 days and restimulated with PMA and ionomycin for 24 h. The secretion of cytokines into the medium was determined by flow cytometry and ELISA. The data shown indicate the results from five to six normal donors. Significance as determined by Student's *t* test for paired samples is shown.

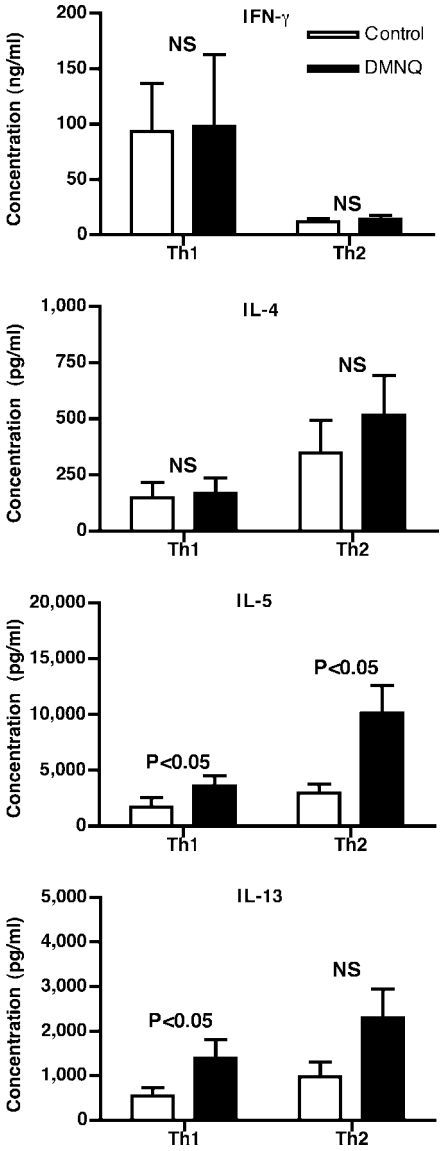

FIGURE 3. Effect of DMNQ on T cells polarized to Th1 or Th2 cytokine secretion. $CD4^+$ peripheral blood T cells were stimulated with anti-CD3/28 in the presence of polarizing cytokines and Abs to generate Th1 and Th2 cells. Five micromolar DMNQ was also present in the indicated cultures. Cytokine concentration in the medium was determined by flow cytometry and ELISA. Data represent the mean ± SEM from five separate determinations. Significance as determined by Student's *t* test for paired samples is shown.

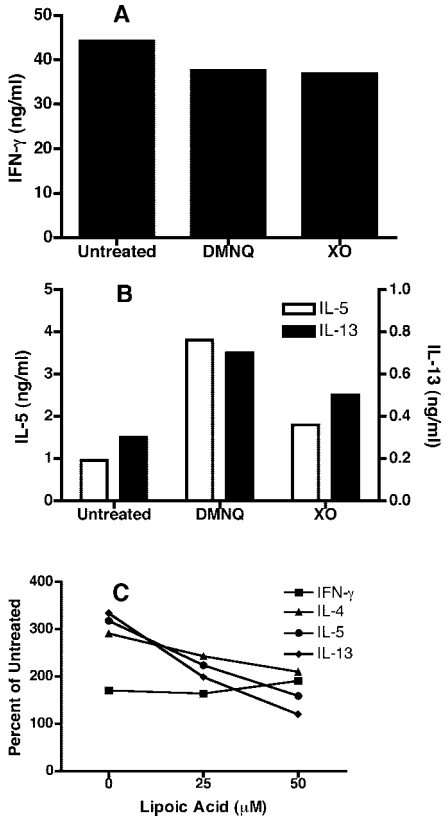

FIGURE 4. Effect of xanthine oxidase and lipoic acid on T cell cytokine production. *A* and *B*, T cells were stimulated with anti-CD3/28 alone, plus 5 μM DMNQ, or with 0.025 U/ml xanthine oxidase (XO) and 1 μM hypoxanthine for 5 days, rested for 2 days, then restimulated with PMA and ionomycin for 24 h. IFN-γ (*A*) and IL-5 (*B*) were determined by flow cytometry. IL-13 (*B*) was determined by ELISA. *C*, T cells were stimulated with anti-CD3/28 alone, plus 5 μM DMNQ, or with DMNQ and α lipoic acid. After 5 days, the cells were rested for 2 days and then restimulated with PMA and ionomycin for 24 h. Cytokine secretion into the medium was determined by flow cytometry. Data represent the percent increase in cytokine production for DMNQ or DMNQ/lipoic acid over control stimulation.

production. Together, these data show that the augmentation of cytokine production in these cultures is due to oxidative stress, and not an unrelated activity of DMNQ.

The effect of repeated T cell stimulation in the presence of low-level oxidative stress is seen in Fig. 5. T cell cultures were stimulated with anti-CD3/28 mAb in the presence of polarizing cytokines or DMNQ for a total of three successive cycles, then restimulated with PMA and ionomycin. This results in the generation of typical Th-polarized cell lines (38), although this stimulation protocol favors Th1 cells. Th1 cell lines produce high levels of IL-2 and IFN-γ but no IL-5 or IL-13 (Fig. 5*A*). Th2-polarized cell lines exhibit the expected, opposite, phenotype. The cell lines generated by repeated stimulation the presence of DMNQ also exhibited the Th2 phenotype producing high levels of IL-5 and IL-13, but little or no IL-2 or IFN-γ. This effect of DMNQ on chronic T cell polarization is also reflected by the percentage of cells that display a Th1 or Th2 phenotype (Fig. 5*B*). Intracellular cytokine staining of T cell lines generated by three weekly rounds of anti-CD3/28 mAb stimulation in the presence of IL-12 and anti-IL-4 demonstrated that >75% of the cells produced high levels of IFN-γ, whereas <1% produced IL-4. Th2-polarized cell lines had 16.7% IFN-γ- and 4.5% IL-4-producing cells, respectively, upon restimulation. Chronic exposure to DMNQ during the 3-wk cycle of stimulation and rest led to the generation of cell lines in which ~17% of cells produced IL-4. Half of these cells also expressed IFN-γ, making the total percentage of IFN-γ-producing cells in the DMNQ-treated cell lines ~25%. In unpolarized T cells, ~30% produce IFN-γ alone after repeated stimulation. Thus, the effect of DMNQ treatment is both to promote the differentiation of T cells to a Th2 phenotype as well as to suppress the generation of Th1 cells.

Chemokine receptor expression in DMNQ-treated T cells

It has been shown that differentiation of T cells into Th1 and Th2 phenotypes is accompanied by changes in certain cell surface markers, particularly chemokine receptors. CXCR3, the receptor for CXCL9 (Mig), CXCL10 (IP-10), and CXCL11 (I-TAC), is highly expressed on Th1 cells and down-regulated on Th2 cells

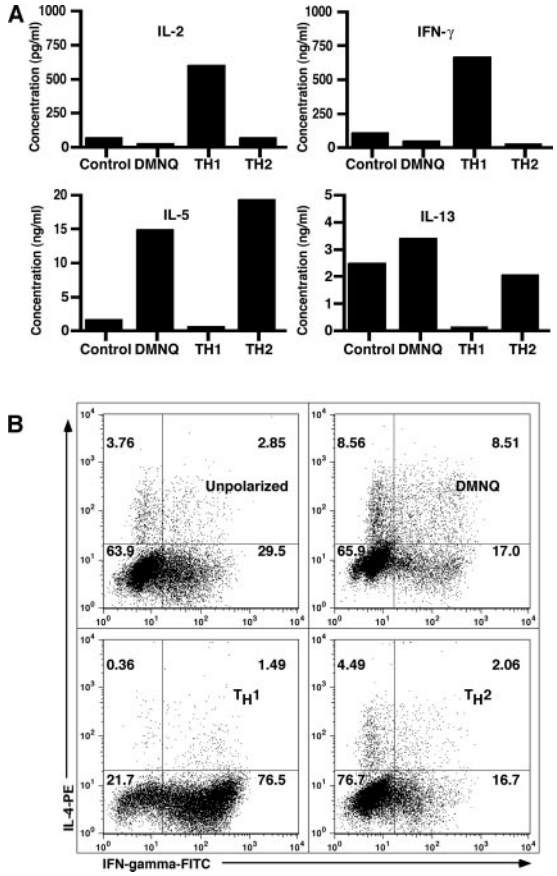

FIGURE 5. Generation of T cell lines in the presence of DMNQ. $CD4^+$ T cells were subjected to three rounds of stimulation with anti-CD3/28 alone, plus DMNQ, or in the presence of polarizing cytokines. Following the third week of stimulation, the cells were restimulated with PMA and ionomycin. Cytokine production in culture supernatants (*A*) was determined before PMA stimulation. Cytokine production by individual cells (*B*) was determined by flow cytometry. The percentage of cells in each quadrant is indicated.

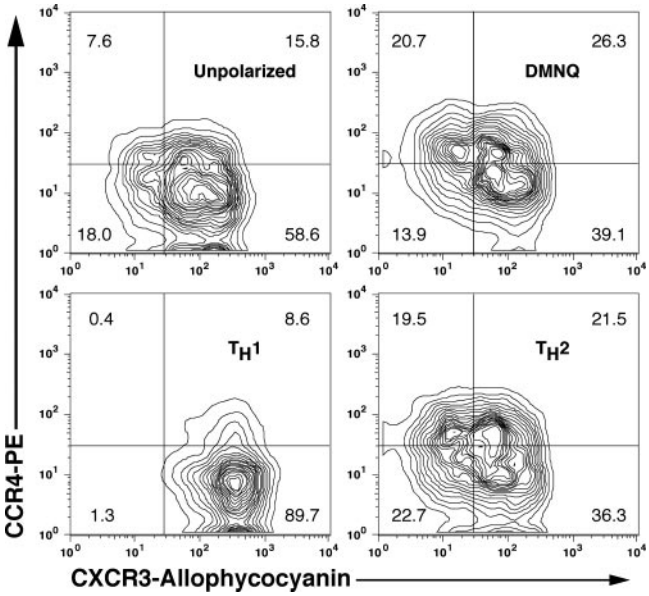

FIGURE 6. Expression of chemokine receptors on polarized T cells. $CD4^+$ T cell lines were generated by repeated anti-CD3/28 stimulation under nonpolarizing conditions, Th1-polarizing conditions, Th2-polarizing conditions, or in the presence of DMNQ. The expression of CXCR3 and CCR4 was determined by flow cytometry. The percentage of cells in each quadrant is given.

(44–46). Conversely, CCR4, the receptor for CCL17 (TARC), is not found on Th1 cells, but is seen on Th2 cells (44, 46–48). An example of this is shown in Fig. 6. T cells that were repeatedly stimulated with anti-CD3/28 mAb in the absence of exogenous cytokines expressed a moderate level of CXCR3. A subset of these cells (~16%) also expressed a low level of CCR4, whereas ~8% expressed CCR4 alone. Th1-polarized cells almost uniformly expressed CXCR3 alone, and at an increased level of surface density. Approximately 40% of Th2-polarized cells expressed CCR4, with half of these cells also expressing CXCR3, albeit at much reduced levels. The T cell lines generated in the continuous presence of DMNQ were quite similar to Th2 cells with regard to chemokine receptor phenotype. Approximately 20% of the cells expressed high levels of CCR4 alone, whereas ~25% expressed both CCR4 and moderate levels of CXCR3. Thus, the T cell differentiation program invoked by chronic oxidative stress extends beyond cytokine synthesis and includes additional functions such as chemokine receptor expression.

Effect of DMNQ on cytokine mRNA levels

A multiplex RNase protection assay was performed to determine whether the polarization of T cells in response to oxidative stress was reflected by cytokine mRNA levels. As shown in Fig. 7*A*, the major cytokine mRNA that is present after 1 day of anti-CD3/28 mAb stimulation is IL-2, regardless of whether the cells are exposed to DMNQ. However, at 3 and 5 days after stimulation, DMNQ exposure led to an increase in the level of mRNA for type 2 cytokines, particularly IL-5, IL-9, and IL-13. At these early time points, there is also an increase in IFN-γ mRNA in cells treated with DMNQ, although to a lesser extent. After a total of 7 days without or with DMNQ, the T cells were restimulated with PMA and ionomycin. Once again, there was an increase in mRNA abundance seen for IL-5, IL-9, and IL-13, with less increase seen for IL-2 and IFN-γ. The relative increase in mRNA expression is greatest for IL-4 and IL-5 as seen in Table I. The ability of DMNQ exposure to increase Th2 mRNA expression is mediated through IL-4. T cells that are stimulated in the presence of anti-IL-4 in addition to DMNQ do not have up-regulated type 2 cytokine mRNA (Fig. 7*B*). This suggests that the effect of oxidative stress is in the initial activation of the IL-4 gene during anti-CD3/28 mAb stimulation, and not a direct effect of DMNQ on later events such as GATA3 activation.

Effect of DMNQ on the phosphorylation of STAT6

The ability of cytokines to polarize T cell responses is mediated through a number of signaling events. The proximal signaling through cytokine receptors requires the participation of specific Janus kinases and associated STAT molecules. Specifically, STAT4 is associated with IL-12-driven polarization to a Th1 phenotype, whereas STAT6 is associated with an IL-4-driven Th2 phenotype (2). Phosphorylation of STAT4 and STAT6 was determined at different time points after anti-CD3/28 mAb stimulation in the absence or presence of DMNQ (Fig. 8*A*). In the presence of the quinone, there is increased and more persistent activation of STAT6 out to day 5 of stimulation. A similar, but smaller effect is seen on the phosphorylation of STAT4. The increased phosphorylation of STAT6 was abrogated by the inclusion of anti-IL-4 in the cultures (Fig. 8*B*). As seen with mRNA levels, these findings suggest that the effect of DMNQ is not to influence signaling

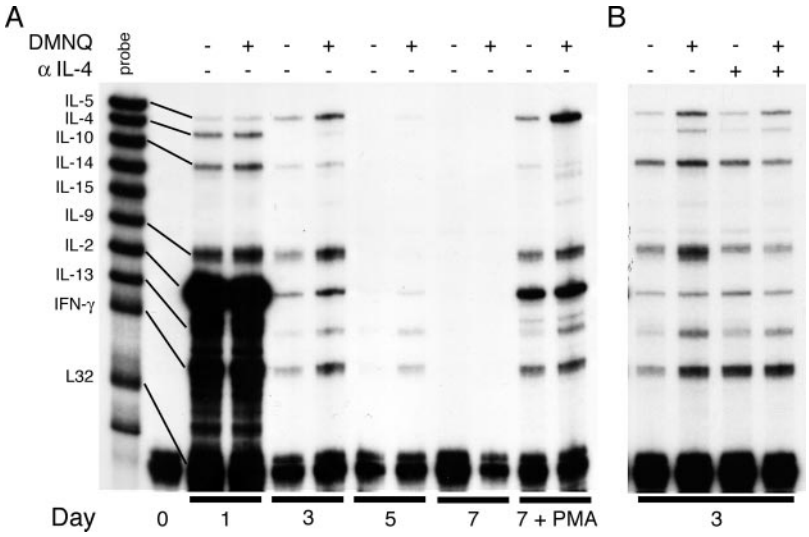

FIGURE 7. Cytokine mRNA production. *A*, CD4$^+$ T cells were stimulated with anti-CD3/28 in the absence or presence of 5 μM DMNQ for the indicated times. Cytoplasmic RNA was prepared from the cells, and the levels of cytokine mRNA were determined by multiplex RNase protection assay. *B*, Cytokine mRNA was quantified from cells stimulated with anti-CD3/28 in the absence or presence of DMNQ, with or without the inclusion of anti-IL-4. Blots are representative of three identical experiments.

events subsequent to the IL-4R (e.g., directly increasing the phosphorylation of STAT6), but to increase the amount of IL-4 that is produced initially in response to anti-CD3/28 mAb stimulation.

Discussion

The present study is the first to provide evidence that DMNQ directly induces oxidative stress that modulates T cell polarization in primary cultures of purified human CD4$^+$ T cells. Our previous studies on oxidative stress and the work of others have been conducted with cell lines (37, 49, 50). Th2 cytokine-producing cells could be generated by exposing cells to low levels of chronic oxidative stress induced by DMNQ. Effector cells differentiated in the presence of DMNQ generated nontoxic low levels of intracellular superoxide anions that modulated both cytokine production and the level of expression of chemokine receptors. T cell effectors exposed to DMNQ tended to produce increased levels of IL-4, IL-5, and IL-13, whereas little or no change was observed in levels of IFN-γ, TNF, or IL-10 in culture supernatants. Thus, these studies provide support for the hypothesis that environmental conditions can strongly influence T cell polarization.

Th2 cell differentiation is dependent on the presence of IL-4 during TCR stimulation (1, 2, 12). A neutralizing Ab to IL-4 prevented DMNQ-mediated STAT6 activation and Th2 polarization. Thus, these studies suggest that small amounts of biologically active IL-4 produced by the memory effector cells within the CD4 T cell population was sufficient to drive DMNQ-mediated expansion of Th2 cytokine-producing cells. Increased numbers of Th2 cytokine-producing cells were observed without a consistent effect on IFN-γ levels in short-term cultures. However, the development of IFN-γ producers was altered such that about half of the IFN-γ producers became IL-4 producers as well after successive rounds of stimulation. We have shown previously an inverse correlation between the strength of TCR triggering, provided by anti-CD3 and anti-CD28, and the differentiation of Th2 cells (38). Thus, the concentration of anti-CD3 mAb used in the current study is optimal for proliferation, yet preferentially drives IFN-γ-producing cells. This is consistent with previous studies demonstrating that Th2-dominated responses were observed under conditions of low doses of Ag or low avidity peptide/MHC class II complexes (51–53). Thus, in the absence of excessive quantities of IL-4 to down-regulate IFN-γ producers, DMNQ augmented Th2 cell numbers and also induced a population of double producers. These findings are consistent with previous studies demonstrating the flexible programming of early memory cells as opposed to late memory cells,

Table I. *Expression of cytokine mRNA*[a]

	Expt. 1		Expt. 2	
Cytokine	Day 3	Day 7 with PMA	Day 3	Day 7 with PMA
IL-4	3.53	4.66	2.80	3.31
IL-5	2.04	2.60	4.00	3.20
IL-9	1.43	1.30	2.46	1.73
IL-13	1.80	1.80	2.63	1.33
IFN-γ	1.68	1.36	2.85	1.40

[a] CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28 in the absence or presence of DMNQ. Cytokine mRNA was quantified by RNase protection assay as shown in Fig. 7. The autoradiographs were digitized. The amount of cytokine mRNA was first normalized to the expression of L32, then the ratio of mRNA in samples with DMNQ to the mRNA in samples without DMNQ was calculated. The results of two experiments are shown.

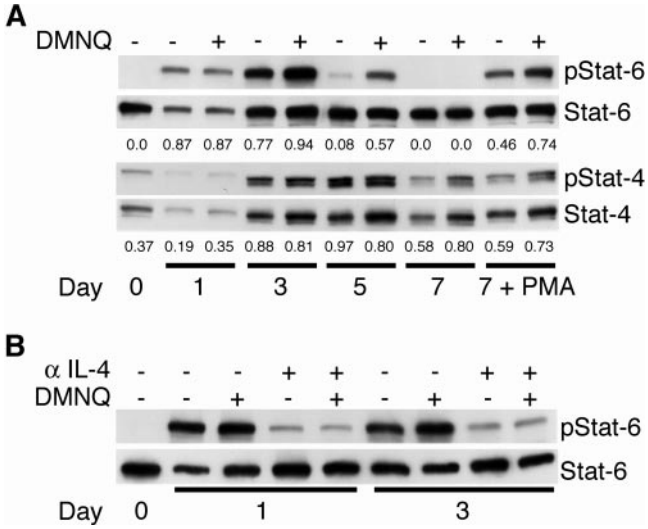

FIGURE 8. Phosphorylation of STAT4 and STAT6. *A*, CD4$^+$ T cells were stimulated as in Fig. 7. Cytoplasmic lysates were analyzed by Western blot using Abs to total or phosphorylated STAT4 and STAT6. Numbers beneath each lane indicate the relative density of the phosphorylated STAT to the total STAT. *B*, Cells were stimulated in the absence or presence of anti-IL-4 as indicated. Blots are representative of three identical experiments.

which appear to be more committed to a subtype and less influenced by the local microenvironment (54–56). The current work does not address the question of whether naive and memory T cells are equally affected by oxidative stress. For example, $CD4^+$ memory T cells can be polarized by CD28 stimulation alone in a PI3K- and MAPK-dependent manner (57). Oxidative stress may be acting in a similar manner by providing Ag-independent activation. Current work is aimed at understanding this and other potential mechanisms to explain our observation.

These studies might also more accurately reflect the microenvironment in vivo. For example, in airway inflammation studies it has been difficult to show that Th1 cytokines are down-regulated in the presence of increased Th2 cytokines (58, 59). Moreover, increased IFN-γ in serum and supernatants of cells from asthmatics have been associated with decreased lung function (60). Increased numbers of IFN-γ-producing cells have been found in the airways of both atopic pediatric and adult patients (61). In fact, several studies have shown that allergen-specific stimulation of peripheral blood T cells from atopic asthma patients induces increased Th1 and Th2 cytokine secretion (61–63). These studies suggest that the proinflammatory effects of IFN-γ might play a role in airway pathogenesis. It suggests that the hypothesis that the dysregulation of cytokines is the result of an imbalance in Th1 and Th2 cells might not account for the complexity of the T cell response in asthmatics. Importantly, such studies suggest that caution is indicated in contemplating treatments focused on increasing IFN-γ levels to reduce Th2 levels.

The effect of DMNQ exposure was to augment expression of mRNA for all type 2 cytokines, including IL-4, IL-5, IL-9, and IL-13. The genes for IL-4, IL-5, and IL-13 have been found to be closely linked on chromosome 5 in the human and chromosome 11 in the mouse (64). Often, the genes for these cytokines have been shown to be coordinately regulated, such that they are rapidly activated by Ag stimulation but transiently maintained without the presence of continued stimulation in the presence of polarizing cytokines (13). In the present studies, higher numbers of IL-4-producing cells were seen in the presence of DMNQ, but only a small increase in IL-4 cytokine production was observed in the supernatants and levels were more variable from donor to donor. However, consistent with the coordinate up-regulation of these cytokines, was the observation that the antioxidant, α lipoic acid, which increases intracellular glutathione, inhibited DMNQ-induced IL-4, IL-5, and IL-13 without a substantial impact on IFN-γ levels. These results were consistent with the assessment of cytokine mRNAs. mRNAs for IL-5 and other Th2-associated cytokines were consistently augmented in the presence of DMNQ. IL-4 mRNA was consistently enhanced at early time points, but somewhat variable expression was observed at later time points. These results reflect the total mRNA of all of the cultured T cells of which IL-4-producing T cells represent a minority. Nonetheless, the results suggest that Th2-associated cytokines and their mRNAs demonstrate persistent enhancement of expression in response to DMNQ.

Early in culture, STAT6 activation was potentiated in the presence of DMNQ and the enhancement of STAT6 activation was again observed upon restimulation. As previously noted, the effects of DMNQ were abrogated by the presence of a neutralizing anti-IL-4 mAb. These results are consistent with the central role of IL-4 in Th2 polarization and suggest that cytokine receptor-induced initiation of STAT6 activation was required for enhancement of IL-4 polarization by DMNQ. A role for oxidative stress in the activation and translocation of NF-κB has been established in several studies (29–31). Although oxidative stress might also influence other cofactors and transcription factors and up-regulate MAPK pathways, the most straightforward interpretation of the current results is that the coordinate up-regulation of STAT6 with increased NF-κB activity results in increased IL-4 production. Thus, a positive feedback loop is established by the interactions of STAT6, IL-4, and DMNQ. It is interesting to note that during peripheral expansion, T cells often become refractory to cytokines as a result of suppressor of cytokine signaling protein up-regulation as well as via additional mechanisms. The current studies suggest that over the course of proliferative expansion and differentiation the daughter cells do not become refractory to DMNQ or downstream effectors.

Finally, it is possible that DMNQ acts via a mechanism other than oxidative stress. Our data showing that lipoic acid reverses the effect of DMNQ argues against that. However, other heterocyclic compounds such as imiquod and resimiquod (R-848) have immunomodulatory effects due to their ability to signal through TLRs (65). TLR2 and TLR4 have both been associated with the induction of Th2 responses (66, 67), as have certain TLR9 ligands (68). We have tested DMNQ in sensitive assays of TLR7- and TLR9-dependent activation of plasmacytoid dendritic cells. We find no direct stimulation of these cells by DMNQ (D. R. Karp, unpublished observation).

These results demonstrate for the first time that DMNQ-induced oxidative stress modulates T cell polarization toward a Th2 phenotype in primary human $CD4^+$ T cells. Thus, oxidative stress could play a role in the development of the T cell repertoire in a number of diseases where oxidative stress or reduced cellular antioxidant capacity might potentially play a pivotal role including asthma and allergies as well as autoimmune diseases such as systemic lupus erythematosus, systemic sclerosis, and rheumatoid arthritis.

Disclosures

The authors have no financial conflict of interest.

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