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A number of lung diseases, including many interstitial lung diseases and HIV infection, are associated with decreases in intracellular thiols. Altered Th1/Th2 T cell balance has also been associated with disease progression in many of the same diseases. IFN-γ and IL-4 are critical effector cytokines of Th1 and Th2 cells, respectively. To determine the effect of thiols on the production of IFN-γ and IL-4 by splenocytes, cells were incubated in the presence and the absence of N-acetylcysteine (NAC) and stimulated with αCD3 or αCD3 and IL-12. Augmenting intracellular soluble thiol pools (~2-fold) with 15 mM NAC blocked induction of IFN-γ and increased production of IL-4 without causing significant changes in intracellular glutathione levels. The effect of NAC on IL-4 production was not linked to an increase in STAT6 phosphorylation, as STAT6 levels were decreased, nor did the increase in IL-4 occur with purified CD4 cells. We found that NAC increased splenocyte IL-4 production via an effect on APCs. We also found that NAC increased two IL-4 relevant transcription factors (AP-1) and NFATc. These studies suggest that increasing intracellular reduced thiol pools decreases IL-12 signaling and IFN-γ production, while increasing IL-4 production. The sum of these effects may contribute to alterations in the balance between Th1 and Th2 responses in lung diseases associated alterations in intracellular thiols. The Journal of Immunology, 2003, 171: S107–S115.
(free NAC and cysteine) without augmenting total GSH content. In stimulated splenocytes, NAC blocked the induction of IFN-γ by αCD3/IL-12. NAC blocked the activation of STAT4, consistent with the effect of NAC on IFN-γ production. In contrast, increasing intracellular thiol pools with NAC increased the production of IL-4 after anti-CD3. This was not consistent with an effect on STAT6 activation (which was inhibited), but was consistent with increased nuclear localization and DNA binding of AP-1 and NFATc2. Purified CD4+ T cells did not alter cytokine production with NAC, suggesting that the NAC-dependent effect was localized in the APC populations. We evaluated splenocytes depleted of CD11b cells and treated with an anti-B7-2 (CD86) and found a block in IL-4 production and in the NAC effect. The obvious candidate for increased IL-4 production is alteration of IL-12. We used an IL-12 knockout animal and found that the NAC-induced increase in IL-4 production was not dependent on IL-12 expression. This study suggests that increasing intracellular thiol pools (free NAC and cysteine) can alter the Th1/Th2 balance by increasing intracellular thiol pools with NAC.

Materials and Methods

Animals

Six- to 8-wk-old female BALB/c wild-type mice were purchased from Harlan (Indianapolis, IN). BALB/c IL-12p35−/− mice (C.129S1(B6)-Il12a tm1Jm) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free environment at the Animal Care Facility at University of Iowa, and maintained on standard mouse chow and water ad libitum. All procedures used in this study were in compliance with Animal Welfare Act regulations and the Guide for the Care and Use of Laboratory Animals.

Abs and reagents

Recombinant murine IL-2 was purchased from Roche (Indianapolis, IN) and recombinant murine cytokines IL-4, IL-12, and IFN-γ were purchased from R&D Systems (Minneapolis, MN). Low endotoxin and azide-free mAb against murine CD3 was purchased from BD Pharmingen (San Diego, CA) and an anti-FITC Multisort Kit, followed by 5% milk in TBST. Membranes were washed four times in TBST. Immunoreactive bands were developed using a chemiluminescent substrate (ECL, Plus or ECL; Amersham Pharmacia Biotech). An autoradiograph was obtained, with exposure times of 10 s to 2 min. Equal loading of the blots is shown by either protein-specific blots (STATs) or total protein stain (NFATc2).

Measurement of intracellular NAC and total intracellular thiol levels

Total intracellular soluble small m.w. thiols (GSH, cysteine, γ-glutamylcysteine) and intracellular NAC levels were assayed following previous published protocols (26–28). Cell pellets were homogenized in 50 mM potassium phosphate buffer (pH 7.8) containing 1.34 mM diethylthiocarbamoylamine-penta-acetic acid. NAC, cysteine, and γ-glutamylcysteine levels in cells were measured following derivatization with N-(1-pyrenyl)maleimide using a 15-cm C18 Reliasil column (Column Engineering, Ontario, Canada) coupled with HPLC with fluorescent detection (27). Total small m.w. soluble intracellular thiols were calculated as the sum of total GSH content determined spectrophotometrically (GSH and glutathione disulfide) plus cysteine and, where relevant, plus NAC, as determined by HPLC. No other soluble thiols reached detectable levels (100 fmol) using this system. The data are expressed as nanomoles of thiol per milligram of cellular protein as previously described (26–28).

Statistical analysis

Data were analyzed with PRISM software (GraphPad, San Diego, CA) using Student’s paired t test with p < 0.05 considered significant.

Results

Stimulation of naive splenocytes with soluble αCD3 or αCD3 plus IL-12 induces production of Th1/Th2 cytokines

Production of IL-4 and IFN-γ by soluble αCD3 or αCD3 and IL-12 was analyzed by ELISA. Naive splenocytes were isolated from BALB/c mice and stimulated with 2.5 μg/ml of soluble αCD3 or soluble αCD3 plus 1 μg/ml of IL-12. Each mode of cell activation resulted in a peak of cytokine synthesis at 48 h. After completion of the experiments, supernatants were collected, and IL-4 or IFN-γ was measured by ELISA. Soluble αCD3 alone stimulated significant production of IL-4 (Fig. 1). Addition of IL-12 as a stimulus slightly decreased the production of IL-4. To stimulate
significantly amounts of IFN-γ, we needed to stimulate the cells with both αCD3 and IL-12 (Fig. 1). Previous studies have shown that a strong T cell stimulus via soluble αCD3 requires coengagement of accessory and costimulatory molecules, while plate-bound αCD3 initiates signal transduction by effectively cross-linking the TCR (29). In our system IFN-γ was induced by immobilized αCD3 alone, but the IL-4 induction with immobilized αCD3 was significantly less (data not shown). Therefore, these studies use soluble αCD3 as a model for stimulation of a Th2 cytokine (IL-4) and soluble αCD3 plus IL-12 as a model for stimulation of a Th1 cytokine (IFN-γ).

NAC inhibits stimulation-induced IFN-γ production and enhances IL-4 production

To study the effects of increasing intracellular thiols on the production of Th1 vs Th2 cytokines, naïve splenocytes were treated with NAC. Briefly, freshly isolated splenocytes were either pre-treated with 15 mM NAC for 45 min or cultured in medium without NAC. After the preincubation period, splenocytes were treated with soluble αCD3 (2.5 μg/ml) or αCD3 plus IL-12 (1 ng/ml) for 48 h in the continued presence or absence of NAC. After completion of the experiments, the supernatants were evaluated for IL-4 and IFN-γ proteins. The effect of NAC on soluble thiol pools was confirmed by measuring intracellular free NAC, total GSH content, and cysteine in treated and untreated cells. Intracellular NAC levels increased from undetectable levels to an average of 4.87 ± 2.15 nmol/mg protein. Treatment with NAC increased cysteine levels from 3.5 nmol/mg protein in untreated cells to 14.1 nmol/mg protein in NAC-treated cells. Interestingly, NAC treatment did not alter total GSH levels, with untreated cells demonstrating 18 nmol/mg protein compared with 20 nmol/mg protein in NAC-treated cells. This suggests that the NAC-dependent alterations shown in this study are due to an effect on soluble thiol levels that do not include alterations in total GSH content. In Fig. 2A, we show that NAC treatment of either the IL-4 relevant stimuli (αCD3) or the IL-12 relevant stimuli (αCD3 plus IL-12) increased total intracellular small m.w. soluble thiols (∼2-fold). Fig. 2B demonstrates that NAC enhances IL-4 production in αCD3-treated splenocytes. This also occurred in the αCD3- plus IL-12-treated cells. In fact, the use of both stimuli with NAC overcame the slight decrease in IL-4 production seen with αCD3 plus IL-12 alone. In contrast, NAC inhibited αCD3- plus IL-12-induced IFN-γ production (Fig. 2C). These data suggest that altering intracellular soluble thiols has opposite effects on production of a Th2 cytokine vs a Th1 cytokine. Therefore, increased thiol levels during stimulation appeared to increase IL-4 and decrease IFN-γ.

APCs are required for the NAC effect on IL-4 and IFN-γ production

In the set of experiments shown in Fig. 3, we fractionated splenocyte populations and evaluated IL-4 and IFN-γ production. In Fig. 3A, CD4^+CD62L^+ T cells were isolated from whole splenocyte cell populations. Because these cells do not respond without a cofactor (provided by the APCs in the whole splenocyte cultures), they were stimulated with αCD3^+αCD28 instead of αCD3 or αCD3 and IL-12. We found that in naïve CD4^+ T cells NAC had no effect on the amounts of IL-4 and IFN-γ produced. In Fig. 3B, we examined the effect of depleting CD4^+ cells. Depletion of CD4^+ cells completely eliminated IL-4 production, while leaving significant IFN-γ production. Having found that purified CD4^+
FIGURE 3. APCs are required for the NAC effect on IL-4 and IFN-γ production. A. CD4+CD62L+ T cells were isolated from mixed splenocyte populations according to procedures detailed in Materials and Methods. Cells (0.5 million/ml) were cultured with and without NAC (15 mM) and with and without αCD3 and αCD28 for 72 h. IL-4 and IFN-γ levels were determined in culture supernatants by ELISA. B. Splenocytes were depleted of CD4+ cells, cultured at 2.5 million/ml with and without NAC (15 mM), and exposed to 2.5 μg/ml of soluble αCD3 or 2.5 μg/ml of soluble αCD3 and 1 ng/ml of IL-12 for 48 h. IL-4 and IFN-γ were analyzed as described above. C. Splenocytes were depleted of CD11b+ cells, cultured at 2.5 million/ml with and without NAC (15 mM), and exposed to 2.5 μg/ml of soluble αCD3 or 2.5 μg/ml of soluble αCD3 and 1 ng/ml of IL-12 for 48 h. IL-4 and IFN-γ were analyzed as described above. D. Naive splenocytes (2.5 million/ml) cultured in the presence and the absence of NAC (15 mM) and exposed to 2.5 μg/ml of soluble αCD3 (IL-4) or 2.5 μg/ml of soluble αCD3 and 1 ng/ml of IL-12 (IFN-γ) with the further addition in some groups of αCD86 (B7-2) for 48 h. IL-4 and IFN-γ were analyzed as described above.
cells did not respond to NAC, we next evaluated the effect of altering APCs in the mixed culture. Depletion of CD11b-expressing cells blocked IL-4 production and significantly decreased IFN-γ production (Fig. 3C). Confirming a role for APCs in the production of both IL-4 and IFN-γ in mixed splenocyte cultures, we found that addition of an Ab to B7-2 (CD86) inhibited both IL-4 and IFN-γ production, and addition of NAC did not override that block (Fig. 3D). As a composite, the data presented in Fig. 3 suggest that the effect of NAC on IL-4 and IFN-γ occurs at the level of the APCs present in the culture.

**NAC-induced increases in IL-4 production are not due to effects on IL-12**

Because of the demonstrated link between IL-12 and IL-4 inhibition (30, 31) and the observation that NAC breaks the IL-12 disulfide bonds resulting in a loss of the p40/p35 heterodimer (32), we wanted to evaluate the role of IL-12 in the NAC-induced increases in IL-4. Splenocyte cultures from IL-12−/− animals were incubated with our standard stimuli (αCD3 or αCD3 plus IL-12) and evaluated for IL-4 production. Fig. 4 demonstrates that alterations in IL-12 levels are not required for the increase in IL-4 production by NAC.

**NAC inhibits αCD3-mediated STAT4 activation**

To assess the effect of αCD3 plus IL-12 on STAT4 activation, naive splenocytes were stimulated with αCD3 (2.5 μg/ml) and IL-12 (1 ng/ml) for various time periods. Whole cell protein was isolated, and STAT4 activity was analyzed using a phospho-specific STAT4 Ab. Fig. 5A demonstrates that IL-12 activates STAT4. To evaluate the effect of NAC on STAT4 activation, freshly isolated splenocytes were stimulated with αCD3 plus IL-12 in the presence or the absence of NAC (15 mM) for different time periods. Whole cell proteins were isolated, and phosphorylated STAT4 was measured. Fig. 5B demonstrates that NAC partially inhibits IL-12-mediated STAT4 activation. This suggests that one-way increased thiol levels down-regulate IFN-γ production is via inhibition of αCD3- plus IL-12-induced STAT4 activation.

**NAC inhibits αCD3-mediated STAT6 activation**

Binding of IL-4 to its receptor induces activation of STAT6 and contributes to further IL-4 production (33). Some studies suggest that STAT6 can be activated with stimuli other than IL-4 (22, 34). To assess the role of αCD3 treatment on STAT6 activation, the following experiment was performed. Naive splenocytes were freshly isolated from BALB/c mice and treated with 2.5 μg/ml of αCD3 for various time periods. Whole cell proteins were then isolated, and STAT6 activation was evaluated using an Ab specific for the phosphorylated form of STAT6. These experiments show that STAT6 is phosphorylated in response to soluble αCD3 (Fig. 6A). To confirm that the induction of STAT6 activation was solely due to αCD3-mediated activation and was not due to the presence of IL-4, these experiments were repeated in the presence of IL-4-neutralizing Ab (10 μl/ml). IL-4-neutralizing Ab did not alter the αCD3-induced STAT6 phosphorylation (data not shown). These observations suggest that TCR ligation alone can induce STAT6 activation. Having found that αCD3-induced STAT6 activation, the effect of increased intracellular thiols on STAT6 activation was

![FIGURE 4](http://www.jimmunol.org/) NAC-induced increases in IL-4 production are not due to effects on IL-12. Splenocytes were isolated from BALB/c IL-12p35−/− mice, cultured at 2.5 million/ml, and exposed to 2.5 μg/ml of soluble αCD3 or 2.5 μg/ml of soluble αCD3 and 1 ng/ml of IL-12 for 48 h. IL-4 was analyzed as described above. The data are representative of two separate experiments.

![FIGURE 5](http://www.jimmunol.org/) STAT4 activation by αCD3 plus IL-12 is blocked by NAC pretreatment. A. Naive splenocytes (2.5 million/ml) were cultured in the presence of soluble αCD3 (2.5 μg/ml) and IL-12 (1 ng/ml) for different time periods (0, 5, 15, and 30 min and 1 and 2 h). Whole cell extracts were prepared and 20 μg of proteins were subjected to SDS-PAGE and Western blot analysis. The blots were analyzed for activated STAT4 using a phospho-specific STAT4 Ab. Ab concentrations of 1/1,000 (primary) and 1/10,000 (secondary) were used. Immunoreactive bands were visualized using chemiluminescence and exposure times of 30 s to 5 min. Equal loading of gels is demonstrated by stripping and reprobing blots for total STAT4. B. Naive splenocytes were cultured as described above in the presence and the absence of NAC (15 mM). Whole cell extracts were analyzed for phosphorylated STAT4 as described above. The figure is representative of at least four independent experiments. The graph depicts densitometry performed on immunoreactive bands and expressed as the fold increase (experimental value/control value) in arbitrary units.
evaluated next. Naïve splenocytes were treated with and without NAC, followed by αCD3 (2.5 μg/ml) for various time periods. Whole cell proteins were isolated, and STAT6 activity was assessed using a phosphorylation-specific STAT6 Ab. Ab concentrations of 1/1,000 (primary) and 1/10,000 (secondary) were used. Immunoreactive bands were visualized using chemiluminescence and exposure times of 30 s to 5 min. Equal loading of gels was demonstrated using with Ponceau S total protein stain. B, Naïve splenocytes were cultured as described above in the presence and the absence of NAC (15 mM). Whole cell extracts were analyzed for phosphorylated STAT6 as described above. The figure is representative of at least four independent experiments. The graph depicts densitometry performed on immunoreactive bands and expressed as the fold increase (experimental value/control value) in arbitrary units.

**FIGURE 7.** NAC increases DNA binding of AP-1 and nuclear localization of NFATc2. A, AP-1 activation in splenocytes (2.5 million/ml) after treatment with NAC (15 mM) and anti-CD3 (2.5 μg/ml) for different time points. Nuclear extracts were isolated, and 5 μg of protein was mixed with 32P-labeled AP-1-specific oligonucleotides and separated on a 10% polyacrylamide gel. Arrowheads indicate the positions of the specific DNA protein complexes. The gel shift is a representative of three independent experiments. B, NFATc2 nuclear localization in presence and the absence of NAC (15 mM). Naïve splenocytes were exposed to 2.5 μg/ml of soluble anti-CD3 for different time periods or were left untreated. Five micrograms of nuclear proteins were subjected to SDS-PAGE, and standard Western analysis was performed. The blots were analyzed for NFATc2 using Ab against total NFATc2. A primary Ab concentration of 1/500 and a secondary Ab of 1/5000 were used. Immunoreactive bands were visualized using chemiluminescence. Equal loading of gels was demonstrated using with Ponceau S total protein stain. The gel is a representative of six independent experiments.
AP-1 levels greater than αCD3 alone. These results are in agreement with previous observations that antioxidants enhance AP-1 binding (35). The NAC effect did not require αCD3, suggesting that NAC provides a ready pool of AP-1 to cooperate with other stimuli-specific effects. NFATc2 has been directly linked to IL-4 production in a recent study by Rengarajan et al. (36). The effect of NAC on NFATc2 was evaluated by treating naive splenocytes with αCD3 with and without NAC for various time points, followed by isolation of nuclear protein. Fig. 7B demonstrates that soluble αCD3 triggers an early activation of NFATc2 and that this activation is enhanced in the presence of NAC. In comparison with the effects of NAC alone on αCD3 binding, NAC alone had no effect on NFATc2 nuclear localization (data not shown). Although αCD3 increased NFkB, there was no effect of NAC on NFkB DNA binding (data not shown). These data suggest that increasing intracellular small m.w. soluble thiols activates at least two of the transcription factors (AP-1 and NFATc2) involved in IL-4 transcription, independent of increased levels of total GSH.

Discussion

In this study the effects of NAC on induction of a marker Th1 cytokine (IFN-γ) and a marker Th2 cytokine (IL-4) were evaluated. We found that increasing intracellular soluble thiols with NAC significantly inhibited IFN-γ production in splenocytes following treatment with αCD3 plus IL-12. In contrast, NAC significantly increased IL-4 production by αCD3 (Fig. 8). Both of these events (altered cytokine production) occurred in the setting of increased thiols (NAC and cysteine) with no changes in total GSH content. The effects of NAC on IL-4 production required APCs and did not occur if purified CD4+CD62L− T cells were stimulated. IL-12 modulation (including a possible extracellular break in the IL-12 disulfide bonds) was not responsible for the effect of NAC on IL-4 production because it occurred in cells from an IL-12 knockout animal. Other possible mechanisms for the alterations in cytokine production include changes in signaling pathways and altered expression or activation of transcription factors. Consistent with an effect of NAC on IL-12 signaling, a decrease in STAT4 activation in the presence of NAC was detected. STAT6 can play a role in IL-4 production in the presence of NAC. In contrast to the coordinated response to NAC of STAT4 and IFN-γ production, NAC increased IL-4 production and inhibited STAT6 activation. Because our results indicate that the NAC-induced increases in IL-4 are independent of STAT6, other transcription factors relevant to IL-4 expression were evaluated. Both AP-1 and NFATc2 have been positively linked to IL-4 production (16, 36, 37). When we evaluated the effect of NAC on AP-1 DNA binding and NFATc2 nuclear translocation, we found that NAC caused a significant increase in the activation of both transcription factors. These data suggest that NAC promotes IL-4 synthesis in a STAT6-independent manner, potentially via an effect on other transcription factors, and that NAC decreases IFN-γ production via inhibition of IL-12-mediated STAT4 activation. These observations are consistent with our hypothesis that an increase in intracellular soluble thiols affects the Th1/Th2 balance in favor of Th2 expression, rather than Th1 expression. The effects of NAC appear to be independent of changes in total GSH content.

A number of studies have indicated that cellular redox status plays a critical role in signal transduction in multiple physiologic and pathophysiologic conditions. Our data suggest that the altered cytokine effects in our mixed cultures are due to an effect on APCs in the cell population. During the inflammatory response, APCs are exposed to increased levels of intra- and extracellular ROS. This initial increased level of ROS has an important effect on the subsequent gene expression. ROS may modulate critical molecules of APC signaling pathways via redox regulation of sensitive (sulf-hydryl groups) signaling molecules (38). GSH is a major intracellular redox buffer and plays a substantial role in protecting cells against oxidative injury. Cysteine (Cys) is a rate-limiting precursor for GSH synthesis, but also has thiol antioxidant activity in its own right. Low m.w. thiols such as 2-ME and NAC have been shown to increase resistance to oxidative stress-mediated apoptosis, increase synthesis of IL-2, and enhance proliferation (39). In this study the demonstrated effects of NAC treatment are due to alterations in intracellular NAC and cysteine levels and not to increases in total GSH. We have not yet determined the mechanism behind the APC-dependent alterations produced by NAC. We have shown that the effect of NAC is not due to an effect on IL-12 (either production in the case of αCD3 alone or dissolution of the disulfide bonds in the systems that include added IL-12). It will be of interest to define the NAC-dependent APC changes that lead to an increased production of IL-4 by CD4+ cells.

Some studies in other systems have found opposite results from our data. P. Jeannin (unpublished observations) has shown in human T cells that NAC increases IL-2 expression, decreases IL-4 expression, and has no effect on IFN-γ expression. In contrast to their study we found increased IL-4 production in NAC-treated cells. The opposite effects seen in the two systems are possibly explained by the fact that the as yet undetermined APC effect in our system seems to override T cell-specific responses.

STAT proteins play a fundamental role in relaying intracellular signals elicited by cytokines (20). STAT4 is crucial for Th1 responses, and IL-12 and IL-18 have been shown to play a central role in cell-mediated immunity via activation of STAT4 (40). STAT4-deficient mice have impaired responses to IL-12 and lack Th1 differentiation (20). STAT4 activation has been shown to contribute to IFN-γ production (40). Our data show that αCD3 alone did not activate STAT4; however, in the presence of IL-12, αCD3 activated STAT4, and this was associated with a 9-fold greater induction of IFN-γ. Our results demonstrate that altering the intracellular soluble thiols decreases both STAT4 activation and IFN-γ production, suggesting that these pathways are under redox regulation. Our data do not address the issue of which cell population is the source of the altered STATs, although we believe that it is probably the T cells.

STAT6 is activated upon exposure of T cells to IL-4 and IL-13 (14, 15). However, there are some reports suggesting that STAT6 can be activated by stimuli other than IL-4 and IL-13, including costimulation by CD28 and IL-2 (22). Although a direct role for STAT6 DNA binding in the IL-4 promoter has been postulated, several studies clearly demonstrate STAT6-independent IL-4 production (21, 41). These data suggest that STAT6 activation is not
essential for IL-4 transcription, which is consistent with our data showing a divergent effect of NAC on STAT4 activation and IL-4 production. In our studies αCD3 stimulation of naive splenocytes activated STAT6. This was inhibited by NAC, and the inhibition did not decrease IL-4 production. IL-4 production, in fact, was increased by NAC pretreatment.

AP-1 is a transcription factor that positively regulates several cytokine genes, including IL-4. Several studies have indicated that cellular redox status affects AP-1 activation (42–45). Further, DNA binding of the AP-1 complex requires that specific cysteine residues become reduced. In our study we showed that NAC strongly enhanced AP-1 DNA binding. The effect of NAC on AP-1 DNA binding is in agreement with previous studies and may explain in part the up-regulation of IL-4 by NAC. The calcium-regulated NFATc2 protein is located in the cytoplasm in resting cells. After Ag stimulation, NFATc2 is dephosphorylated by the phosphatase calcineurin and rapidly translocated into the nucleus, where it interacts with other transcription factors to induce cytokine production (46). This was inhibited by NAC, and the inhibition may explain in part the enhanced expression of IL-4 by NAC. The calcium-regulated NFATc2 protein is located in the cytoplasm in resting cells. After Ag stimulation, NFATc2 is dephosphorylated by the phosphatase calcineurin and rapidly translocated into the nucleus, where it interacts with other transcription factors to induce cytokine production (46). This was inhibited by NAC, and the inhibition may explain in part the enhanced expression of IL-4 by NAC. We thank Susan A. Walsh for expert technical assistance with the measurement of cellular thiols.

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References


