T cell surface redox levels determine T cell reactivity and arthritis susceptibility

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Rats and mice with a lower capacity to produce reactive oxygen species (ROS) because of allelic polymorphisms in the *Ncf1* gene (which encodes neutrophil cytosolic factor 1) are more susceptible to develop severe arthritis. These data suggest that ROS are involved in regulating the immune response. We now show that the lower capacity to produce ROS is associated with an increased number of reduced thiol groups (−SH) on T cell membrane surfaces. Artificially increasing the number of reduced thiols on T cells from animals with arthritis-protective *Ncf1* alleles by glutathione treatment lowered the threshold for T cell reactivity and enhanced proliferative responses *in vitro* and *in vivo*. Importantly, T cells from immunized congenic rats with an E3-derived Ncf1 allele (DA.*Ncf1^E3* rats) that cannot transfer arthritis to rats with an arthritis-associated Dark Agouti (DA)-derived mutated Ncf1 allele (DA.*Ncf1^DA* rats) became arthritogenic after increasing cell surface thiol levels. This finding was confirmed by the reverse experiment, in which oxidized T cells from DA.*Ncf1^DA* rats induced less severe arthritis compared with controls. Therefore, we conclude that ROS production as controlled by *Ncf1* is important in regulating surface redox levels of T cells and thereby suppresses autoreactivity and arthritis development.

Ncf1 | reactive oxidative species | p47 phox | NADPH | glutathione

Reactive oxygen species (ROS) are generally thought to be harmful and to play a disease-enhancing role in autoimmune diseases such as arthritis (1, 2). However, we have found that a decreased capacity to produce ROS because of polymorphisms in *Ncf1* increases susceptibility for autoimmunity and arthritis (3, 4). *Ncf1* encodes neutrophil cytosolic factor 1 (Ncf1, also known as p47phox), the activating protein in the NADPH oxidase complex that produces ROS upon activation. In the rat, a SNP in the *Ncf1* gene was identified by positional cloning to be one of the strongest genes in regulating both oxidative burst and arthritis (3). In the mouse, a spontaneous mutation was identified that affects splicing and results in expression of truncated, less functional Ncf1 protein (5), which also resulted in increased arthritis and autoimmunity (4). Hence, it was clear that the *Ncf1* gene that controls oxidative burst also controlled the autoimmune response and severity of arthritis in both rats and mice.

It has been shown that arthritis as induced by immunization with pristane in rats and collagen in mice expressing polymorphic *Ncf1* is T cell dependent. In the rat model, only T cells from DA.*Ncf1^DA* rats [Dark Agouti (DA) rats with the mutated *Ncf1^DA* allele from the DA rat] can transfer disease to naïve DA.*Ncf1^DA* recipients, whereas T cells from the congenic DA.*Ncf1^E3* rats (DA rats with the WT Ncf1^E3 allele from the E3 rat) cannot (3, 6). In the mouse model, a mutation in *Ncf1* results in an increased delayed-type hypersensitivity response and serum levels of anti-collagen type II (CII) IgG antibodies (4), indicating enhanced activation of autoreactive T cells. Thus, Ncf1 somehow influences autoreactive T cells during immune priming to become arthritogenic, either by means of cell–cell contact or by means of the cellular milieu.

To function properly, cells need to maintain an adequate redox balance (7). Recent work indicates a role for reduced cell surface thiols (−SH) as targets of redox regulation in the immune system

(8). Such redox-sensitive moieties at the cell surface mostly are in an oxidized state, probably because they are exposed to the oxidizing extracellular environment (9). Changes in redox balance of extracellular proteins can result in modified receptor activation or in modification of proteins that act as redox sensors (10, 11). A decrease in NADPH oxidase function, resulting in decreased ROS production, might interfere with cell surface redox levels of immune cells (12), resulting in defective immune regulation at a certain time point during the immune response.

T cell function is markedly influenced by alterations in the redox balance. It is known that lymphocytes require a reducing milieu for optimal proliferation and activation (13, 14). Exposure to ROS has been demonstrated to down-regulate T cell activity (14, 15), and a decrease in intracellular redox levels impairs T cell function (16). Hence, our aim was to determine whether a decreased capacity to produce ROS affects the redox balance of T cells and thereby affects the downstream effector mechanisms associated with the identified *Ncf1* polymorphism, controlling arthritis severity.

Results

T Cells from *Ncf1* Mutated Rats Have Higher Levels of Reduced Cell Surface Thiols. To explain how oxidative burst influences T cell activation, we investigated whether the redox balance in T cells is disturbed in animals with a decreased NADPH oxidase function. It has been observed that ROS production by the NADPH oxidase complex and the redox balance are linked (12). First, we tested the ability of T cells to exert oxidative burst compared with granulocytes. Peripheral blood from DA.*Ncf1^DA* and DA.*Ncf1^E3* rats was stained for intracellular ROS with DHR123 (dihydrorhodamine 123) and cell-specific markers with or without stimulation by phorbol 12-myristate 13-acetate (PMA). T cells did not burst upon PMA stimulation, in contrast to granulocytes (Fig. 1a). When staining for Ncf1 expression, a clear difference was observed between the strains when looking at neutrophils, in contrast to T cells, where no Ncf1 staining was detected as compared with the control (in which an irrelevant first antibody was used) (Fig. 1b).

Next, we investigated the cell surface and intracellular redox levels of T cells in the different strains. Cell surface redox levels were determined by flow cytometry after staining the cells for reduced thiol (−SH) groups and cell-specific markers (16). T cells in blood from *Ncf1* mutated DA.*Ncf1^DA* rats were shown to have a higher number of cell surface thiols compared with those from Ncf1 WT DA.*Ncf1^E3* rats (Fig. 1c). Higher levels of T cell surface thiols
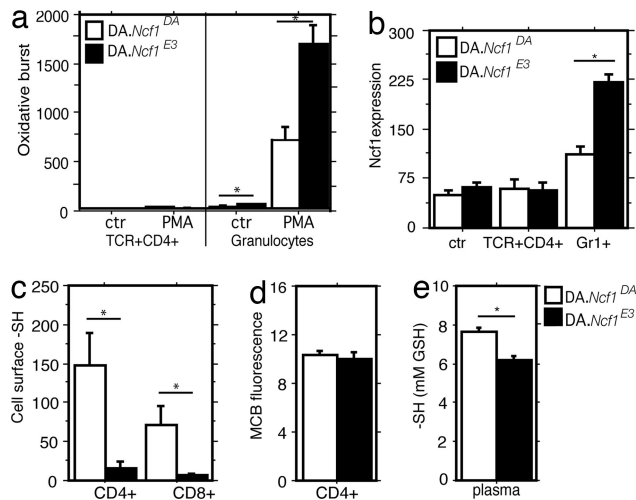
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Fig. 1. *Ncf1* mutated rats and mice have increased numbers of cell surface thiol groups. (*a*) T cells do not exert oxidative burst upon PMA stimulation, in contrast to granulocytes (Gr-1$^+$ cells). DHR123 staining in blood TCR$^+$CD4$^+$ cells and granulocytes was determined by flow cytometry after stimulation with PMA or without stimulation. (*b*) Ncf1 expression was determined by flow cytometry and expressed in geomean. (*c*) The relative number of cell surface thiol (−SH) groups was determined by Alexa Fluor 633-maleimide staining and expressed in geomean. Rats with a polymorphism in *Ncf1* and lower ROS production (DA.*Ncf1^{DA}*) have higher numbers of cell surface thiol groups compared with congenic rats expressing WT *Ncf1* (DA.*Ncf1^{E3}*). (*d*) Intracellular thiol levels were determined by staining with MCB, detected by FACS, and expressed in geomean. No differences in intracellular GSH levels were observed. (*e*) Plasma thiol groups were determined with 5,5′-dithiobis(2-nitrobenzoic acid) and expressed in relative numbers compared with a standard of GSH. Plasma of mutated rats contained more reduced −SH groups. Means ± SEM are shown. The number of animals per group ranged from three to five per experiment; at least three experiments were performed. ∗, *P* < 0.05.

in DA.*Ncf1^{DA}* rats were also observed in spleen and inguinal lymph node (LN) T cells (not shown). Other cell types showed tendencies to have increased amounts of cell surface thiols in *Ncf1* mutated animals (macrophages, neutrophils, and B cells), although these differences were not significant. Intracellular thiol levels of T cells were determined by flow cytometry with monochlorobimane (MCB), reacting with intracellular reduced glutathione (GSH) (17). No difference in intracellular thiol levels between the strains was detected in T cells (Fig. 1*d*). In contrast, the extracellular milieu was shown to be genetically controlled. Plasma of DA.*Ncf1^{DA}* rats was shown to contain more reduced thiol groups on plasma proteins as compared with DA.*Ncf1^{E3}* rats (Fig. 1*e*) as determined with an assay using 5,5′-dithiobis(2-nitrobenzoic acid) (9, 13). Similar data were obtained for the Ncf1 mutated mice (see Fig. 7, which is published as supporting information on the PNAS web site).

The Number of Cell Surface Thiols Can Be Artificially Increased. To study the effect of higher numbers of cell surface thiols on T cell function, we wanted to change this number artificially. Both GSH and *N*-acetylcysteine (NAC) have been found to increase the number of cell surface −SH groups on human cells (9). Heparinized rat blood was treated with 4 mM GSH or 4 mM NAC (16) and stained for cell-specific markers and reduced surface thiols (−SH). No hemolysis was performed, because that influenced the staining of surface −SH. Both GSH and NAC increased the number of T cell surface thiols (Fig. 2 *a* and *b*). GSH did not influence intracellular redox levels, whereas NAC increased the number of intracellular thiols (Fig. 2*c*) (9). Because we were interested in studying the effect of increasing only the cell surface thiols, GSH was used throughout the remaining experiments. The concentration that we used (4 mM) resulted in an increase in cell surface thiol
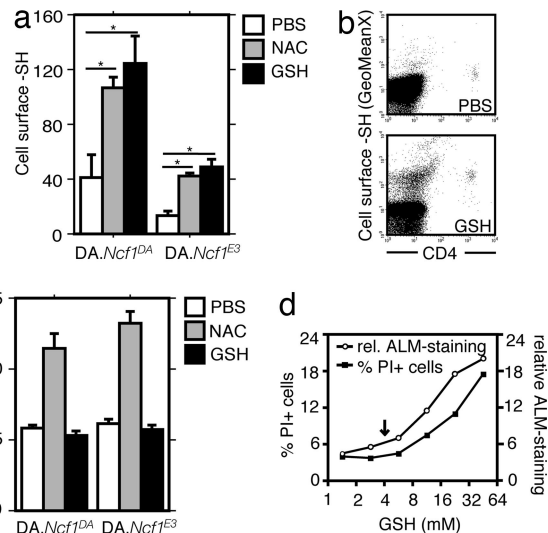

Fig. 2. The number of cell surface thiols can be increased by GSH. (*a* and *b*) Treatment with GSH or NAC increases TCR$^+$CD4$^+$ T cell surface thiol levels in rats with decreased (DA.*Ncf1^{DA}*) and normal (DA.*Ncf1^{E3}*) ROS production. Number of cell surface −SH groups is expressed in geomean. (*c*) Treatment with GSH does not affect intracellular thiol levels, in contrast to NAC, as determined by staining with MCB. Means ± SEM are shown. Four animals per group were used per experiment; at least three experiments were performed. ∗, *P* < 0.05. (*d*) GSH treatment does not affect viability of blood cells at the concentration of 4 mM as determined by titration of GSH and double staining with Alexa Fluor 633-maleimide and PI. A representative experiment is shown.

levels that was comparable to the difference present between DA.*Ncf1^{DA}* and DA.*Ncf1^{E3}* T cells. We have tested and found GSH not to be toxic to the cells at this concentration; only at a concentration >16 mM did the percentage of dead [propidium iodide (PI) positive] cells increase (Fig. 2*d*). Similar data were obtained for mice (not shown).

Increasing the Number of Cell Surface −SH Groups on T Cells Increases Activation and Proliferation. Next, we investigated whether the higher number of cell surface thiols on T cells influences T cell activation and proliferation. We used an established system with mouse hybridoma T cells and spleen-derived antigen-presenting cells (APCs). HCQ10 T hybridoma cells (18), which recognize the immunodominant CII peptide bound to the class II molecule H2-A^q, or APCs were treated with PBS or with 4 mM GSH to increase the number of cell surface −SH groups. Higher numbers of cell surface thiols on T cells were shown to increase IL-2 production, whereas a higher number of thiol groups on APCs did not (Fig. 3*a*). In the absence of CII, no differences in IL-2 production were observed between GSH- and PBS-treated conditions. These data indicate that the number of cell surface thiols on T cells determines their proliferative response *in vitro*.

To investigate the role of T cell surface thiols *in vivo*, we used the rat model, because it was shown before that T cells from DA rats could transfer pristane-induced arthritis (3). Spleen cells and LN CD4$^+$ T cells were isolated from immunized DA.*Ncf1^{E3}* rats. Cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and treated with 4 mM GSH to obtain similar numbers of cell surface thiol groups as T cells from DA.*Ncf1^{DA}* rats. The rats were killed 12 and 36 h after i.v. injection; significant numbers of CFSE$^+$ cells were found in mediastinal LNs (draining the injection site) and spleen but not in blood, inguinal LNs (draining the joints), bone marrow, or thymus. It was shown that, at both time points, in spleen and draining LNs, the intensity of CFSE staining of both CFSE$^+$/PI$^-$ (living) cells and CFSE$^+$/T cell antigen receptor (TCR)$^+$ cells was lower when GSH-treated cells were injected than
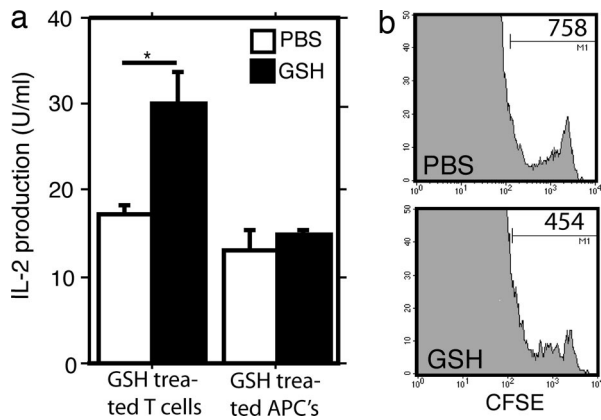
Fig. 3. T cells with increased numbers of surface thiols are more activated. (*a*) *In vitro* T cell activation after increasing the number of cell surface thiols was studied in a mouse model with hybridoma T cells recognizing CII. When cell surface thiol numbers on T cells were increased by GSH (GSH-treated T cells), T cells produced more IL-2, which depended on T cell APC interaction. This was not the case if APCs were treated with GSH (GSH-treated APCs). Experimental values minus control values (T cells and APCs without antigen) are shown. (*b*) These data were confirmed *in vivo* in the rat: GSH treatment results in increased T cell proliferation *in vivo*; the geomean of CFSE staining of $CD4^+$ and PI^- (living) cells was 758 in the rats receiving PBS-treated cells compared with 454 in the GSH group. Means with SDs are shown for three experiments. *b* shows a representative experiment of four.

Fig. 4. T cells from DA.$Ncf1^{E3}$ rats can only transfer arthritis when cell surface thiol numbers are increased. (*a*) Spleen cells from 14-day immunized DA.$Ncf1^{DA}$ rats can transfer arthritis to naïve recipient DA.$Ncf1^{DA}$ rats. DA.$Ncf1^{E3}$ spleen cells can only transfer arthritis when the number of cell surface thiols is increased by GSH treatment. (*b*) This effect is mediated by $CD4^+$ cells; a transfer with purified $CD4^+$ T cells from inguinal LNs provides similar results. (*c*) Number of cell surface thiols of all TCR^+CD4^+ cells in recipients did not differ after transfer. (*d*) In the allotransfer experiment, all cells positive for the donor MHC class I ($RT1Aa^+$; M1) were $CD4^+$; $RT1Aa^+$cells gated by M1 are shown in *Right*. This finding confirms that CD4 T cells are responsible for transferring disease. Means (with SEM) are shown for representative experiments with five to eight rats per group. All experiments showed similar significant results. ∗, $0.05 > P > 0.005$; ∗∗, $0.005 > P > 0.0005$; ∗∗∗, $P < 0.0005$.

when PBS-treated cells were injected, indicating higher levels of proliferation (Table 1 and Fig. 3*b*). In line with this, we observed that 12 h after injection, the percentage of CFSE-positive cells amongst all spleen or LN cells was higher in the GSH-treated group. Similar results were obtained when purified LN $CD4^+$ T cells were transferred (Table 1). These results indicate that GSH-treated $CD4^+$ T cells proliferated more, confirming the *in vitro* data obtained with mouse cells.

Increasing the Number of Cell Surface Thiol Groups on T Cells Increases Arthritogenicity. The next question to be answered was whether DA.$Ncf1^{E3}$ T cells with increased numbers of cell surface $-SH$ could induce arthritis upon adoptive transfer. Spleen cells from 14-day immunized DA.$Ncf1^{E3}$ rats were treated with GSH or PBS, and 35×10^6 cells were injected i.v. into naïve DA rats; development of arthritis was followed (3). Remarkably, T cells from DA.$Ncf1^{E3}$ rats that normally cannot transfer arthritis became arthritogenic by increasing the number of cell surface thiol groups (Fig. 4*a*). Disease incidence induced by GSH-treated DA.$Ncf1^{E3}$ cells was 100% in all experiments. To confirm that $CD4^+$ T cells were the responsible cells for the observed effect as shown before (6), the experiment was repeated with purified $CD4^+$ T cells from inguinal LNs from
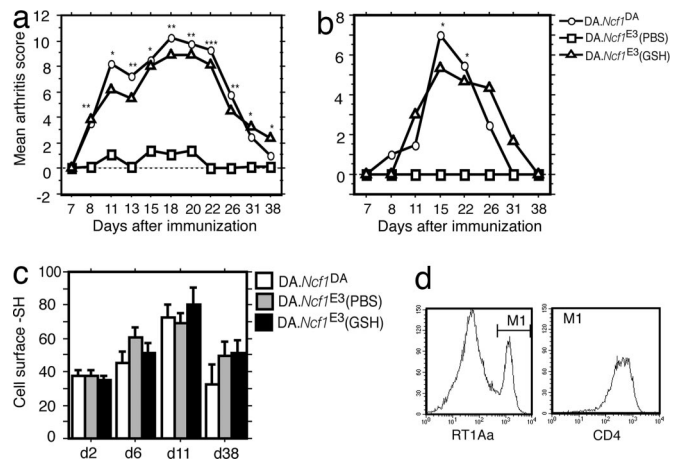
immunized DA.$Ncf1^{E3}$ rats. Injection of 15 million GSH-treated $CD4^+$ DA.$Ncf1^{E3}$ T cells induced arthritis as well (Fig. 4*b*).

After transfer, the number of cell surface thiols in blood was followed over time. However, no significant increase in the cell surface thiol levels of T cells or any other cell type was observed (Fig. 4*c*). Apparently, the number of transferred T cells was too low to change the number of $-SH$ groups on all T cells. In plasma, no differences in $-SH$ group numbers were observed either (not shown).

GSH-Treated T Cells Become Effector Cells. Next, we wanted to study the survival and functional state of the injected GSH-treated donor cells. Because CFSE staining diluted out to background levels within 2 days, an allelic marker was used to follow the transferred cells for a longer time. A similar transfer protocol was used as described for the CFSE transfer, but the cells were now injected in irradiated congenic DA.1I rats, which do not express the MHC class I molecule RT1A^a (6). Rats were killed at day 7 after transfer, at

Table 1. T cells with increased numbers of surface thiols proliferate better *in vivo*

Treatment	Spleen cell transfer			Lymph node $CD4^+$ transfer		
	$CFSE^+PI^-$	$CFSE^+TCR^+$	Percent*	$CFSE^+PI^-$	$CFSE^+TCR^+$	Percent*
PBS	$221 \pm 84^†$	412 ± 17	0.280 ± 0.030	175	216	0.020
GSH	$59 \pm 16^‡$	$219 \pm 119^‡$	$0.500 \pm 0.056^‡$	65	149	0.040

Concanavalin A cultured T cells from spleen or purified TCR^+CD4^+ T cells from inguinal LNs from immunized DA.$Ncf1^{E3}$ rats were CFSE-labeled and subsequently treated with PBS or GSH before i.v. injection in DA.$Ncf1^{DA}$ rats. After 12 h, $CFSE^+TCR^+$ cells were found in the spleen (shown in the table) and draining LNs (data not shown). GSH-treated cells had lower levels of CFSE staining, whereas the relative number of cells in the spleen was higher than when PBS-treated cells were injected, indicating that GSH-treated cells proliferated more efficiently than PBS-treated cells. Means $\pm$ SD are shown from one representative experiment of four with three rats (spleen transfer) or one rat (LN transfer).
*Percentage of CFSE-positive cells amongst all cells.
†Geomean (*x*) of CFSE staining of the depicted population ($CFSE^+PI^-$ or $CFSE^+TCR^+$).
‡$P < 0.05$.

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Table 2. T cells with increased numbers of surface thiols become effector cells

Treatment	Blood	Spleen	Inguinal LNs	Draining LNs	Thymus	Joint
PBS, day 7	24.0 ± 20.51*	0.43 ± 1.04	7.96 ± 5.93	29.69 ± 31.25	15.28 ± 2.37	5.28 ± 1.71
GSH, day 7	55.67 ± 5.51	23.14 ± 9.34†	53.75 ± 3.07†	77.79 ± 17.20†	19.55 ± 3.71	7.27 ± 3.32
PBS, day 12	6.02 ± 2.08	22.74 ± 7.72	16.50 ± 18.11	26.46 ± 18.47	5.23 ± 1.04	13.74 ± 2.62
GSH, day 12	20.85 ± 5.87	27.70 ± 5.68	58.13 ± 6.77†	9.79 ± 5.60	3.29 ± 0.34	27.33 ± 3.67†

Concanavalin A cultured T cells from spleens from immunized DA.$Ncf1^{E3}$ rats (RT1A^{a+}) were treated with PBS or GSH before i.v. injection into irradiated (6 Gy) allogeneic DA.1I rats (RT1A^{a-}). After 7 and 12 days, RT1A^{a+}-positive cells were found in draining (mediastinal) LNs, inguinal LNs, spleen, thymus, and arthritic joints. The percentage of RT1A^{a+} CD4$^+$TCR$^+$ cells amongst CD4$^+$TCR$^+$ cells is shown ±SD. GSH-treated cells proliferated significantly more than PBS-treated cells in draining and inguinal LNs and homed more to the joints. Only rats that received GSH-treated cells developed arthritis. Mean ± SD is shown for four to five rats per group.
*Percentage of RT1A^{a+} CD4$^+$TCR$^+$ cells amongst all CD4$^+$TCR$^+$ cells.
†$P < 0.05$.

disease onset, or at day 12, at maximal disease score. Blood, spleens, draining (mediastinal) and inguinal LNs, thymi, and hind-paw ankle and toe joints were harvested, and the number of RT1A^{a+} CD4$^+$TCR$^+$ cells amongst total CD4$^+$TCR$^+$ cells was determined by flow cytometry. Indeed, this experiment confirmed and extended the short-term CFSE experiment; 7 days after transfer, GSH-treated T cells had expanded significantly more in inguinal and draining LNs and also in inguinal LNs at day 12. The GSH-treated T cells also reached the joints in higher numbers than PBS-treated cells (Table 2). Gating on all donor (RT1A^{a+}) cells (gate M1) showed that these cells were mainly CD4$^+$ T cells, confirming that CD4$^+$ T cells are indeed the cells that proliferate upon transfer (Fig. 4d). Hence, the GSH-treated cells survive equally well, are still present in higher numbers compared with PBS-treated cells during disease, are relatively expanded in the joint-draining LNs, and reach the synovia.

The next issue to clarify was whether the CD4$^+$RT1A^{a+} double-positive donor T cells maintained their increased levels of cell surface −SH *in vivo*. The cells were isolated from various tissues 12 days after injection, and, surprisingly, a maintained higher level of −SH groups was observed on donor T cells isolated from blood and inguinal LNs compared with host T cells. However, in cells from spleen, thymus, draining LNs, or affected joints, these levels had reverted to normal at this time point (Fig. 5a). To investigate the longevity of GSH treatment in an *in vitro* system, we treated the HCQ10 hybridoma T cells with GSH or PBS and then labeled them with CFSE. The increased level of cell surface −SH staining on the GSH-treated cells was maintained until the third division, when it started to decrease, reaching similar levels as in the PBS-treated group after six divisions (Fig. 5 b and c). These data show that the reduced T cell membrane status could be maintained through several rounds of cell division and also *in vivo* in the blood and LNs but not when the T cells finally arrive in the joints.

Decreasing the Number of Cell Surface Thiol Groups on T Cells Decreases Arthritogenicity. To confirm that the observed effects of GSH treatment were indeed mediated by means of increasing cell surface −SH groups and not by a side effect of GSH, we performed the reverse experiment. First, it was investigated whether treatment with oxidized glutathione (GSSG) resulted in a decrease in T cell surface −SH groups, which was shown to be the case (Fig. 6a). To investigate whether this decrease in cell surface −SH resulted in decreased T cell activation in the mouse system, we compared IL-2 production by HCQ10 T hybridoma cells after GSSG or PBS treatment. It was shown that less IL-2 was produced when T cells were treated with GSSG, indicating that decreasing cell surface −SH suppresses T cell activation (Fig. 6b). In line with this, we observed *in vivo* that, in rats, a decrease in T cell surface −SH due to GSSG treatment led to significantly less severe arthritis in a spleen cell transfer from immunized DA.$Ncf1^{DA}$ rats to DA.$Ncf1^{DA}$ rats (Fig. 6c).

Discussion

Here, we provide evidence for a previously uncharacterized mechanism whereby ROS produced by the NADPH oxidase complex
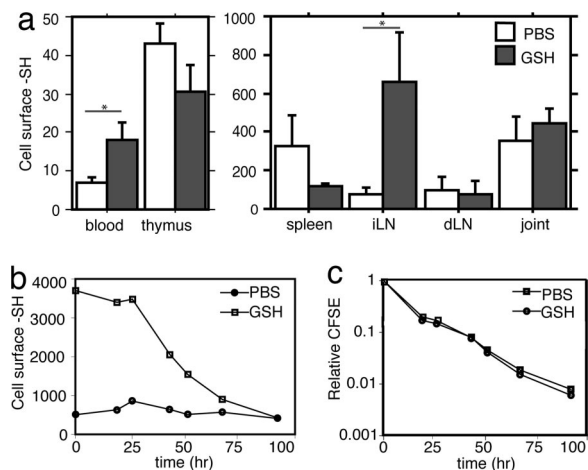

Fig. 5. GSH-treated T cells become effector cells. (a) *In vivo*, cell surface −SH groups of CD4$^+$RT1A^{a+} cells in tissues from the transfer experiment retained high levels of −SH groups in blood and inguinal LNs but not in other tissues. To investigate the longevity of GSH treatment on T cells, HCQ10 T cells were cultured in 2-mercaptoethanol-free medium after treatment with PBS or 4 mM GSH and labeling with CFSE. The number of cell surface −SH groups and the relative CFSE staining intensity were followed over time. (b) Cell surface −SH group levels were higher in the GSH-treated groups and only started to decrease after approximately two divisions, reaching normal levels after six divisions. (c) No differences in proliferation kinetics were observed. Representative experiments of two to three are shown. *, $P < 0.05$.
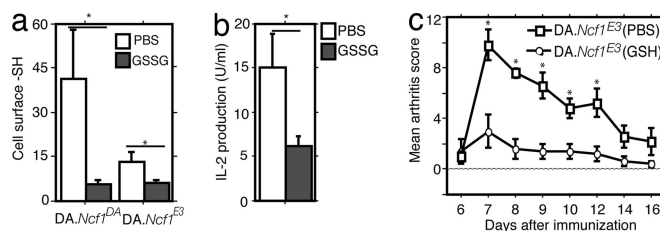

Fig. 6. Decreasing the number of cell surface thiol groups on T cells decreases arthritogenicity. (a) Treatment of blood with GSSG decreases the levels of cell surface −SH groups on CD4$^+$ T cells. (b) In the mouse T cell activation assay, treatment of the T hybridoma cells with GSSG leads to a decrease in IL-2 production. The shown values are corrected for control values in the absence of antigen. GSSG treatment by itself, in the absence of antigen, did not decrease levels of IL-2 production. (c) In a spleen cell transfer from DA.$Ncf1^{DA}$ rats to DA.$Ncf1^{DA}$ recipients, GSSG treatment of cells led to decreased arthritis severity compared with PBS-treated cells. Means with SDs are shown. *, $P < 0.05$.

determine the T cell surface redox level, thereby controlling T cell reactivity and the development of arthritis. Although our findings oppose the current dogma that ROS are attenuating an immune response, we earlier provided genetic evidence that a decrease in ROS production by the NADPH oxidase complex increases arthritis severity in both rats and mice. In search of a mechanism by which a decrease in ROS could operate in regulating the immune response, we studied T cell activation by the redox balance in our animal models.

It has previously been shown that T cell maturation and proliferation are influenced by ROS (19). Although most previous reports focused on the intracellular redox balance, some of them showed that a reducing extracellular environment increases T cell responsiveness *in vitro* and *in situ* (8, 9, 16, 20). We now show that an increase in cell surface thiols directly increases T cell activation and proliferation both *in vitro* and *in vivo* and thereby determines T cell arthritogenicity. Although the control of redox levels is a complex issue to study and results depend on many factors (such as the anatomical compartment studied and the experimental setup), we had the advantage of having identified a SNP in the *Ncf1* gene that indirectly affected T cell surface thiol levels, enabling us to use well defined inbred rat and mouse strains that differed only in this functional polymorphism.

It is an interesting question: How is the membrane redox status of T cells determined by ROS? Although others reported expression of a functional NADPH oxidase complex in T cells (21, 22), we could detect neither Ncf1 expression nor oxidative burst in T cells from our rats or mice. We do not exclude the occurrence of constitutive low levels of ROS in T cells (23), but the very low background ROS levels we observed did not differ between T cells derived from the different animal strains and thus seem to be NADPH oxidase independent. This observation leads to the hypothesis that the number of T cell surface −SH groups is determined by other cells that do produce ROS, such as APCs or neutrophils (24). Changes in cell surface −SH might occur during direct interaction between T cells and APCs during antigen presentation (13) or during thymic selection (25) or without cell contact by means of the local environment (26).

The first and most likely possibility is that T cell membranes normally become oxidized through interactions with Ncf1-expressing APCs (13), which fails in the animals with a reduced capacity to burst. Interaction between T cells and APCs occurs at several stages during the immune response: upon thymic selection, during migration into the peripheral lymphoid tissues, and during antigen presentation (4). We observed that if a T cell had increased levels of cell surface −SH groups, increased IL-2 production occurred only in the presence of antigen and APCs, suggesting that T cell surface redox levels are determined during T cell–APC interaction. Critical molecules or proteins on the T cell surface present in the immunological synapse may normally be oxidized to prevent T cell activation. This oxidation might be especially important during thymic selection, where T cells are activated by the TCR–MHC class II interaction, but they should not go into the periphery with an activated phenotype. The −SH groups on cell surface proteins are maintained in the reduced state by electron and hydrogen shuffling between redox active residues (27). It has been shown that the microenvironment of the cell surface supports redox reactions in and between certain surface proteins (27), thereby allowing regulatory processes by means of these proteins. One of several proteins that are susceptible for redox regulation is CD4 itself (11). It might be possible that the CD4–MHC class II interaction is altered upon redox changes in CD4 (28), resulting in different signal transductions into the cell and different outcomes of thymic selection or antigen presentation.

The second possibility is that T cells obtain increased cell surface thiol numbers in the periphery (for example, during trafficking in the blood). We show that plasma of animals with polymorphic *Ncf1* contained higher levels of −SH groups than that of WT animals,

which might be due to a decreased level of systemic ROS released during phagocytosis, thymic selection, or antigen presentation. However, the observation that T cells do not proliferate better when they are in close contact with APCs in the absence of peptide makes this possibility less likely.

As the present experiments clearly show, the redox levels of T cell surfaces could be maintained several cycles of division after transfer and also lead to enhanced T cell activation *in vivo*. However, arthritis developed only several days after the cell surface redox levels already decreased on the donor T cells. In addition, donor T cells that migrated to the joints as effector cells, before arthritis developed, did not maintain this increased level of cell surface −SH. These findings argue that the redox levels regulate immune activation rather than direct effector functions in the joints, which might be underscored by the previous finding that plasma of rheumatoid arthritis patients contains significantly higher levels of ROS as compared with matched controls (29, 30). Also, in arthritic rats, the level of ROS in spleen, thymus, and lymphocytes was increased (30, 31). These findings seem to oppose our results but probably just reflect the fact that Ncf1 function is also enhanced as a result of the inflammatory process. In the effector phase of disease, in which joints are destroyed and accumulation of ROS-producing cells in the joint has taken place, the physiological buffering capacity is exceeded, and the result is local and systemic oxidative stress, when ROS possibly could be harmful rather than beneficial. Cell surface −SH groups might therefore be used as an arthritis predicting parameter rather than as a diagnostic tool.

In conclusion, we show here that the number of thiol groups on the cell surface of T cells is regulated by means of ROS, produced by the NADPH oxidase complex, and that the level of T cell surface −SH groups influences activation, proliferation, and arthritis development.

Methods

Animals. DA (DA.*Ncf1^DA^*; originally obtained from Zentralinstitut fur Versuchstierzucht, Hannover, Germany), congenic DA.*Ncf1^E3^* (3), and DA.1I (6) rat strains were established in our colony for >13 backcross generations and share the DA rat gene background. Animals were kept in the animal house of Medical Inflammation Research under conventional conditions. All animal experiments were approved by the Malmö/Lund ethical committee (license nos. M70/01 and M70/04).

Arthritis Induction and Evaluation. Arthritis was induced in 8- to 14-week-old rats by injecting 200 μl (disease) or 500 μl (T cell transfer) of pristane (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich) s.c. at the base of the tail. Arthritis development was monitored by using a macroscopic scoring system: Each swollen or red toe, midfoot digit, or knuckle received 1 point, and each swollen ankle received 5 points, resulting in a maximal score of 60 per rat.

Antibodies. Anti-rat TCR (R73), anti-rat Gr-1 (HIS48), anti-rat CD4 (OX-35), anti-rat CD8 (341), anti-rat RT1A^a (C3), anti-mouse CD3 (135–2C11), anti-mouse CD4 (H129.19), anti-mouse granulocyte (Rb6), anti-mouse macrophage (F4/80), and anti-mouse dendritic cells (N4.18) (all from Pharmingen) labeled with different fluorochromes or biotin that was detected with streptavidin-fluorochrome conjugates (Pharmingen) were used.

Flow Cytometry. The relative number of cell surface thiol groups on different cell types was determined with Alexa Fluor 633 coupled to maleimide (ALM-633; Molecular Probes) as described in ref. 16. Fifteen microliters of heparinized blood was incubated with 1.5 μl of ALM-633 diluted in PBS (10 μM) for 15 min on ice. Then, cells were stained with antibodies directed against different surface antigens. A total of 250,000–500,000 cells was acquired on a FACScan sorter (Becton Dickinson). The geomean of ALM-633 staining per cell type was used to express the relative number of cell

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surface $-SH$ groups. To measure intracellular GSH, cells were stained with cell-specific markers, and, after washing, they were incubated with 40 μM MCB (Molecular Probes) diluted in PBS; the reaction was stopped after exactly 20 min with 50% FCS, and cells were washed and kept on ice until analysis (20). To determine burst capacity, cells were stained for cell surface markers and taken up in 200 μl of DMEM (GIBCO). DHR123 (25 μl) (Molecular Probes) in DMEM (final concentration of 3 μM) was added and incubated for 10 min at 37°C. Then, 25 μl of PMA (Sigma) in DMEM (final concentration of 200 ng/ml) was added to stimulate burst. After 20 min at 37°C, cells were washed, and the geomean of DHR123 staining per cell type was determined.

Ncf1 expression was determined by intracellular staining with rabbit anti-E3 rat Ncf1. This rabbit polyclonal antibody was elicited with a peptide of WT Ncf1 and results in lower staining levels in DA rats than in E3 rats. Blood was stained with antibodies against CD4, TCR, and Gr-1. After washing, cells were fixed and permeabilized with Cytofix/Cytoperm (Becton Dickinson) and washed twice in Perm/Wash (Becton Dickinson). Then, cells were stained with anti-Ncf1 or an irrelevant control antibody detected by goat anti-rabbit IgG (DAKO). The geomean of Ncf1 staining per cell type was determined by flow cytometry.

Measurement of Plasma $-SH$ Groups. Plasma was diluted 1/2 in PBS, and an equal amount of 400 μM 5,5′-dithiobis(2-nitrobenzoic acid) was added. OD_{450} was measured, and the relative number of plasma thiols was calculated according to a GSH standard.

GSH and NAC Treatment. To increase the number of cell surface thiol groups, cells were treated with 4 mM GSH (Sigma) or 4 mM NAC (Sigma) in PBS. To decrease cell surface thiol groups, 2 mM GSSG (Sigma) diluted in PBS was used. All treatments were done for 15 min on ice.

T Cell Activation Assays. APCs were isolated from naïve mouse spleen suspensions as follows. Cells were taken up in 2 ml of 3:1 PBS:Optiprep (Axis-shield, Oslo) and overlayered with 2.5 ml of 1:4.2 Optiprep:Diluent C [0.88% (wt/vol) NaCl/1 mM EDTA/ 0.5% (wt/vol) BSA (Sigma)/10 mM Hepes-NaOH, pH 7.4] and 1.5 ml of PBS. After centrifugation at $600 \times g$ for 15 min, the upper cell layer was washed and taken up in DMEM with 10% FCS, 2.4 mg/ml Hepes, 3.9 μg/ml 2-mercaptoethanol, and penicillin/ streptomycin ($DMEM^{+++}$). These cells were macrophages (F4/ 80^+) and dendritic cells ($N4.18^+$) with a minor contamination of lymphocytes (maximal 8%) as determined by FACS. T cell HCQ.10 hybridoma cells (50×10^3) were cocultured with 50×10^4 APCs and 10 μg/ml rat CII in a total volume of 125 μl. T cells or APCs were treated with 4 mM GSH or NAC in PBS or PBS only and washed before adding them to the plates. Increases in cell surface thiol

numbers were confirmed by flow cytometry. After 24 h at 37°C, 75 μl of supernatant was taken and assayed for IL-2 concentration by ELISA.

IL-2 ELISA. Rat anti-mouse IL-2 (Pharmingen) was coated on ELISA plates (Costar, Corning) in PBS (pH 9) for 2 h at 37°C. Then, 150 μl of 3% BSA in PBS was added and incubated for 1 h at 37°C. After washing with ELISA buffer (1.3 M NaCl/0.1 M Tris/0.1% Tween 20, pH 7.4), 75 μl of supernatant was added to the plates and incubated for 2 h at room temperature. IL-2 was detected with biotinylated rat anti-mouse IL-2 (Pharmingen) and subsequently with europium-labeled streptavidin diluted in assay buffer (Wallac, Gaithersburg, MD) for 30 min at room temperature. After washing, plates were developed with 50 μl of enhancement solution (Wallac). The level of fluorescence was detected with a multilabel counter (VICTOR 1420, Wallac). Recombinant mouse IL-2 was used as a standard.

T Cell Transfer. Rats were immunized with 500 μl of pristane. At day 14, spleen cells and inguinal LN $CD4^+$ T cells (purified by panning; >94% $CD4^+$ T cells) were cultured for 48 h in $DMEM^{+++}$ with 3 μg/ml concanavalin A (Sigma) at 37°C (6). Then, cells were treated with 4 mM GSH in PBS or PBS as a control (in 5 ml for 175×10^6 cells) for 15 min on ice. After washing three times in PBS, 35×10^6 cells in PBS were injected i.v. in naïve DA rats, and disease development was scored. For *in vivo* T cell proliferation studies, cells were labeled with 0.5 μM CFSE before GSH treatment. CFSE staining and increased extracellular reduction were confirmed by FACS. GSH treatment did not affect CFSE staining. Twelve and 36 h after injection, a set of rats was killed, and tissues were harvested and analyzed for CFSE staining amongst living cells (PI^-) and TCR^+ (R73-PerCP) cells. For the allotransfer experiment, DA.1I rats were irradiated with 6 Gy before transfer. Spleen cells were obtained and treated as described above but were not labeled with CFSE. Rats were killed at days 7 and 12 after transfer, and the percentage of $RT1A^{a+}$ $CD4^+TCR^+$ cells amongst $CD4^+TCR^+$ cells was determined by FACS.

Statistics. Differences between groups were analyzed with the Mann–Whitney U test, considering $P < 0.05$ as significantly different. Group sizes ranged between 3 and 10 samples or animals per group per experiment; two to five experiments were performed.

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