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

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Abbreviations: GSH, glutathione (reduced); GSSG, glutathione (oxidized); LN, lymph node; NAC, N-acetyl-cysteine; RA, rheumatoid arthritis; ROS, reactive oxygen species

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Abstract

Rats and mice with a lower capacity to produce reactive oxygen species (ROS) due to allelic polymorphisms in the *Ncf1* gene are more susceptible to develop severe arthritis. This suggests 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 a 'wildtype' E3-derived *Ncf1* allele (DA.$Ncf1^{E3}$ rats) that cannot transfer arthritis to rats with an arthritis-associated 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 where 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 to regulate surface redox levels of T cells and thereby suppresses autoreactivity and arthritis development.

Introduction

Reactive oxygen species (ROS) are generally thought to be harmful and to play a disease enhancing role in autoimmune diseases like arthritis (1, 2). However, we have found that a decreased capacity to produce ROS due to polymorphisms in *Ncf1*, increases susceptibility for autoimmunity and arthritis (3, 4). *Ncf1* encodes the neutrophil cytosolic factor 1 (Ncf1, alias p47phox), the activating protein in the NADPH oxidase complex that produces ROS upon activation. In the rat, a single nucleotide polymorphism (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 (PIA) and collagen in mice (CIA) expressing polymorphic *Ncf1*, is T cell dependent. In the rat model, only T cells from DA.*Ncf1*^{DA} rats (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} (DA rats with the wildtype Ncf1^{E3} allele from the E3 rat) cannot (3,6). In the mouse model, a mutation in *Ncf1* results in an increased DTH response and serum levels of anti-collagen type II IgG antibodies (4), indicating enhanced activation of autoreactive T cells. Thus, Ncf1 somehow influences autoreactive

T cells during immune priming to become arthritogenic, either via cell-cell contact or via 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 if the redox balance in T cells is disturbed in animals with a decreased NADPH oxidase function. It has been described 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 to granulocytes. Peripheral blood from DA.*Ncf1*^{DA} and DA.*Ncf1*^{E3} rats was stained for intracellular ROS with DHR123 and cell specific markers, with or without stimulation by 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 to the control, where 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 flowcytometry 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 as compared to those from Ncf1 wildtype DA.*Ncf1*^{E3} rats (Fig 1c). Higher levels of T cell surface thiols in DA.*Ncf1*^{DA} rats were also observed in spleen and inguinal lymph (LN) node T cells (not shown). Other cell types showed tendencies to have increased amounts of cell surface thiols in *Ncf1* mutated animals (macrophages, neutrophils, B-cells), although these were not significantly different. Intracellular thiol

levels of T cells were determined by flowcytometry with monochlorobimane (MCB), reacting with intracellular GSH (17). No difference in intracellular thiol levels between the strains was detected in T cells (Fig 1d). 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 to DA.*Ncf1*^{E3} rats (Fig 1e) as determined with an assay utilizing 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (9, 13). Similar data were obtained for the Ncf1 mutated mice (see supplementary Fig 1a-e).

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 reduced glutathione (GSH) and N-acetyl-cysteine (NAC) are described to increase the amount of cell surface -SH groups on human cells (9). Heparinized rat blood was treated with 4 mM of GSH or 4 mM of NAC (16) and stained for cell specific markers and reduced surface thiols (-SH). No hemolysis was performed, since that influenced the staining of surface –SH. Both GSH and NAC increased the number of T cell surface thiols (Fig 2a, b). GSH, however, did not influence intracellular redox levels whereas NAC increased the number of intracellular thiols (Fig 2c) (9). Since we were interested in studying the effect of increasing only the cell surface thiol number, GSH was used throughout remaining experiments. The used concentration of 4 mM resulted in an increase in cell surface thiol 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 the used concentration; only above 16 mM the

percentage of dead (PI-positive) cells increased (Fig 2d). Similar data were obtained for mouse (not shown).

Increasing the number of cell surface -SH on T cells increases activation and proliferation

Next, we investigated if 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 APC's. HCQ10 T hybridoma cells (18), recognizing the immunodominant collagen type II (CII) peptide bound to the class II molecule H2-A^q, or APC's were either treated with PBS or with 4 mM GSH to increase the amount 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 APC's did not (Fig 3a). In 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, since it was shown before that T cells from DA rats could transfer pristane induced arthritis (PIA) (3). Spleen cells and LN $CD4^+$ T cells were isolated from immunized DA.*Ncf1*^{E3} rats. Cells were labeled with 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 sacrificed 12 and 36 hours after i.v. injection; significant numbers of $CFSE^+$ cells were found in mediastinal LN (draining the injection site) and spleen, but not in blood, inguinal LN (draining the joints), bone marrow or thymus. It was shown that at both time

points in spleen and draining LN the intensity of CFSE staining of both $CFSE^+/PI^-$ (living) cells and $CFSE^+/TCR^+$ cells were lower when GSH treated cells were injected then when PBS treated cells were injected, indicating higher levels of proliferation (Table 1 and Fig. 3b). In line with this, we observed that twelve hours 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 lymph node 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 if DA.*Ncf1*^{E3} T cells with increased numbers of cell surface –SH could induce arthritis upon adoptive transfer. Spleen cells from 14 days immunized DA.*Ncf1*^{E3} rats were treated with GSH or PBS and $35x10^6$ cells were injected i.v. into naïve DA rats and 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 4a). 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, 19), the experiment was repeated with purified $CD4^+$ T cells from inguinal LN from immunized DA.*Ncf1*^{E3} rats. 15 million of GSH treated $CD4^+$ DA.*Ncf1*^{E3} T cells induced arthritis as well (Fig 4b).

After transfer, the number of cell surface thiols in blood was followed in time. However, no significant increase in numbers of cell surface thiol levels of T cells or any other cell type was observed (Fig. 4c). Apparently the number of transferred T cells was

too low to change the number of –SH 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. Since CFSE staining diluted out to background levels within two 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 $RT1A^{a}$ (19). Rats were sacrificed at day 7 after transfer, at disease onset or at day 12, at maximal disease score. Blood, spleens, draining (mediastinal) and inguinal LN, thymi and hindpaw ankle and toe joints were harvested and the number of $RT1A^{a+}$ $CD4^{+}TCR^{+}$ cells amongst total $CD4^{+}TCR^{+}$ cells was determined by flowcytometry. 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 LN, and in inguinal LN also 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) it was shown that these 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 number compared to PBS treated cells during disease, are relatively expanded in the joint draining lymph nodes 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 LN, compared to host T cells. However, in cells from spleen, thymus, draining lymph nodes 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 6 divisions (Fig 5b, c). These data shows that the reduced T cell membrane status could be maintained through several rounds of cell division and also *in vivo* in the blood and lymph nodes, but not when they 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 via increasing cell surface –SH groups and not by a side effect of GSH we performed the reverse experiment. First it was investigated if treatment with oxidized glutathione (GSSG) resulted in a decrease in T cell surface –SH groups. This was shown to be the case (Fig 6a). To investigate if 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

We here provide evidence for a new mechanism whereby ROS produced by the NADPH oxidase complex determine the T cell surface redox level, thereby controlling T cell reactivity and the development of arthritis. Although our findings are opposite to 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 for a mechanism how 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 (20). 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, 21). 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 like the anatomical compartment studied and experimental set-up, we had the advantage of having identified

a single nucleotide polymorphism in the *Ncf1* gene that indirectly affected T cell surface thiol levels. This enabled us to use well-defined inbred rat and mouse strains, differing only in this functional polymorphism.

It is an interesting question how the membrane redox status of T cells is determined by ROS. Although others reported expression of a functional NADPH oxidase complex in T cells (22, 23), we could neither detect Ncf1 expression nor oxidative burst in T cells from our rats or mice. We do not exclude occurrence of constitutive low levels of ROS in T cells (24) 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 leads to the hypothesis that the number of T cell surface –SH groups is determined by other cells that do produce ROS, like APC's or neutrophils (25). This might occur via direct interaction between T cells and APC's during antigen presentation (13) or during thymic selection (26) or without cell contact via the local environment (27).

The first and most likely possibility is that T cell membranes normally become oxidized through interactions with Ncf1-expressing APC's (13), which fails in the animals with a reduced capacity to burst. Interaction between T cells and APC's 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, an increased IL-2 production occurred only in presence of antigen and APC's, 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 rendered oxidized to

prevent T cell activation. This might especially be 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 (28). It has been shown that the micro-environment of the cell surface supports redox reactions in and between certain surface proteins (28), thereby allowing regulatory processes via 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 (29), resulting in different signal transduction into the cell and different outcome 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 wildtype animals. This might be due to a decreased level of systemic ROS released during e.g. phagocytosis, thymic selection or antigen presentation. However, the observation that T cells do not proliferate better when they are in close contact with APC's in 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 for that the redox levels regulate immune activation rather than direct effector functions in the joints. This might be underscored by the previous finding that plasma of RA patients contains significantly higher levels of ROS as compared to matched controls (30, 31)). Also in arthritic rats the level of ROS in spleen, thymus and lymphocytes was increased (31, 32). These findings seem opposite to 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, where 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 rather be used as an arthritis predicting parameter than as a diagnostic tool.

In conclusion, we here show that the number of thiol groups on the cell surface of T cells is regulated via 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 more than 13 backcross generations and share the DA rat gene background. Animals were kept in the animal house of Medical Inflammation Research (www.inflam.lu.se) under conventional conditions. All animal experiments were approved by the Malmö/Lund ethical committee (license M70/01 and M70/04).

Arthritis induction and evaluation

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

Antibodies

Anti-rat T cell receptor (R73), anti-rat Gr1 (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), anti-mouse-DC (N4.18) (all from Pharmingen, San Jose, CA), labeled with different fluorochromes or biotin that was detected with streptavidin-fluorochrome conjugates (Pharmingen) were used.

Flowcytometry

The relative number of cell surface thiol groups on different cell types was determined with Alexa-633 coupled to maleimide (ALM-633: Molecular Probes, Leiden, The Netherlands), as described before (16). 15 µl of heparinized blood was incubated with 1.5

µl of in DMSO dissolved ALM-594 diluted in PBS (10 µM) for 15' on ice. Then cells were stained with Ab directed against different surface antigens. 250,000-500,000 cells were acquired on a FACScan (Becton Dickinson, Mountain View, CA). GeoMean of ALM-633 staining per celltype was used to express the relative number of cell surface –SH groups. To measure intracellular GSH, cells were stained with cell-specific markers and after washing incubated with 40 µM MCB (Molecular Probes) diluted in PBS, the reaction was stopped after exactly 20 min with 50% FCS, washed and kept on ice till analysis (21). To determine burst capacity, cells were stained for cell surface markers and taken up in 200 µl DMEM (Gibco, Paisly, UK). 25 µl of DHR123 (Molecular Probes) in DMEM (final concentration 3µM) was added and incubated for 10 minutes at 37°C. Then 25 µl of PMA (Sigma, St Louis, MO) in DMEM (final concentration 200 ng/ml) was added to stimulate burst. After 20 minutes at 37°C, cells were washed and GeoMean of DHR123 staining per celltype was determined.

Ncf1 expression was determined by intracellular staining with rabbit-anti-E3 rat Ncf1. This rabbit polyclonal was elicited with a peptide of wildtype 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 (BD) and washed twice in Perm/Wash (BD). Then cells were stained with anti-Ncf1 or an irrelevant control antibody, detected by Goat-anti-Rabbit IgG (DAKO, Glostrup, Denmark). GeoMean of Ncf1 staining per celltype was determined by flowcytometry.

Measurement of plasma –SH groups

Plasma was diluted 1/2 in PBS and equal amount of 400 μM DTNB was added. OD450 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 reduced glutathione (GSH; Sigma) or 4 mM N-acetylcysteine (NAC; Sigma) in PBS. To decrease cell surface thiol groups 2 mM of oxidized glutathione (GSSG; Sigma) diluted in PBS was used. All treatments were done for 15' on ice.

T cell activation assays

APC's were isolated from naïve mouse spleen suspensions as follows: cells were taken up in 2 ml of 3:1 PBS:Optiprep (Axis-shield, Oslo, Norway) and overlayered with 2,5 ml 1:4,2 Optiprep:Diluent C (0.88% w/v NaCl, 1mM EDTA, 0.5% w/v BSA (Sigma), 10 mM Hepes-NaOH pH7.4) and 1,5 ml of PBS. After centrifugation at 600G for 15', the upper cell layer was washed and taken up in DMEM with 10% FCS, 2.4mg/ml HEPES, 3.9 μg/ml β-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. $50x10^3$ T cell HCQ.10 hybridoma cells were cocultured with $50x10^4$ APC's 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. Increase in cell surface thiol numbers was confirmed by flowcytometry. After 24 hrs 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 Inc., Corning, NY) in PBS pH 9 for 2 hrs at 37°C. Then 150 µl 3% BSA in PBS was added and incubated for 1 hr 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 supernatant was added to the plates and incubated for 2 hrs at RT. Il-2 was detected with biotinylated rat-anti-mouse IL-2 (Pharmingen), and subseuently with Europium-labeled streptavidine diluted in Assay buffer (Wallac, Turlu, Finland) for 30 min at RT. After washing, plates were developed with 50 µl enhancement solution (Wallac). The level of fluorescence was detected with a multilabel counter (VICTOR^{TM} 1420, Wallac). Recombinant mouse IL-2 was used as standard.

T cell transfer

Rats were immunized with 500 µl pristane. At day 14, spleen cells and inguinal LN $CD4^+$ T cells (purified by panning; >94% $CD4^+$ T cells) were cultured for 48 hrs in DMEM+++ with 3µg/ml Concanavaline A (ConA; Sigma) at 37°C (6). Then cells were treated with 4 mM GSH in PBS or PBS as a control, in 5 ml for $175x10^6$ cells, for 15' on ice. After washing 3x in PBS, $35x10^6$ 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. 12 and 36 hours after injection a set of rats was sacrificed, 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 prior to transfer. Spleen cells were obtained and treated as described above, but not labeled with CFSE. Rats were sacrificed at day 7 and 12 after transfer and the percentage of $RT1A^{a+}$ $CD4^{+}TCR^{+}$ cells amongst $CD4^{+}TCR^{+}$ cells were determined by FACS.

Statistics

Differences between groups were analyzed with 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, 2-5 experiments were performed.

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Figure Legends

Fig 1. *Ncf1 mutated rats and mice have increased numbers of cell surface thiol groups*

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 flowcytometry after or without stimulation with PMA (a). Ncf1 expression was determined by flowcytometry and expressed in GeoMeanX (b). The relative number of cell surface thiol (-SH) groups was determined by Alexa633-Maleimide staining and expressed in GeoMeanX. Rats with a polymorphism in *Ncf1* and lower ROS production (DA.$Ncf1^{DA}$) have higher numbers of cell surface thiol groups compared to congenic rats expressing wildtype *Ncf1* (DA.$Ncf1^{E3}$)(c). Intracellular thiol levels were determined by staining with monochlorobimane (MCB), detected by FACS and expressed in GeoMean. No differences in intracellular GSH levels were observed (d). Plasma thiol groups were determined with DTNB and expressed in relative numbers compared to a standard of GSH. Plasma of mutated rats contained more reduced –SH groups (e). Means ± S.E.M. are shown. The number of animals per group ranged from 3 to 5 per experiment, at least three experiments were done. * $p<0.05$.

Fig 2. *The number of cell surface thiols can be increased by GSH*

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 (a, b (DA.$Ncf1^{DA}$)). Amount of cell surface –SH is expressed in GeoMean. Treatment with GSH does not affect intracellular thiol levels in contrast to NAC, as determined by staining with MCB

(c). Means ± S.E.M. are shown. 4 animals per group were used per experiment, at least three experiments were done. * $p<0.05$. GSH treatment does not affect viability of blood cells at the used concentration of 4 mM as determined by titration of GSH and doublestaining with Alexa633-maleimide and propidium iodide (PI) (d). A representative experiment is shown.

Fig 3. *T cells with increased number of surface thiols are more activated*

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 was dependent on T cell APC interaction (a). This was not the case if APC's were treated with GSH (GSH treated APC's)(a). Experimental values minus control values (T cells and APC's without antigen) are depicted. These data were confirmed *in vivo* in rat: GSH treatment results in increased T cell proliferation *in vivo*; the GeoMeanX of CFSE staining of $CD4^+$ and Propidium $Iodide^-$ (PI^-; living) cells was 758 in the rats receiving PBS treated cells compared to 454 in the GSH group (b). Means with standard deviations are shown of 3 experiments. b shows a representative experiment out of 4.

Fig 4. *T cells from DA.Ncf1^{E3} rats can only transfer arthritis when cell surface thiol numbers are increased*. Spleen cells from 14 days 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 (a). This is mediated by $CD4^+$ cells; a transfer with purified $CD4^+$ T cells from inguinal

LN provides similar results (b). Number of cell surface thiols of all TCR^+CD4^+ in recipients did not differ after transfer (c). 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 the right histogram (d). This confirms that CD4 T cells are responsible for transferring disease. Means (with S.E.M) are shown of representative experiments with 5-8 rats per group. All experiments showed similar significant results. *0.05>p>0.005, **0.005>p>0.0005, ***p<0.0005.

Fig 5. *GSH treated T cells become effector cells*

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 LN, but not in other tissues (a). To investigate the longevity of GSH treatment on T cells, HCQ10 T cells were cultured in 2-ME 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 in time. Cell surface –SH group levels were higher in the GSH treated groups and only started to decrease after approximately 2 divisions, reaching normal levels after 6 divisions (b). No differences in proliferation kinetics were observed (c). Representative experiments out of 2-3 are shown. * p<0.05

Fig 6. *Decreasing the number of cell surface thiol groups on T cells decreases arthritogenicity.*

Treatment of blood with GSSG decreases the levels of cell surface –SH groups on $CD4^+$ T cells (a). In the mouse T cell activation assay, treatment of the T hybridoma cells with

GSSG leads to a decrease in IL-2 production (b). The shown values are corrected for control values in absence of antigen. GSSG treatment by itself, in absence of antigen did not decrease levels of IL-2 production. In a spleen transfer from DA.*Ncf1*^{DA} rats to DA.*Ncf1*^{DA} recipients, GSSG treatment of cells led to decreased arthritis severity as compared to PBS treated cells (c). Means with standard deviations are shown. *$p<0.05$.

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

ConA cultured T cells from spleen or purified $TCR^{+}CD4^{+}$ T cells from inguinal LN 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 hours, $CFSE^{+}TCR^{+}$ cells were found back in spleen (table) and draining LN (not shown). GSH treated cells had left lower levels of CFSE staining whereas the relative number of cells in the spleen was higher than when PBS treated cells were injected. This indicates that GSH treated cells proliferated more efficiently than PBS treated cells. Mean ± S.D. are shown from 1 representative experiment out of 4 with 3 (spleen transfer) or 1 (LN transfer) rats.

	Spleen cell transfer			**Lymph node CD4 transfer**		
Treatment	CFSE+PI-	CFSE+TCR+	%[a]	CFSE+PI-	CFSE+TCR+	%
PBS	221±84[b]	412±17	0.280±0.030	175	216	0.020
GSH	59±16*	219±119*	0.500±0.056*	65	149	0.040

[a] % of CFSE positive cells amongst all cells

[b] GeoMeanX of CFSE staining of the depicted population (CFSE+PI- or CFSE+TCR+)

* $p<0.05$

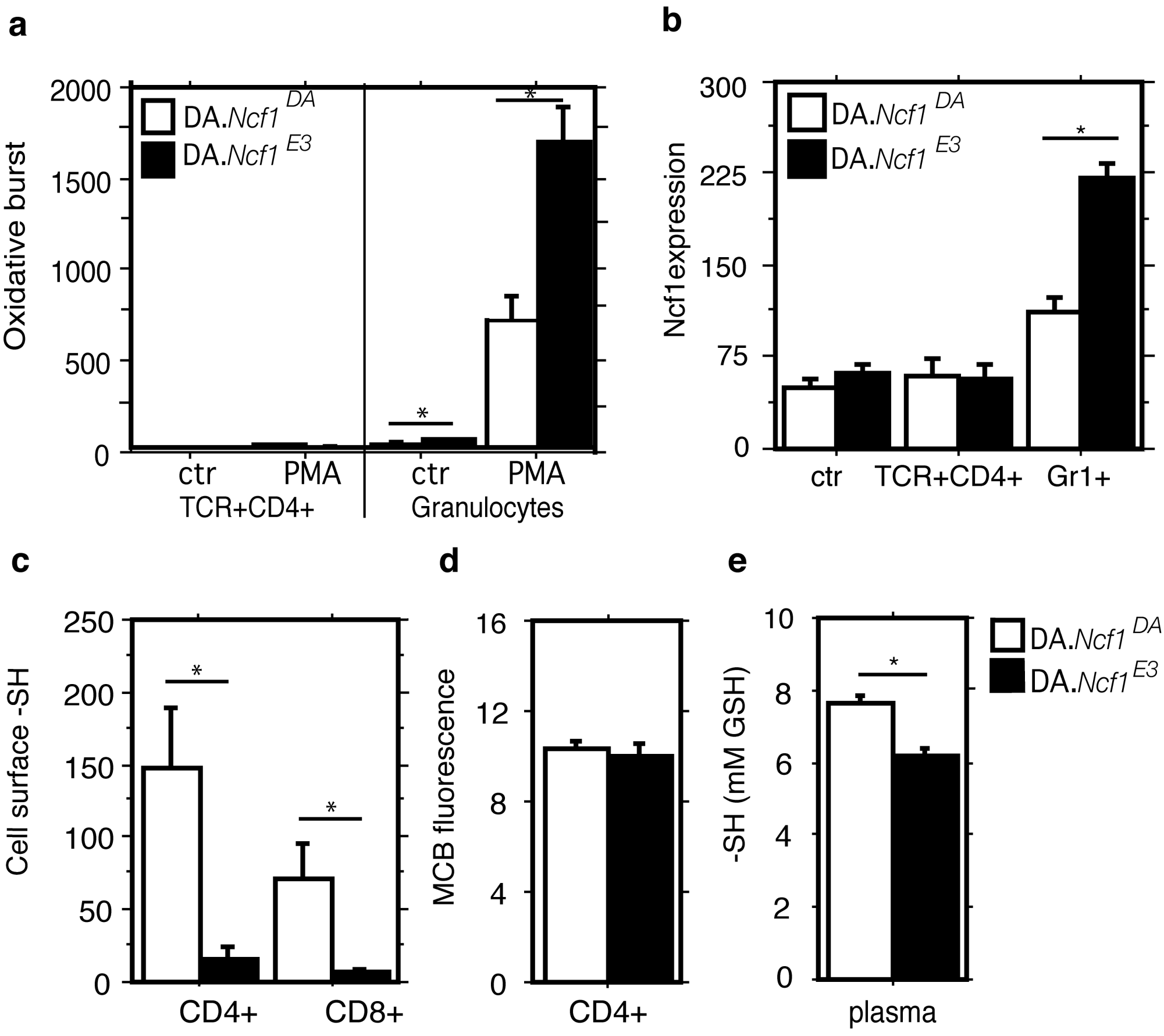
Table 2: *T cells with increased numbers of surface thiols become effector cells.*

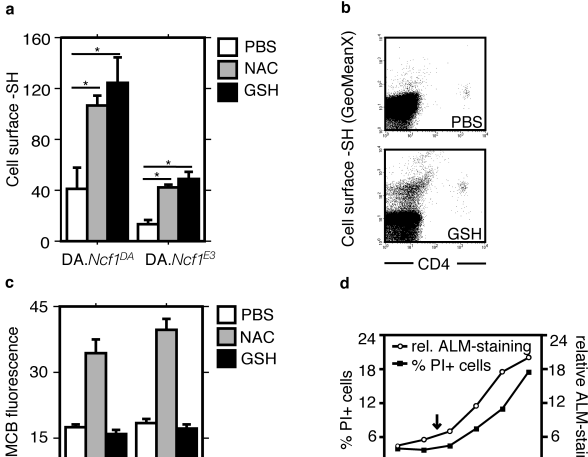
ConA cultured T cells from spleens from immunized DA.*Ncf1*^{E3} rats (RT1A^{a+}) were treated with PBS or GSH before i.v. injection into irradiated (6 Gy) allogeneic DA.1I rats (RT1A^{a-}). After 7 and 12 days, RT1A^{a+} positive cells were found back in draining (mediastinal) LN, inguinal LN, spleen, thymus and arthritic joints. In the table the percentage of RT1A^{a+} CD4^{+}TCR^{+} cells amongst CD4^{+}TCR^{+} cells is shown +/- standard deviation. GSH treated cells proliferated significantly more than PBS treated cells in draining and inguinal LN and homed more to the joints. Only rats that received GSH treated cells developed arthritis. Mean ± S.D. is shown of 4-5 rats per group.

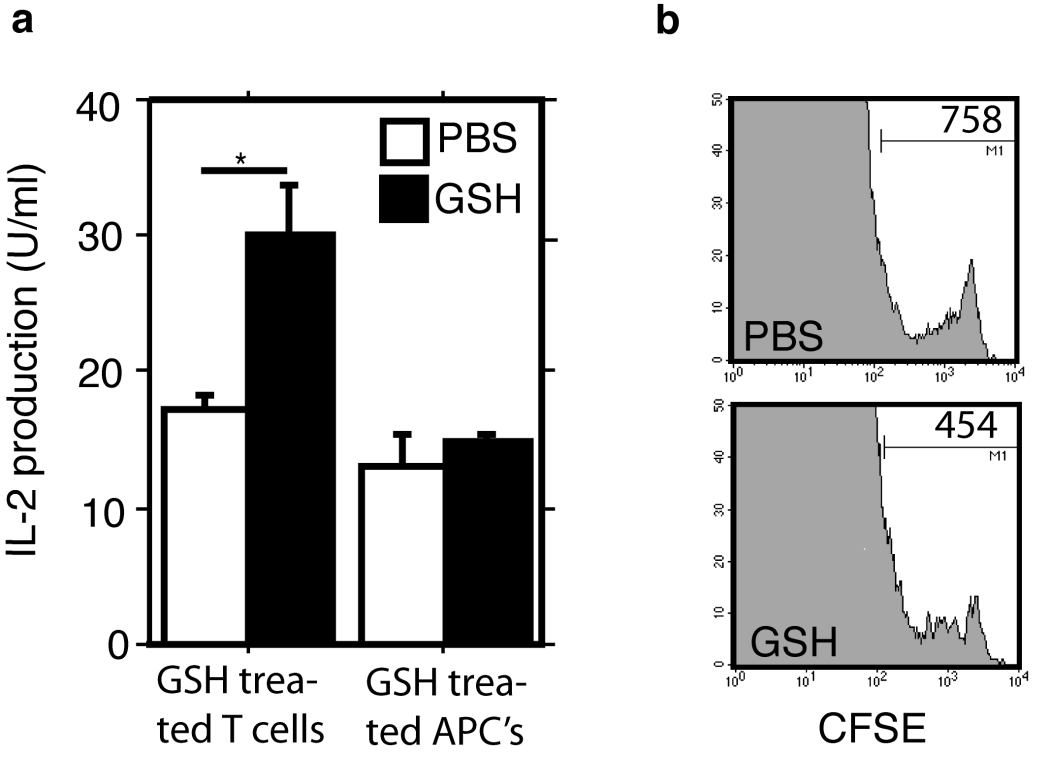
	blood	**spleen**	**Inguinal LN**	**Draining LN**	**thymus**	**joint**
PBS d7	24.0 ± 20.51[a]	0.43 ± 1.04	7.96 ± 5.93	29.69 ± 31.25	15.28 ± 2.37	5.28 ± 1.71
GSH d7	55.67 ± 5.51	23.14 ± 9.34*	53.75 ± 3.07*	77.79 ± 17.20*	19.55 ± 3.71	7.27 ± 3.32
PBS d12	6.02 ± 2.08	22.74 ± 7.72	16.50 ± 18.11	26.46 ± 18.47	5.23 ± 1.04	13.74 ± 2.62
GSH d12	20.85 ± 5.87	27.70 ± 5.68	58.13 ± 6.77*	9.79 ± 5.60	3.29 ± 0.34	27.33 ± 3.67*

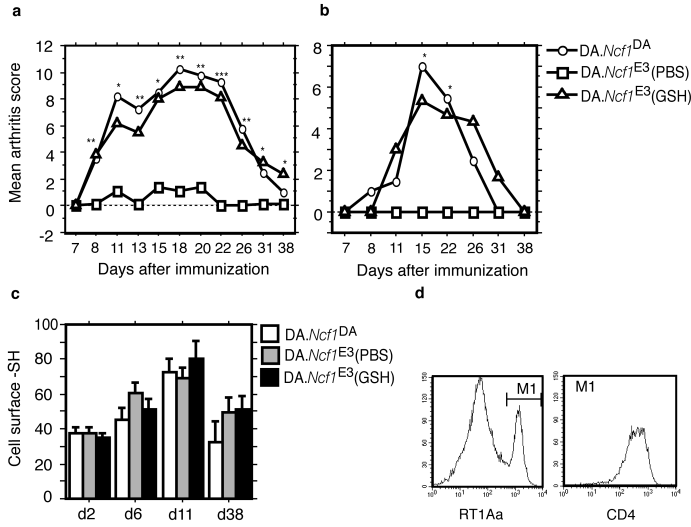
[a] % of RT1A^{a+} CD4^{+}TCR^{+} cells amongst all CD4^{+}TCR^{+} cells

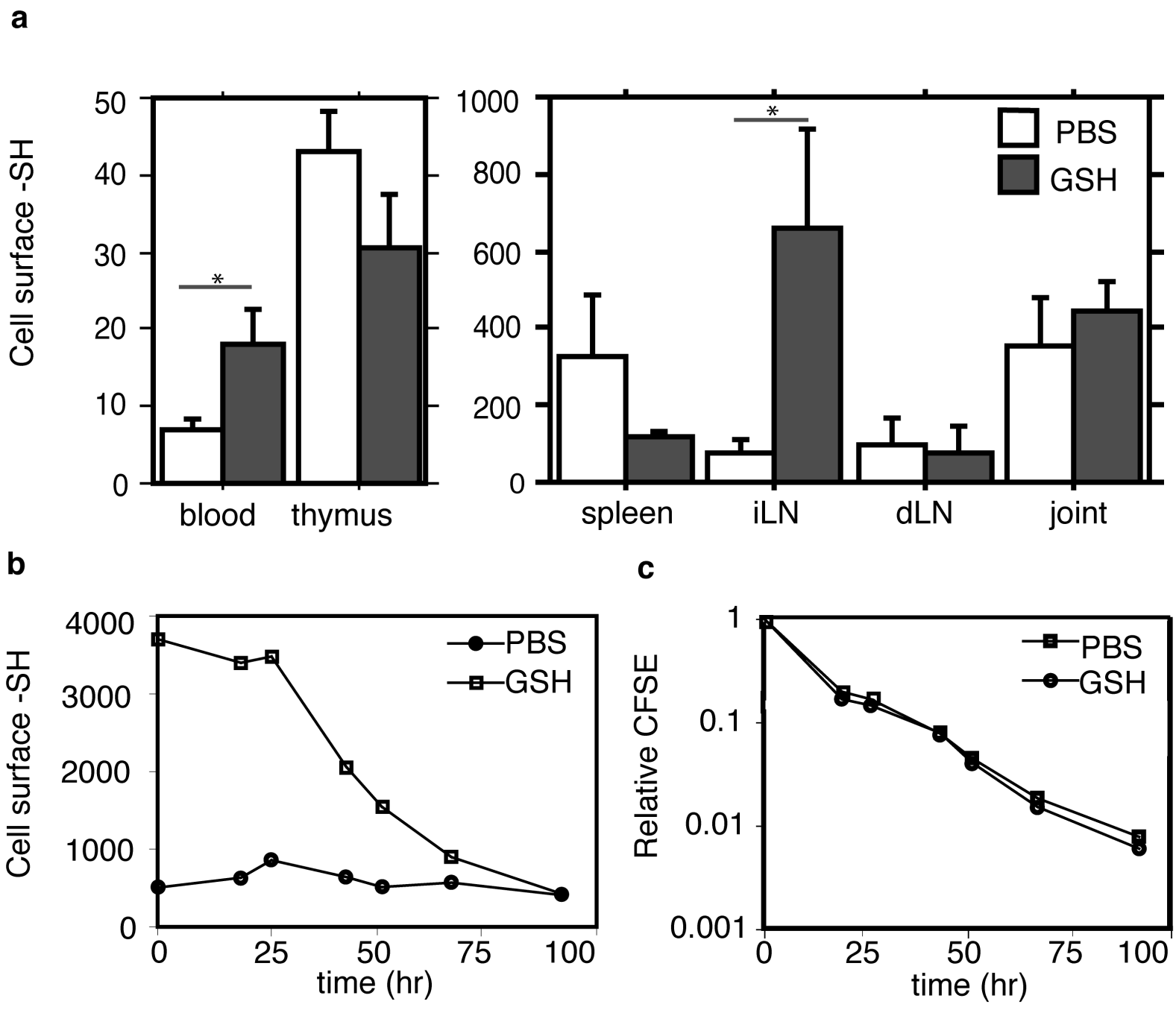
* $p<0.05$

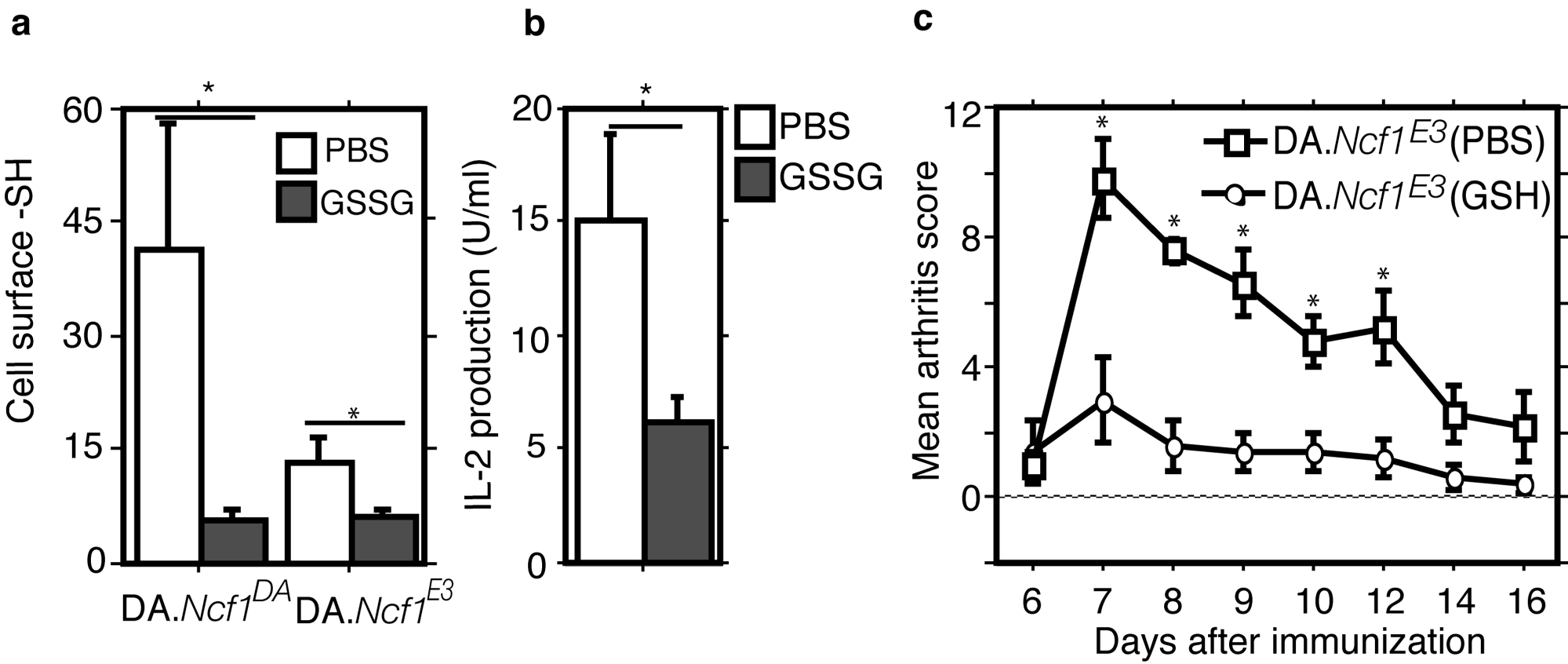

a
Oxidative burst
2000
1500
1000
500
0
DA.Ncf1 DA
DA.Ncf1 E3
*
*
ctr
PMA
TCR+CD4+
ctr
PMA
Granulocytes
b
Ncf1expression
300
225
150
75
0
DA.Ncf1 DA
DA.Ncf1 E3
*
ctr
TCR+CD4+
Gr1+
c
Cell surface -SH
250
200
150
100
50
0
*
*
CD4+
CD8+
d
MCB fluorescence
16
12
8
4
0
CD4+
e
-SH (mM GSH)
10
8
6
4
2
0
*
plasma
DA.Ncf1 DA
DA.Ncf1 E3


a
160
120
80
40
0
Cell surface -SH
PBS
NAC
GSH
DA.Ncf1DA
DA.Ncf1E3
b
Cell surface -SH (GeoMeanX)
PBS
GSH
CD4
c
45
30
15
0
MCB fluorescence
PBS
NAC
GSH
DA.Ncf1DA
DA.Ncf1E3
d
24
18
12
6
0
% PI+ cells
rel. ALM-staining
% PI+ cells
relative ALM-staining
1
2
4
8
16
32
64
GSH (mM)


a
b
40
30
20
10
0
IL-2 production (U/ml)
*
PBS
GSH
GSH trea-
ted T cells
GSH trea-
ted APC's
758
M1
PBS
454
M1
GSH
CFSE


a
b
c
d
Mean arthritis score
Days after immunization
Days after immunization
DA.Ncf1DA
DA.Ncf1E3(PBS)
DA.Ncf1E3(GSH)
Cell surface -SH
d2
d6
d11
d38
M1
M1
RT1Aa
CD4

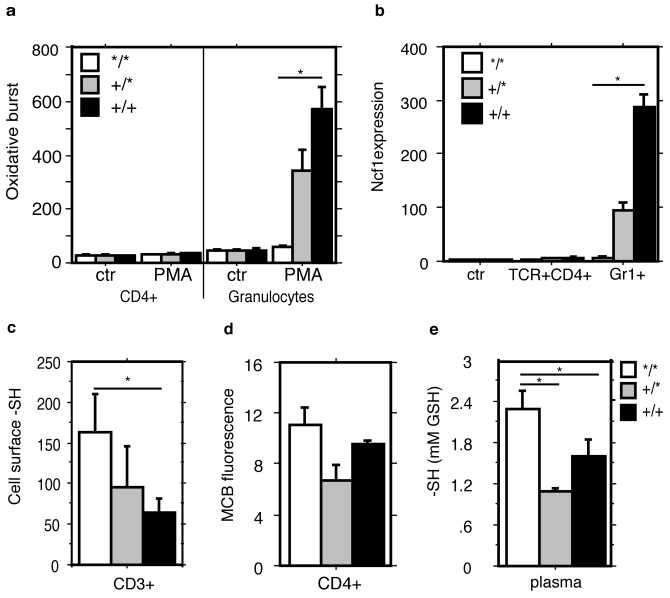

a
Cell surface -SH
50
40
30
20
10
0
1000
800
600
400
200
0
*
*
PBS
GSH
blood
thymus
spleen
iLN
dLN
joint
b
Cell surface -SH
4000
3000
2000
1000
0
PBS
GSH
0
25
50
75
100
time (hr)
c
Relative CFSE
1
0.1
0.01
0.001
PBS
GSH
0
25
50
75
100
time (hr)


a
b
c
Cell surface -SH
IL-2 production (U/ml)
PBS
GSSG
DA.Ncf1DA
DA.Ncf1E3
Mean arthritis score
DA.Ncf1E3(PBS)
DA.Ncf1E3(GSH)
Days after immunization

Supplementary text to supplementary Fig1

Littermate offspring from intercrossed B10.Q.Ncf1+/* mice (+ refers to wildtype and * refers to the mutation in exon 8 of the *Ncf1* gene; described previously by Huang et al. (5) and Hultqvist et al. (4)) was used for experiments. Oxidative burst was determined by staining with DHR123 after or without stimulation with PMA. As shown in Suppl Fig 1a, granulocytes from Ncf1 wildtype mice (B10.Q.Ncf1+/+; +/+) exerted significant higher levels of oxidative burst as compared to Ncf1 mutated mice (B10.Q.Ncf1*/*; */*). No oxidative burst was detected in T cells. Ncf1 expression was determined by intracellular staining with a mouse-anti-human Ncf1 antibody, cross-reacting with mouse (clone D-10; Santa Cruz Biotechnology, Santa Cruz, CA). It was shown, that granulocytes from Ncf1 wildtype mice expressed significantly higher levels of Ncf1 as compared to Ncf1 mutated mice, whereas T cells of none of the mice expressed Ncf1 (Suppl Fig 1b). Cell surface –SH group levels were determined by staining with Maleimide-Alexa-633 (16). T cells from mice expressing mutated Ncf1 had higher levels of cell surface –SH groups (Suppl Fig 1c), whereas intracellular thiol levels did not differ (Suppl Fig 1d; expressed in MCB fluorescence). The number of –SH groups in plasma, was determined with DTNB and is expressed as the number of –SH groups comparable to the indicated concentration of a GSH standard in mM. Ncf1 mutated mice had higher levels of –SH groups in plasma as compared to wildtype or heterozygous mice (Suppl. Fig 1e). So a decreased ability to exert oxidative burst due to a mutation in Ncf1, results in increased levels of cell surface –SH groups on T cells and increased levels of –SH groups in plasma of Ncf1 mutated

mice. These data are similar to those obtained in rats with a polymorphism in Ncf1 (Fig1).


a
b
c
d
e
Oxidative burst
800
600
400
200
0
/
+/*
+/+
*
ctr
PMA
ctr
PMA
CD4+
Granulocytes
Ncf1expression
400
300
200
100
0
ctr
TCR+CD4+
Gr1+
Cell surface -SH
250
200
150
100
50
0
CD3+
MCB fluorescence
16
12
8
4
0
CD4+
-SH (mM GSH)
3
2.4
1.8
1.2
.6
0
plasma