ORIGINAL RESEARCH COMMUNICATION

Regulation of T Cell Function by Reactive Nitrogen and Oxygen Species in Collagen-Induced Arthritis

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Abstract

Aims: In this study, we investigate the role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in autoimmune diseases. We focus on oxidative regulation at the interaction between antigen-presenting cells (APCs) and T cells, and consequent effect of ROS and RNS on type II collagen (CII)-induced arthritis (CIA) model in mice.

Results: Mice deficient in ROS and peroxide, due to a mutation in *Ncf1* gene, develop an exaggerated CIA and a stronger T cell response to CII. In contrast, nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME) was found to protect against CIA. The most pronounced protective effect was observed when L-NAME treatment started immediately after CII immunization. Ten days after immunization, the CII-reactive T cell-proliferative response was greater in *Ncf1*-mutant mice that were treated with L-NAME. T cells from L-NAME-treated mice, primed with CII, showed lower interleukin-2 secretion in response to CII *in vitro*. Moreover, inhibition of RNS production resulted in dysregulation of NOS1 (neuronal) expression in CII-reactive T cells.

Innovation and Conclusion: The results support that deficiency of a paracrine factor as ROS and peroxide released by APC leads to pronounced activation of T cells and enhanced arthritis. An intrinsic factor might be RNS produced by NOS1, which likely enhanced T cell activation in an autocrine manner. *Antioxid. Redox Signal.* 32, 161–172.

Keywords: reactive nitrogen species, reactive oxygen species, NOS1, NCF1, arthritis, autoimmunity

Introduction

PROFESSIONAL PHAGOCYTES, including neutrophils and macrophages, are highly effective in the recognition and processing of pathogens by releasing a package of reactive oxygen species (ROS) and reactive nitrogen species (RNS) through the NADPH oxidase 2 (NOX2) complex and nitric oxide synthase (NOS), respectively (8). The original product of the NOX2 complex activation is superoxide anion radicals, leading to accumulation of more long-lived peroxide (42). NOS-derived nitric oxide (NO) is a substrate of superoxide anion to produce peroxynitrite (37). NO and its metabolites are called RNS. Compared with ROS, the role of RNS is often underappreciated in the event of oxidative burst, a triggered rapid release of radicals by phagocytes.

Oxidative burst plays a dual role in protecting against infections through professional phagocytes. Excessive ROS/

RNS can cause permanent damages to cellular proteins, lipid, and DNA, but beneficial for the killing of phagocytized bacteria and fungi (37). A human disease that illustrates the crucial role of induced oxidative burst is chronic granulomatous

Innovation

The present study for the first time showed that reactive nitrogen species (RNS) are pathogenic in type II collagen (CII)-induced arthritis during the priming phase. In contrast to the well-known protective role of reactive oxygen species (ROS) from antigen-processing cells, an autocrine pathway of RNS was suggested to increase CII-reactive T cell activation in ROS-deficient mice with the *Ncf1* polymorphism, leading to enhanced arthritis.

disease (CGD). CGD is caused by mutations in genes encoding the NOX2 complex subunits. These mutations result in increased both infections and susceptibility to autoimmune diseases (17).

Oxidative burst can have a profound effect on adaptive immunity. For example, neutrophil cytosolic factor 1 (NCF1) (alias p47^{PHOX}) is a subunit of the NOX2 complex. NCF1 deficiency, which may be caused by polymorphism or spontaneous mutations, can result in a lower oxidative burst and the development of more severe autoimmune disease in rats (12, 26), mice (11), and humans (27, 28, 41). The ROS-derived hydrogen peroxide has been shown to play a protective role in downregulation of T cell activation by antigen-presenting cells (APCs) in animal models of autoimmune diseases, including rheumatoid arthritis (RA) (6). The other arm of oxidative stress is RNS, but its regulatory role in autoimmune diseases and the adaptive immune system is not well known.

Type II collagen (CII)-induced arthritis (CIA) in mice is the most commonly used animal model of RA (9). It is an optimal model to study the mechanisms of adaptive immune response to a defined autoantigen that leads to arthritis development. The same determinant core of CII was identified as CII residues 259–273 (CII_{259–273}) in glycosylated form for autoreactive T cells in both patients with RA and mice with CIA (1). T cells expressed no or very low level of NCF1 or exerted an oxidative burst, when induced by inflammatory stimuli (7). In contrast, phagocytes, APCs, and B cells express NCF1. They produced ROS/RNS when stimulated by inflammatory factors or upon interactions with T cells (7). Macrophage-restricted NCF1 expression restored arthritis resistance to the level of wild-type mice by dampening T cell activation in the CIA model (7, 30). Therefore, the CIA model was used to investigate a potentially interactive role of RNS.

High amounts of RNS are produced by murine macrophages in an inflammatory tissue or during an immune response (35). The availability of NOS inhibitors and mouse strains deficient in inducible NOS (NOS2) has improved our understanding of RNS and opens the possibility to unravel their immune regulatory functions (18). Cuzzocrea *et al.* reported that mice treated with an NOS2 inhibitor GW274150, similar to *Nos2*-knockout mice, had a delayed development of clinical signs and attenuated severity of CIA, compared with wild-type *C57BL/6J* mice (4). However, Sakaguchi *et al.* showed that

administration of the NOS2 inhibitor 1400W exhibited no effect on arthritic inflammation in *DBA/1J* mice (33).

These conflicting results can be due to many reasons. For instance, mouse strains used in these studies were different. It is known that macrophages from *C57BL/6J* mice produce a higher level of RNS in response to lipopolysaccharide than macrophages from *DBA/1J* mice (22). Furthermore, *C57BL/6J* mice do not develop a classical CIA compared with *DBA/1J* mice (2, 13). The susceptibility of *C57BL/6J* mice bearing the major histocompatibility complex (MHC) H-2^b haplotype to arthritis is highly variable as it is known to be triggered by contaminations in the CII preparations. T cell recognition of the glycosylated CII epitopes is not seen in *C57BL/6J* mice (2). In the current study, aimed to clarify the roles of ROS and RNS in CIA development, we used *B10Q* and *B6NQ* mouse strains bearing MHC-II Aq molecules, based on the *C57BL* genetic background, allowing induction of classical and well-defined CIA (2).

This study focused on the interactions between ROS and RNS. Their effects were investigated on CII-reactive T cell activation and the development of CIA in mice with genetic disruption of *Ncf1* gene. Reduction of RNS production was achieved by using NG-nitro-L-arginine methyl ester (L-NAME) as a NOS inhibitor (38), and by investigating mice with deficiency of NOS2 and NCF1.

Results

Treatments with NG-nitro-L-arginine methyl ester ameliorate CIA

ROS and RNS can be produced in excessive quantities by activated macrophages under inflammatory conditions. In CIA, activated macrophages process and present CII to T cells (7, 30). *Ncf1*-mutant macrophages enhanced T cell activation during both priming and effector phases of CIA (30). Here, we investigated the effects of inhibiting NOS on CIA in ROS-sufficient and ROS-deficient mice. This was achieved by the intraperitoneal administration of NG-nitro-L-arginine methyl ester (L-NAME) every other day immediately after CII immunization, until the end of arthritis experiment, that is, the 60th day post-immunization (DPI). Compared with the phosphate-buffered saline (PBS)-treated mice, L-NAME treatment resulted in a reduction in clinical scores of arthritis in both *Ncf1* wild-type (*B10Q.Ncf1*^{+/+}) and *Ncf1*-mutant

FIG. 1. Treatments of L-NAME suppress CIA in mice. (A) *Ncf1* wild type mice (*B10Q.Ncf1*^{+/+} genotype) with intraperitoneal injection of L-NAME ($n = 13$, of which 9 mice got arthritis) developed the less severe arthritis in comparison to wild-type mice receiving PBS ($n = 16$, of which 15 mice got arthritis). *Ncf1*-mutant mice (*B10Q.Ncf1*^{*/*} genotype) with intraperitoneal injection of L-NAME ($n = 17$, of which 16 mice got arthritis) developed less severe arthritis in comparison to *Ncf1*-mutant mice receiving PBS ($n = 16$, all of mice got arthritis). The treatment was performed every other day from 0 to 60 DPI. (B) A representative image shows the distribution of ROS and RNS in both *B10Q.Ncf1*^{+/+} and *B10Q.Ncf1*^{*/*} mice at 50 DPI. A representative image of the naive mice is included for comparison. (C) A positive correlation exists between the L-012-derived optical intensity and the clinical score of arthritis. Each spot ($n = 85$) came from an imaging of the *B10Q.Ncf1*^{+/+} mouse after immunization with CII. The same mice were imaged at 10, 45, and 50 DPI. The number was 53 of mice with arthritis score ≥ 1 . **** $p < 0.0001$. (D) The optical radiance at paws and hind legs was detected from *Ncf1* wild type and *Ncf1*-mutant mice. (E) The representative imaging of each group shows the H&E staining of joints between tibia and talus in mice at 60 DPI, coupled with the naive mice. (F) H&E staining of joints was performed in mice at 60 DPI. Typical ankle joints of CIA mice displayed bone erosion and massive cell infiltration into the synovia, compared with normal histologic architecture from the naive mouse. Mice with CIA receiving L-NAME treatments showed a smaller articular cavity, pannus formation, and bone/cartilage destruction in the mouse in comparison to mice with CIA receiving PBS treatments. (G) The serum levels of anti-CII IgG, IgG₁, and IgG_{2b} antibodies were determined at 60 DPI. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as determined by the Mann-Whitney *U* test. CIA, CII-induced arthritis; CII, type II collagen; DPI, day post-immunization; H&E, hematoxylin and eosin; IgG, immunoglobulin G; L-NAME, NG-nitro-L-arginine methyl ester; PBS, phosphate-buffered saline; RNS, reactive nitrogen species; ROS, reactive oxygen species. Color images are available online.

(*B10Q.Ncf1^{*/*}*) mice (Fig. 1A), whereas an effect on the day of arthritis onset or incidence did not reach statistical significance ($p < 0.05$) (Supplementary Fig. S1A).

The typical day of arthritis onset for *B10Q.Ncf1^{*/*}* mouse strain is 20–25 DPI. The onset day is after 35 DPI for *B10Q.Ncf1^{+/+}* mouse strain. To investigate the role of RNS during the onset phase of CIA, we treated the mice with L-NAME and NG-nitro-D-arginine methyl ester (D-NAME) from 18 DPI to 60 DPI (Supplementary Fig. S1B). To investigate the role of RNS during the effector phase of CIA, we also studied the effect of NOS inhibition after the booster immunization through L-NAME treatments between 35 and 60 DPI (Supplementary Fig. S1C). Neither the disease onset nor severity was regulated by NOS inhibition in both *Ncf1* wild-type and *Ncf1*-mutant mice if L-NAME treatments were given after the priming phase of CIA at 18 DPI (Supplementary Fig. S1B, C).

In summary, we showed that RNS exhibited a pathogenic role during the initiation of CIA. The suppressive effect of L-NAME on arthritis was more pronounced in *Ncf1*-mutant mice.

RNS production correlates with severity of arthritis

To visualize the effects of L-NAME on RNS depletion during the course of arthritis development in Figure 1A, we performed optical imaging of the same mice at 10, 45, and 50 DPI, using chemiluminescent probe L-012 previously shown to be highly sensitive to peroxynitrite (43). Figure 1B shows a representative image of the whole-body distribution of L-012-detectable RNS in both *Ncf1* wild-type and *Ncf1*-mutant mice with arthritis at 50 DPI, together with a pair of naive mice. Only a few photons arising from the random occurrence were detected in *Ncf1*-mutant mice (*B10Q.Ncf1^{*/*}*), and its optical distribution was used for determination of sample-specific background signaling and optical calibration. This observation reflected peroxynitrite deficiency, that is, the NOX2 complex deficiency in *Ncf1*-mutant mice. The result is consistent with the fact that *Ncf1*-mutant neutrophils and macrophages fail to produce ROS in response to PMA stimulation (31).

In *Ncf1* wild-type (*B10Q.Ncf1^{+/+}*) mice with CIA, we observed that the mean radiance of both forepaws and hind paws correlated well with clinic score of arthritis severity per mouse (Fig. 1C). Figure 1D illustrates that L-NAME administration reduced the RNS level at forepaws and hind paws of *B10Q.Ncf1^{+/+}* mice.

In summary, we provided direct optical evidence of a long-term overall reduction in RNS production at the inflammatory joints of *Ncf1* wild-type mice treated with L-NAME.

*L-NAME reduces joint inflammation and the CII antibody response of *Ncf1*-mutant mice to the level of wild-type mice*

To confirm the effects on joint inflammation by L-NAME, both the left hind paw and serum were collected from each mouse at 60 DPI. We examined the histopathological changes in ankle joints and anti-CII antibody titers in serum. Figure 1E shows the representative hematoxylin and eosin (H&E) staining of ankle joints between tibia and talus in *B10Q.Ncf1^{*/*}* and *B10Q.Ncf1^{+/+}* mice. Overall, L-NAME-treated *B10Q.Ncf1^{*/*}* mice developed milder joint inflammation with a lower histology score than the PBS-treated *B10Q.Ncf1^{*/*}* mice (Fig. 1F). The L-NAME-treated *B10Q.Ncf1^{*/*}* mice also developed

lower levels of anti-CII antibodies in the serum (immunoglobulin G [IgG], IgG1, and IgG2b) (Fig. 1G), demonstrating an immune-suppressive effect of inhibiting NOS in CIA.

In summary, these results demonstrated that while ROS were protective in the CIA model, lowering of RNS reduced the inflammatory response to CII in *Ncf1*-mutant mice to the level of *Ncf1* wild-type mice.

RNS enhance CII-specific immune response at the priming phase of CIA

Oral treatment of NOS2 inhibitors was administrated by previous studies of CIA (4, 33). In the following experiments, we performed oral administration instead of intraperitoneal administration. Here, we aimed to evaluate the effects of both L-NAME and its inactive enantiomer D-NAME, given in the drinking water to *Ncf1*-mutant mice (*B10Q.Ncf1^{*/*}*) between day 0 and day 40, which approximately corresponds to the period of immune priming to CII and the disease in CIA. We observed that L-NAME-treated mice developed milder arthritis than D-NAME-treated mice (Fig. 2A). Clinical phenotypes were consistent with the above result (Fig. 1A). To evaluate the levels of both serum NO (nitrate–nitrite) and anti-CII antibodies (IgG, IgG1, and IgG2b) before the development of any established CIA, we collected serum just before or at the clinical onset of CIA, that is, 21 DPI. The assessment showed that L-NAME reduced the levels of both serum NO (Fig. 2B) and anti-CII antibodies at the early stage of CIA induction (Fig. 2C).

In summary, oral administration of L-NAME inhibited RNS production in blood, suppressed the immune response as determined by the level of anti-CII IgG antibodies at the priming phase, and protected the *Ncf1*-mutant mice against disease.

NOS2-independent RNS enhance T cell activation by ROS-deficient APCs

To identify the effect of ROS and RNS on antigen-specific T cell activation at the priming stage of CIA, we investigated the interleukin-2 (IL-2) production of CII-reactive HCQ3 T hybridoma cells stimulated by the naive APCs. HCQ3 T cells expressed an $\alpha\beta$ T-cell receptor (TCR) specific for CII and the post-translational modification variant of CII_{259–273} peptide (3, 32). Treatment with L-NAME suppressed IL-2 production in the presence of APC from *Ncf1*-mutant mice (*B6NQ.Ncf1^{*/*}.Nos2^{-/-}* and *B6NQ.Ncf1^{*/*}.Nos2^{+/+}*) (Fig. 3A, B). To investigate the role of NOS2, which is expressed in macrophages and mediates NO production, we analyzed splenocytes from the naive *B6NQ.Ncf1^{+/+}.Nos2^{-/-}* mice and their *B6NQ.Ncf1^{+/+}.Nos2^{+/+}* littermates, as well as the naive *B6NQ.Ncf1^{*/*}.Nos2^{-/-}* mice and their *B6NQ.Ncf1^{*/*}.Nos2^{+/+}* littermates. Surprisingly, NOS2 deficiency did not confer any significant benefit on IL-2 production (Fig. 3C).

To test whether NOS2 could influence arthritis, the mice were immunized with CII. NOS2 deficiency did not impact the susceptibility to CIA (Fig. 3D). With the ROS-sufficient APC from *B10Q* mice with the wild-type *Ncf1* gene, L-NAME had no effect on IL-2 production, regardless of NOS2 expression (Supplementary Fig. S2). It confirmed again the previous result of the APC-produced ROS having a protective role in the induction of CIA (30). In summary, the results demonstrated that 24 h after the initial antigen stimulation presented by APC from the naive NCF1-deficient mice, IL-2

FIG. 2. RNS modulate the immune response to CII in *Ncf1*-mutant mice. The *Ncf1*-mutant mice (*B10Q.Ncf1*^{*/*} genotype) were treated with D-NAME ($n=11$, all mice developed arthritis) and L-NAME ($n=13$, of which 10 mice developed arthritis) in the drinking water from 0 to 40 DPI. We immunized the mice with CII at day 0 and collected the blood sample by submandibular bleeding at day 21. (A) The clinical scores of arthritis were suppressed by L-NAME compared with D-NAME. (B) The serum levels of NO (nitrate and nitrite) were determined at 21 DPI. (C) The serum levels of IgG, IgG₁, and IgG_{2b} anti-CII antibodies were titrated at 21 DPI. * $p < 0.05$ and ** $p < 0.01$ as determined by the Mann-Whitney *U* test. D-NAME, NG-nitro-D-arginine methyl ester. Color images are available online.

production of CII-specific T cells was mediated by NOS inhibitors, regardless of NOS2 expression in the APC. Accordingly, NOS2 did not influence CIA development.

Expression of constitutive NOS in the APC is not regulated by L-NAME

Apart from NOS2, we therefore investigated whether the RNS reduction was associated with constitutive NOS1 (neuronal) and NOS3 (endothelial) in the APC. This was achieved by measuring the expression of NOS1 and NOS3 in the feedback response to L-NAME, using transgenic mice of *B10NQ.Ncf1*^{*/*}.HCQ3^{tg/+}.CD45.1^{tg/+} strain expressing $\alpha\beta$ TCR (HCQ3) specific for CII (32). To inhibit NOS, L-NAME was added to drinking water for 10 days after immunization. At day 10, we determined NOS expression in lymph nodes and spleen cells by flow cytometric analysis. The phenotype was a mean fluorescence intensity (MFI) of anti-NOS1 or NOS3 conjugated to phycoerythrin (PE) in a population of cells. However, we found no expressional shift in myeloid or B cells from L-NAME-treated mice (Supplementary Figs. S3 and S4). Likewise, there was no difference in MHC-II gene expression in the same cell populations of APCs (Supplementary Figs. S3 and S4).

In summary, administrations of L-NAME and D-NAME in drinking water did not alter NOS1, NOS3, or MHC-II expression by APCs during priming phase of CIA.

NOS1 and Ki67 expression in T cells was enhanced by L-NAME

Our results thus indicate that NOS expression in T lymphocytes should be of the prime interest to be defined to ex-

plain the effect of L-NAME on the immune responses to CII. To verify this hypothesis, we studied the expression of NOS1 and NOS3 in CII-reactive T cells (CD3⁺CD4⁺CD45.1⁺) in the above experiments with TCR transgenic and *Ncf1*-mutant mice treated with L-NAME or D-NAME from day 0 to day 10 after immunization of CII. Interestingly, we observed that L-NAME resulted in only an increased NOS1 expression (mean, 247.1), when compared with D-NAME (mean, 217.2), as determined by MFI in CD4 T cells (Fig. 4C).

Next, we assessed *in vivo* proliferation of T cells from L-NAME- and D-NAME-treated mice at 10 DPI. Our results show that the frequency of Ki67⁺ cells in CD4 T cells was significantly higher in the L-NAME group (mean, 25.1%) than in the control (mean, 22.1%) (Fig. 4D). These data suggest that NOS inhibitor benefited antigen-specific proliferation of T cells by modulating the intrinsic factor NOS1 expression *in vivo*. In addition, we determined FOXP3 expression in lymph nodes and spleen cells of *Ncf1*-mutant transgenic mice at 10 DPI; the frequency of regulatory T cells (CD4⁺CD25⁺FOXP3⁺) in CD4 T cells and FOXP3 expression in regulatory T cells were not affected by L-NAME treatment when compared with D-NAME treatment (Supplementary Fig. S5A).

Furthermore, we performed the antigen recall assay to examine the effect of L-NAME in memory maintenance. At 10 DPI with CII, the cell suspensions of the spleen and lymph nodes collected from immunized mouse were reactivated *in vitro* by CII. We used the nonmodified (naked-CII_{259–273}) and glycosylated CII peptides (Gal-CII_{259–273}) and concanavalin A (ConA) as controls for CII. We observed that L-NAME treatment resulted in a reduction in IL-2 production, in response to CII and ConA stimulation (Fig. 4E). However,

FIG. 3. CII-specific IL-2 production is increased by NOS2-independent NO. CII-specific T cells came from the HCQ3 hybridomas cell line, and IL-2 production in cell culture supernatant was assayed after incubation of T cells and APCs for 24 h, in the presence of PBS, D-NAME, or L-NAME. (A) The APCs were collected from the naive *Ncf1*-deficient mice (*B6NQ.Ncf1*^{+/+}.*Nos2*^{+/+} genotype, *n* = 8) and (B) their littermates *Ncf1*-*Nos2* double-gene-deficient mice (*B6NQ.Ncf1*^{+/+}.*Nos2*^{-/-} genotype, *n* = 8). (C) The APCs were collected from the naive *Nos2* wild type mice (*B6NQ.Ncf1*^{+/+}.*Nos2*^{+/+} genotype, *n* = 8) and their littermates *Nos2*-deficient mice (*B6NQ.Ncf1*^{+/+}.*Nos2*^{-/-} genotype, *n* = 8) in the presence of PBS. (D) *Nos2* deficiency had no effect on the development of CIA. The clinical scores were shown in *B6NQ.Ncf1*^{+/+}.*Nos2*^{-/-} (*n* = 22, of which 21 mice got arthritis) mice and their littermates *B6NQ.Ncf1*^{+/+}.*Nos2*^{+/+} mice (*n* = 18, of which 16 mice got arthritis), together with *B6NQ.Ncf1*^{+/+}.*Nos2*^{-/-} (*n* = 14, and all got arthritis) mice and their littermates *B6NQ.Ncf1*^{+/+}.*Nos2*^{+/+} mice (*n* = 13, and all got arthritis). These mice were immunized once with CII at day 0. The asterisk by the value denotes a significant difference between two groups of mice receiving L-NAME and PBS (Mann-Whitney *U* test), and differences between two groups of mice receiving L-NAME and D-NAME that are statistically significant are marked with a hashtag. *p < 0.05, #*p* < 0.05 and **p < 0.01 as determined by the Mann-Whitney *U* test. APCs, antigen-presenting cells; IL-2, interleukin-2; NO, nitric oxide; NOS, nitric oxide synthase. Color images are available online.

we could not find any statistical difference in secreted IL-17 in the above cell culture supernatant (Supplementary Fig. S5B). Additionally, we did not observe any expressional difference of either BCL2 in APCs (Supplementary Fig. S5C) or xanthine oxidoreductase (XOR) in APCs and T cells (Supplementary Fig. S5D) that could influence the observed effects.

In summary, *in vivo* administration of L-NAME in *Ncf1*-mutant mice led to increased expression of NOS1 and Ki67 in antigen-specific CD4 T cells, and these activated T cells produced less IL-2 during *in vitro* stimulation with recall antigens (Fig. 5).

Discussion

The association between the *Ncf1* gene polymorphism and autoimmune diseases has been shown in both rodents and humans (42). The disease association with a single nucleotide polymorphic mutation in *Ncf1* gene was first shown in rats (12, 26). Mice with a natural point mutation in the *Ncf1* gene were also found to develop more severe arthritis and lupus (11, 16). Subsequent studies in humans demonstrated a strong association in humans between a coding single nucleotide polymorphism in the *NCF1* gene and subsequent lower production of ROS, and a higher risk of developing diseases, such systemic lupus erythematosus (27, 41). A copy number variation in the *NCF1* gene was

also associated with RA (28). In this study, we showed a dramatic protective effect on arthritis by L-NAME in *Ncf1*-mutant mice. Future studies investigating the possibility of such treatment in patients expressing a polymorphic quantitative trait nucleotide in the *NCF1* gene with a lower production of ROS will be interesting.

In studies of the NCF1/NOX2 complex deficiency in autoimmunity, the role of RNS has not yet been clearly addressed. Complete deficiency of NCF1 or the NOX2 complex leads to the development of CGD, with both infection sensitivity and increased autoimmune manifestations (17). Nevertheless, it remains unclear what is the molecular control of enhanced susceptibility to autoimmune disease in the absence of NOX2-mediated protection. A basic problem is to determine the role of radical and the nonradical reactive species in oxidative burst. It is known that the commercial probe dihydrorhodamine 123 (DHR) is not specific for ROS detection in clinical or laboratory samples because DHR reacts with RNS as well (14, 15). Additionally, the effect of *Ncf1*-mutant gene might be compensated by elevated expression of other genes. The compensation response could be dysregulated NOS expression. In this study, we demonstrate that a pathogenic role of RNS might be amplified in mice with a mutant *Ncf1* gene.

Recently, we showed that the *Ncf1* mutation not only led to a deficiency of the NOX2-derived ROS but also resulted in a higher level of RNS in the mannan-induced psoriatic arthritis

FIG. 4. NOS1 expression and CII-reactive T cell activation are regulated by L-NAME. TCR transgenic and *Ncf1*-mutant mice (*B10NQ.Ncf1^{+/+}.HCQ3^{tg/+}.CD45.1^{tg/+}* genotype, $n = 10$ randomly divided into two groups for L-NAME or D-NAME treatments) were immunized with CII. Single cell suspensions were prepared from pooled spleen and inguinal lymph nodes from individual mouse. FACS plot of T cells was derived from cell suspensions at 10 DPI. (A) A representative Zebra plot of single cell suspensions stained for CD3, CD4, CD45.1, CD25, and FOXP3, showing populations of regulatory T cells. (B) The absolute number of CII-reactive CD4 T cells ($CD3^+CD4^+CD45.1^+$) was from a cell counter, and the frequency of CD4 cells in single cell suspensions was measured by using FACS analysis. (C) The intensity of NOS1 and NOS3 expression per CD4 T cell is shown as MFI. (D) The frequency of Ki67-positive cells in CD4 T cells was determined by FACS analysis. (E) The level of IL-2 production in culture supernatant was determined after incubation for 72 h. Each symbolic marker denotes one sample per mouse in the graph. * $p < 0.05$ and ** $p < 0.01$ as determined by the Mann-Whitney *U* test. ConA, concanavalin A; FACS, fluorescence-activated cell sorter; MFI, mean fluorescence intensity; TCR, T-cell receptor. Color images are available online.

(MIP) model (43). The NOS2-dependent RNS from the resident macrophage were found essential to drive the psoriatic disease *via* the IL-1 α signaling (43). Of interest is that the disease severity of MIP was not modulated by neutralizing the IL-1 β signaling. However, IL-1 β signaling was shown pathogenic in *Ncf1*-mutant mice in K/BxN serum-induced arthritis model that mimics the effector phase of RA, and NOS2 deficiency did not affect the severity of joint swelling (10).

In the present study, we observed that L-NAME treatment protected *Ncf1*-mutant mice against CIA if treatments were given during the priming phase but not during the effector phase. In contrast to a pathogenic role of NOS2 previously shown in MIP, which is a disease independent of MHC-II-restricted T cells (43), we now show that only NOS inhibition, but not NOS2 deficiency, is in fact associated with CII-reactive

T cell proliferation and activation and consequently suppressed arthritis development. Therefore, the regulation of constitutive NOS in T cells might explain the protection against CIA afforded by L-NAME treatment. Tracking of NOS1 and NOS3 in APCs and T cells is therefore an emerging issue in ROS-deficient mice. Overall inhibition of the NOS activity with L-NAME has been shown to induce the protein synthesis of NOS1 and NOS3 by activating *de novo* pathway as a negative feedback loop (36). García-Ortiz and Serrador suggested that NOS3 expression of human T cells is important in antigen processing and presentation (5). In the present study, we found that the protein expression of NOS1 in antigen-primed T cells, but not NOS3, was affected by L-NAME treatment *in vivo*.

In contrast to CII-reactive T cells, protein levels of NOS1 and NOS3 did not significantly change in APCs. Of importance,

FIG. 5. The schematic summary of CII-reactive T cells activation regulated by ROS and RNS at the APC–T cell interaction in paracrine and autocrine manners. (A) The schematic plot shows that CII-reactive T cell produces IL-2 in the presence of ROS released by the NCF1-sufficient APC. **(B)** The schematic plot shows that the NCF1-deficient APC stimulates CII-reactive T cell to produce IL-2 in the absence of NCF1-derived ROS, and its NOS1-derived RNS production can be suppressed by using NOS inhibitor L-NAME. NCF1, neutrophil cytosolic factor 1. Color images are available online.

MFI of NOS1 staining in CD4 T cells was more than twofolds higher than that in dendritic and B cells. It is possible that NOS1 regulates CII-reactive T cell during antigen presentation by releasing RNS in an autocrine manner. This is different from ROS signaling that regulates antigen processing in the APC (40) and impacts T cell activation by modifying T cell-surface thiol expression during antigen presentation in a paracrine manner (6). Subsequent studies will be very interesting to identify molecular mechanisms in mice model with specific NOS1 deficiency or overexpression in T cells.

The downstream of antigen-specific RNS signaling on T cell differentiation and activation has been an open question in common in the development of autoimmune disease. First, RNS were shown important to determine FOXP3 (19, 25) and IL-17 (24, 39) expression in models of autoimmune diseases. However, we did not observe a significant change in the frequency of regulatory T cells in CD4 T cells or the FOXP3 expression between these two groups of *Ncf1*-mutant mice during the priming phase. Second, it has been shown that the autophagy is important for antigen processing and presentation, and increased autophagy flux of CD4 T cells plays a role in the development of CIA (21, 34). However, we did not observe any expressional difference of BCL2 as a negative regulator of autophagy in APCs between two groups of mice during the priming phase. The increase of NOS1 expression in CII-reactive T cells might function as a negative regulator in the hyperactivation or apoptosis resistance to overactive or self-reactive immune responses.

An alternative possibility is that other sources of induced ROS in *Ncf1*-mutant mice might account for increased susceptible to autoimmunity, such as the enzyme XOR in purine metabolism. Our previous study showed that oral administration of L-NAME resulted in an increase of XOR expres-

sion in the liver (29). In the present study, we did not find any evident expressional difference of XOR in APCs and T cells that could influence the observed effects.

Apparently, ROS and RNS act differently in activating adaptive immune responses. Both ROS and RNS are short-lived free radicals, and real-time detection is challenging (15). There are studies of RNS released by L-NAME *in vitro*, due to the nitro group in its structure and Fenton chemistry, as well as the concentration of L-NAME and its prolonged persistent effect (20, 38). In other words, IL-2 production as the signature cytokine of selected CII-reactive T cells will provide indirect quantification of ROS/RNS effects (3). In our previous study (6), the paracrine-derived ROS signaling from the APC was shown to suppress the IL-2 production of T cells response to CII through reducing T cell surface thiol.

In the present study, we have showed that inhibition of NOS, most likely in the responding T cells, reduced IL-2 production due to stimulation with NCF1-deficient APCs. The production of IL-2 was comparably low with NCF1-sufficient APCs, and in this more normal situation, NOS inhibitor was no longer protective. In short, the present results may thereby open a new therapeutic possibility in autoimmune disease, by targeting RNS signaling in low ROS-producing individuals.

Materials and Methods

Animals

Founders of *B10Q* (*C57/B10.Q/rhd*) (*B10Q.Ncf1^{+/+}*) and *B6NQ* (*C57/B6N.Q/rhd*) (*B6NQ.Ncf1^{+/+}*) mice have been fully backcrossed and maintained in our laboratory (*rhd*). A point mutation in the *Ncf1* gene (*m1j*) (*Ncf1* protein also denoted p47phox) in the *B10Q* mice, designated as *B10Q.Ncf1^{m1j/m1j}* or *B10Q.Ncf1^{*/*}*, impairs the expression of the *Ncf1* gene, thereby totally blocking the function of the NOX2 complex. *Nos2*-knockout mice (*B6.129P2-Nos2^{tm1Lau/J}*) were obtained from the Jackson Laboratory (18) and were crossed to our *B6NQ* mice to get the *B6NQ.Ncf1^{+/+}.Nos2^{-/-}* mice with *B6NQ.Ncf1^{+/+}.Nos2^{+/+}* littermate controls in experiments. *B6NQ.Ncf1^{+/+}.Nos2^{-/-}* mice were crossed with *B6NQ.Ncf1^{*/*}* mice to generate *B6NQ.Ncf1^{*/*}.Nos2^{-/-}* mice with *B6NQ.Ncf1^{*/*}.Nos2^{+/+}* littermate controls. A male transgenic mouse of *B10NQ.HCQ3^{tg/+}.CD45.1^{tg/+}* (3) was used to cross with *B10Q.Ncf1^{*/*}* mice to collect the *B10Q.Ncf1^{*/*}.HCQ3^{tg/+}.CD45.1^{tg/+}* mice. The primers for *Nos2* genotyping are as follows: 5'-ACA TGC AGA ATG AGT ACC GG-3' (common), 5'-TCA ACA TCT CCT GGT GGA AC-3' (wild type), 5'-AAT ATG CGA AGT GGA CCT CG-3' (mutant).

All mice in this study bear the H2-A^q haplotype (9). Mice were housed under specific pathogen-free conditions in individual ventilated cages with wood shaving bedding, with a paper napkin as enrichment, and in a climate-controlled environment with 12-h light/dark cycles. All experiments were performed in male mice that were between 8 and 9 weeks old. Each adult mouse weighed ~25 g. The animal study protocols were approved by Stockholm regional animal ethics committee, Sweden (N490/12, N35/16).

Antibodies

The following antibodies were purchased from Bio-Legend, as TCR- β (clone: H57-597, PerCP/Cyanine5.5), CD11b (clone: M1/70, Pacific Blue), CD11c (clone: N418,

PE-Cyanine7), Gr1 (clone: RB6-8C5, APC), I-A/I-E (clone: M5/114.15.2, PerCP/Cyanine5.5), and BCL2 (clone: BCL/10c4, PE-Cyanine7). The following antibodies were purchased from BD Pharmingen, as CD16/CD32 (clone: 2.4G2, purified), Ly6C (clone: AL-21, FITC), CD19 (clone: 1D3, Pacific Blue), CD3 ϵ (clone: 145-2C11, FITC, or PerCP/Cyanine5.5), CD4 (clone: RM4-5, Pacific Blue, or FITC), CD25 (clone: PC61, PE, or PE-Cyanine7), NOS3 (clone: 33/eNOS, PE), and IL-17A (clone: TC11-18H10, APC). Antibodies for FOXP3 (clone: FJK-16s, APC) were purchased from eBioscience. Anti-XOR polyclonal antibody (Catalog No. PA5-26285) was purchased from Thermo Fisher Scientific. Anti-NOS1 (neuronal) polyclonal antibody (Catalog No. ab1376) was purchased from the Abcam. Antibodies were used according to the instructions from the manufacturers, and the stock solution was diluted at 1:200 for flow cytometric staining.

Collagen-induced arthritis

Each male mouse was first injected with 100 μ g of rat CII, which was emulsified in complete Freund's adjuvant (Catalog No. DF0638-60-7, Difco; Nordic BioLabs, Sweden) at the base of the tail (22). After 35 days, mice were boosted with 50 μ g of rCII in incomplete Freund's adjuvant (Catalog No. DF0639-60-6, Difco; Nordic BioLabs). Mice were scored every 2 or 3 days for inflammation in the peripheral joints; one point was given for each swollen and red toe or joint, and five points for a swollen ankle, adding up to a max score of 60 points per mouse (7).

Blocking NO production

NOS can be inhibited by L-NAME in an enantiomer-specific manner. The inhibitory effect could be reversed by L-arginine but not by D-arginine, leading to the use of D-stereoisomers of L-arginine analogues as negative controls (23). Thus, the D-NAME can be used as negative controls in this study, similar to PBS. In this study, two approaches were used in the administration of NOS inhibitor. In the first approach, mice were injected intraperitoneally with 100 μ L volume of L-NAME (Sigma-Aldrich Co., Sweden) or D-NAME (Sigma-Aldrich Co.) as indicated after CII immunization and were compared with mice injected with the same volume of PBS. Both L-NAME and D-NAME were dissolved in PBS. The dose of L-NAME or D-NAME was 4.3 mg/100 μ L/mouse/time, pH 7.4. In the second approach, mice received oral administration of the same dose of L-NAME as intraperitoneal injection. Stock solutions of L-NAME and D-NAME were diluted into the drinking water, both at a final concentration of 416 mg/L.

In vivo whole-body optical imaging

The used imaging system was PhotonIMAGER™ (Bio-space Lab, Paris, France), including a 4-view mirror and 510–560 nm/50 nm bandpass filter. The luminescent probe L-012 was purchased from Wako Chemical (Nordic BioLabs) and dissolved in water HiPerSolv CHROMANORM® for high-performance liquid chromatography. For *in vivo* imaging, the mice were immobilized with intraperitoneal injection of both ketamine (50 mg/kg; Orion Corporation, Finland) and Dormitor (1 mg/kg; Orion Corporation) dissolved together in PBS

(1 $\times$; Life Technologies Europe BV, Sweden). An optical scanning was performed in the mice under anesthesia, immediately after intraperitoneal injection of L-012 (1 mg/mouse) for 20 min. The mice were awoken by use of Antisedan (1 mg/kg; Orion Corporation) at the end of each experiment.

Serum NO (NO₂⁻/NO₃⁻) detection

Blood (100 μ L) from the mouse tail vein was taken to measure serum nitrite/nitrate levels. The blood was gently shaken for 15 min and centrifuged at 1500 g/min for 30 min. The obtained serum samples were stored at -80°C until analysis. A commercial NO (NO₂/NO₃) research kit (Catalog No. ADI-917-010; Enzo Life Sciences, Inc.) was used to determine the level of serum NO by a microplate reader (Synergy 2; BioTek, Inc., Winooski, VT).

Histology

Mice were sacrificed at the end of the arthritis experiment. Left hind paw without skin per mouse was fixed for 5 days in 4% phosphate-buffered formaldehyde, decalcified with ethylenediaminetetraacetic acid, and embedded in paraffin. Tissue sections were stained with H&E. H&E slides were assessed with the identity of mice obscured. Histology scores were given to synovium, cartilage, bone, and joint architecture. For synovium, 0, 1, or 2 points were given to each of the following five criteria, depending on the severity, hyperplasia, infiltration, angiogenesis, pannus formation, and necrosis. For cartilage erosions, 0, 1, or 2 points were given to each of the following two criteria: erosion and new formation. For bone erosions, 0, 1, or 2 points were given to each of the following two criteria: erosion and new bone formation. For joint destruction, 0, 3, or 6 points were given to each of the following two criteria: destruction of joints and ankylosis. The score for synovium is 10, cartilage 4, bone 4, and joint structure 12, and adding up is to 30 points in total.

CII-specific IL-2 production with hybridoma cells

All cells were cultured in DMEM+Glutamax-I (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin, and 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES). To assay the functions of APCs with CII-specific T cells from HCQ.3 hybridomas (23), spleen cells were collected from naive mice as the APCs. 1×10^6 APCs were incubated with 1×10^5 HCQ.3 T cells and an indicated concentration of CII in the presence of 0.4 mM of L-NAME or D-NAME per well and cultured for 24 h. The supernatant was then collected to determine the level of IL-2 production in enzyme-linked immunosorbent assay (ELISA) with a microplate reader (Synergy 2; BioTek, Inc.). The purified anti-mouse IL-2 antibodies (clone: JES6-1A12) were used to coat the 96-well plate at 4°C overnight. The secondary antibodies were biotin anti-mouse IL-2 antibodies (clone: JES6-5H4).

Antigen recall assay

All cells were cultured in DMEM+Glutamax-I (Gibco) supplemented with 10% heat-inactivated FBS, penicillin/streptomycin, and 10 mM HEPES. The transgenic mice of *B10Q.Ncf1^{*/*}.HCQ3^{tg/+}.CD45.1^{tg/+}* were treated with L-NAME or D-NAME after immunization of CII. Both

the spleen and two inguinal lymph nodes per mouse were collected and pooled together at day 10 after immunization. 1×10^6 Cells were incubated with indicated concentration of triggers per well and cultured for 72 h. The ConA (CAS No. 11028-71-0; Sigma–Aldrich Co.) ($3 \mu\text{g/mL}$) was used as the positive control. The supernatant was then collected to determine the level of IL-2 and IL-17 production in ELISA with a microplate reader (Synergy 2; BioTek, Inc.). The purified anti-mouse IL-2 (clone: JES6-1A12) and IL-17 (clone: TC11-18H10.1) antibodies were used to coat the 96-well plate at 4°C overnight. The secondary antibodies were biotin anti-mouse IL-2 (clone: JES6-5H4) and IL-17 (clone: TC11-8H4) antibodies.

Flow cytometry

Flow cytometry was performed on single-cell suspensions from spleens and inguinal lymph nodes. The cell density was counted by using Sysmex KX-21N automated hematology analyzer (Sysmex Corporation, NY). Single cells were then stained with a LIVE/DEAD® Fixable Dead Cell Stain (Catalog No. L10119; Thermo Fisher Scientific, Inc.). After an anti-mouse CD16/CD32 Fc block, extracellular antigens were stained for 20 min at 4°C in PBS with 1% FBS. For intracellular IL-17 staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm solution (Catalog No. 554714; BD Biosciences, CA). For intracellular Foxp3 staining, cells were fixed and permeabilized by Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent solutions (Catalog No. 00-5521-00; eBioscience) and Permeabilization Buffer (Catalog No. 00-8333-56; eBioscience). Samples were acquired using BD LSR II flow cytometer. The workstation is managed by FACSDiva software version 8.0 (BD Biosciences), and the data were analyzed using the FlowJo software version 10.5.3 (TreeStar, Inc., OR).

Statistics

Statistical analyses were performed with GraphPad Prism software, version 8.2.1 (GraphPad Software, San Diego, CA). Unless otherwise stated, the Mann–Whitney *U* test was used to compare the means of two groups. Prism computes an approximate *p* value for Spearman nonparametric correlation between arthritis score and optical radiance. All results are shown as mean $\pm$ standard error of the mean. *p* Value <0.05 was considered as significant: *p <0.05 , **p <0.01 , ***p <0.001 , and ****p <0.0001 .

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Authors' Contributions

J.Z. designed the research, performed the experiments, including acquiring and analyzing data, and wrote and revised the article. A.C.Y.Y. performed experiments, analyzed data, and revised the article. R.H. designed the research, analyzed data, and revised the article.

Author Disclosure Statement

The authors declare that they have no relevant financial, personal, or professional relationships to disclose, which

could be perceived as a conflict of interest or as potentially influencing or biasing the authors' work.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5

References

- Bäcklund J, Carlsen S, Höger T, Holm B, Fugger L, Kihlberg J, Burkhardt H, and Holmdahl R. Predominant selection of T cells specific for the glycosylated collagen type II epitope (263-270) in humanized transgenic mice and in rheumatoid arthritis. *Proc Natl Acad Sci U S A* 99: 9960–9965, 2002.
- Bäcklund J, Li C, Jansson E, Carlsen S, Merky P, Nandakumar K-S, Haag S, Ytterberg J, Zubarev RA, and Holmdahl R. C57BL/6 mice need MHC class II Aq to develop collagen-induced arthritis dependent on autoreactive T cells. *Ann Rheum Dis* 72: 1225–1232, 2013.
- Corthay A, Bäcklund J, Broddefalk J, Michaëlsson E, Goldschmidt TJ, Kihlberg J, and Holmdahl R. Epitope glycosylation plays a critical role for T cell recognition of type II collagen in collagen-induced arthritis. *Eur J Immunol* 28: 2580–2590, 1998.
- Cuzzocrea S, Chatterjee PK, Mazzon E, McDonald MC, Dugo L, Di Paola R, Serrano I, Britti D, Caputi AP, and Thiemermann C. Beneficial effects of GW274150, a novel, potent and selective inhibitor of iNOS activity, in a rodent model of collagen-induced arthritis. *Eur J Pharmacol* 453: 119–129, 2002.
- García-Ortiz A and Serrador JM. Nitric oxide signaling in T cell-mediated immunity. *Trends Mol Med* 24: 412–427, 2018.
- Gelderman KA, Hultqvist M, Holmberg J, Olofsson P, and Holmdahl R. T cell surface redox levels determine T cell reactivity and arthritis susceptibility. *Proc Natl Acad Sci U S A* 103: 12831–12836, 2006.
- Gelderman KA, Hultqvist M, Pizzolla A, Zhao M, Nandakumar KS, Mattsson R, and Holmdahl R. Macrophages suppress T cell responses and arthritis development in mice by producing reactive oxygen species. *J Clin Invest* 117: 3020–3028, 2007.
- Gordon S. Phagocytosis: an immunobiologic process. *Immunity* 44: 463–475, 2016.
- Holmdahl R, Andersson M, Goldschmidt TJ, Gustafsson K, Jansson L, and Mo JA. Type II collagen autoimmunity in animals and provocations leading to arthritis. *Immunol Rev* 118: 193–232, 1990.
- Huang Y-F, Lo P-C, Yen C-L, Nigrovic PA, Chao W-C, Wang W-Z, Hsu GC, Tsai Y-S, and Shieh C-C. Redox

- regulation of pro-IL-1 β processing may contribute to the increased severity of serum-induced arthritis in NOX2-deficient mice. *Antioxid Redox Signal* 23: 973–984, 2015.
11. Hultqvist M, Olofsson P, Holmberg J, Backstrom BT, Tordsson J, and Holmdahl R. Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced oxidative burst due to a mutation in the *Ncf1* gene. *Proc Natl Acad Sci U S A* 101: 12646–12651, 2004.
  12. Hultqvist M, Sareila O, Vilhardt F, Norin U, Olsson LM, Olofsson P, Hellman U, and Holmdahl R. Positioning of a polymorphic quantitative trait nucleotide in the *Ncf1* gene controlling oxidative burst response and arthritis severity in rats. *Antioxid Redox Signal* 14: 2373–2383, 2011.
  13. Inglis JJ, Criado G, Medghalchi M, Andrews M, Sandison A, Feldmann M, and Williams RO. Collagen-induced arthritis in C57BL/6 mice is associated with a robust and sustained T-cell response to type II collagen. *Arthritis Res Ther* 9: R113, 2007.
  14. Jourdain D, Jourdain FL, Kutchukian PS, Musah RA, Wink DA, and Grisham MB. Reaction of superoxide and nitric oxide with peroxynitrite: implications for peroxynitrite-mediated oxidation reactions *in vivo*. *J Biol Chem* 276: 28799–28805, 2001.
  15. Kalyanaraman B, Darley-Usmar V, Davies KJA, Dennery PA, Forman HJ, Grisham MB, Mann GE, Moore K, Roberts LJ, and Ischiropoulos H. Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. *Free Radic Biol Med* 52: 1–6, 2012.
  16. Kelkka T, Kienhöfer D, Hoffmann M, Linja M, Wing K, Sareila O, Hultqvist M, Laajala E, Chen Z, Vasconcelos J, Neves E, Guedes M, Marques L, Krönke G, Helminen M, Kainulainen L, Olofsson P, Jalkanen S, Lahesmaa R, Souto-Carneiro MM, and Holmdahl R. Reactive oxygen species deficiency induces autoimmunity with type 1 interferon signature. *Antioxid Redox Signal* 21: 2231–2245, 2014.
  17. Kuhns DB, Hsu AP, Sun D, Lau K, Fink D, Griffith P, Huang DW, Priel DAL, Mendez L, Kreuzburg S, Zerbe CS, De Ravin SS, Malech HL, Holland SM, Wu X, and Gallin JI. NCF1 (p47phox)-deficient chronic granulomatous disease: comprehensive genetic and flow cytometric analysis. *Blood Adv* 3: 136–147, 2019.
  18. Laubach VE, Shesely EG, Smithies O, and Sherman PA. Mice lacking inducible nitric oxide synthase are not resistant to lipopolysaccharide-induced death. *Proc Natl Acad Sci U S A* 92: 10688–10692, 1995.
  19. Lee S-W, Choi H, Eun S-Y, Fukuyama S, and Croft M. Nitric oxide modulates TGF- β -directed signals to suppress Foxp3+ regulatory T cell differentiation and potentiate Th1 development. *J Immunol Baltim Md 1950* 186: 6972–6980, 2011.
  20. Liu T, Zhang M, Mukosera GT, Borchardt D, Li Q, Tipple TE, Ishtiaq Ahmed AS, Power GG, and Blood AB. L-NAME releases nitric oxide and potentiates subsequent nitroglycerin-mediated vasodilation. *Redox Biol* 26: 101238, 2019.
  21. van Loosdregt J, Rossetti M, Spreafico R, Moshref M, Olmer M, Williams GW, Kumar P, Copeland D, Pischel K, Lotz M, and Albani S. Increased autophagy in CD4⁺ T cells of rheumatoid arthritis patients results in T-cell hyperactivation and apoptosis resistance: Immunomodulation and immune therapies. *Eur J Immunol* 46: 2862–2870, 2016.
  22. Mills CD, Kincaid K, Alt JM, Heilman MJ, and Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* 164: 6166–6173, 2000.
  23. Moore PK, al-Swayeh OA, Chong NW, Evans RA, and Gibson A. L-NG-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br J Pharmacol* 99: 408–412, 1990.
  24. Niedbala W, Besnard A-G, Jiang HR, Alves-Filho JC, Fukada SY, Nascimento D, Mitani A, Pushparaj P, Alqahtani MH, and Liew FY. Nitric oxide-induced regulatory T cells inhibit Th17 but not Th1 cell differentiation and function. *J Immunol* 191: 164–170, 2013.
  25. Niedbala W, Cai B, Liu H, Pitman N, Chang L, and Liew FY. Nitric oxide induces CD4+CD25+ Foxp3 regulatory T cells from CD4+CD25⁺ T cells via p53, IL-2, and OX40. *Proc Natl Acad Sci U S A* 104: 15478–15483, 2007.
  26. Olofsson P, Holmberg J, Tordsson J, Lu S, Åkerström B, and Holmdahl R. Positional identification of *Ncf1* as a gene that regulates arthritis severity in rats. *Nat Genet* 33: 25–32, 2003.
  27. Olsson LM, Johansson ÅC, Gullstrand B, Jönsen A, Saevarsdottir S, Rönnblom L, Leonard D, Wetterö J, Sjöwall C, Svenungsson E, Gunnarsson I, Bengtsson AA, and Holmdahl R. A single nucleotide polymorphism in the *NCF1* gene leading to reduced oxidative burst is associated with systemic lupus erythematosus. *Ann Rheum Dis* 76: 1607–1613, 2017.
  28. Olsson LM, Nerstedt A, Lindqvist A-K, Johansson ÅCM, Medstrand P, Olofsson P, and Holmdahl R. Copy number variation of the gene *NCF1* is associated with rheumatoid arthritis. *Antioxid Redox Signal* 16: 71–78, 2012.
  29. Peleli M, Zollbrecht C, Montenegro MF, Hezel M, Zhong J, Persson EG, Holmdahl R, Weitzberg E, Lundberg JO, and Carlström M. Enhanced XOR activity in eNOS-deficient mice. *Free Radic Biol Med* 99: 472–484, 2016.
  30. Pizzolla A, Gelderman KA, Hultqvist M, Vestberg M, Gustafsson K, Mattsson R, and Holmdahl R. CD68-expressing cells can prime T cells and initiate autoimmune arthritis in the absence of reactive oxygen species. *Eur J Immunol* 41: 403–412, 2011.
  31. Pizzolla A, Hultqvist M, Nilsson B, Grimm MJ, Eneljung T, Jonsson I-M, Verdrengh M, Kelkka T, Gjerdtsson I, Segal BH, and Holmdahl R. Reactive oxygen species produced by the NADPH oxidase 2 complex in monocytes protect mice from bacterial infections. *J Immunol* 188: 5003–5011, 2012.
  32. Raposo B, Merky P, Lundqvist C, Yamada H, Urbanaviciute V, Niaudet C, Viljanen J, Kihlberg J, Kyewski B, Ekwall O, Holmdahl R, and Bäcklund J. T cells specific for post-translational modifications escape intrathymic tolerance induction. *Nat Commun* 9: 353, 2018.
  33. Sakaguchi Y, Shirahase H, Ichikawa A, Kanda M, Nozaki Y, and Uehara Y. Effects of selective iNOS inhibition on type II collagen-induced arthritis in mice. *Life Sci* 75: 2257–2267, 2004.
  34. Sarkar S, Korolchuk VI, Renna M, Imarisio S, Fleming A, Williams A, Garcia-Arencibia M, Rose C, Luo S, Underwood BR, Kroemer G, O’Kane CJ, and Rubinsztein DC.

- Complex inhibitory effects of nitric oxide on autophagy. *Mol Cell* 43: 19–32, 2011.
35. Stuehr DJ and Marletta MA. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proc Natl Acad Sci U S A* 82: 7738–7742, 1985.
  36. Suda O, Tsutsui M, Morishita T, Tanimoto A, Horiuchi M, Tasaki H, Huang PL, Sasaguri Y, Yanagihara N, and Nakashima Y. Long-term treatment with N(omega)-nitro-L-arginine methyl ester causes arteriosclerotic coronary lesions in endothelial nitric oxide synthase-deficient mice. *Circulation* 106: 1729–1735, 2002.
  37. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, and Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39: 44–84, 2007.
  38. Viteček J, Lojek A, Valacchi G, and Kubala L. Arginine-based inhibitors of nitric oxide synthase: therapeutic potential and challenges. *Mediators Inflamm* 2012: 1–22, 2012.
  39. Yang J, Zhang R, Lu G, Shen Y, Peng L, Zhu C, Cui M, Wang W, Arnaboldi P, Tang M, Gupta M, Qi C-F, Jayaraman P, Zhu H, Jiang B, Chen S, He JC, Ting AT, Zhou M-M, Kuchroo VK, Morse HC, Ozato K, Sikora AG, and Xiong H. T cell–derived inducible nitric oxide synthase switches off T_H 17 cell differentiation. *J Exp Med* 210: 1447–1462, 2013.
  40. Yang M, Haase C, Viljanen J, Xu B, Ge C, Kihlberg J, and Holmdahl R. Cutting Edge: processing of oxidized peptides in macrophages regulates T cell activation and development of autoimmune arthritis. *J Immunol* 199: 3937–3942, 2017.
  41. Zhao J, Ma J, Deng Y, Kelly JA, Kim K, Bang S-Y, Lee H-S, Li Q-Z, Wakeland EK, Qiu R, Liu M, Guo J, Li Z, Tan W, Rasmussen A, Lessard CJ, Sivits KL, Hahn BH, Grossman JM, Kamen DL, Gilkeson GS, Bae S-C, Gaffney PM, Shen N, and Tsao BP. A missense variant in NCF1 is associated with susceptibility to multiple autoimmune diseases. *Nat Genet* 49: 433–437, 2017.
  42. Zhong J, Olsson LM, Urbonaviciute V, Yang M, Bäckdahl L, and Holmdahl R. Association of NOX2 subunits genetic variants with autoimmune diseases. *Free Radic Biol Med* 125: 72–80, 2018.
  43. Zhong J, Scholz T, Yau ACY, Guerard S, Hüffmeier U, Burkhardt H, and Holmdahl R. Mannan-induced Nos2 in macrophages enhances IL-17–driven psoriatic arthritis by innate lymphocytes. *Sci Adv* 4: eaas9864, 2018.

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Abbreviations Used

APCs = antigen-presenting cells
 CGD = chronic granulomatous disease
 CIA = CII-induced arthritis
 CII = type II collagen
 CII_{259–273} = CII residues 259–273
 ConA = concanavalin A
 D-NAME = NG-nitro-D-arginine methyl ester
 DHR = dihydrorhodamine
 DPI = day post-immunization
 ELISA = enzyme-linked immunosorbent assay
 FBS = fetal bovine serum
 H&E = hematoxylin and eosin
 HEPES = (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
 IgG = immunoglobulin G
 IL-2 = interleukin-2
 L-NAME = NG-nitro-L-arginine methyl ester
 MHC = major histocompatibility complex
 MFI = mean fluorescence intensity
 MIP = mannan-induced psoriatic arthritis
 NCF1 = neutrophil cytosolic factor 1
 NO = nitric oxide
 NOS = nitric oxide synthase
 NOX2 = NADPH oxidase 2
 PBS = phosphate-buffered saline
 PE = phycoerythrin
 RA = rheumatoid arthritis
 RNS = reactive nitrogen species
 ROS = reactive oxygen species
 TCR = T-cell receptor
 XOR = xanthine oxidoreductase