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NADPH Oxidase Deficiency Regulates Th Lineage Commitment and Modulates Autoimmunity

Hubert M. Tse,*1,2 Terri C. Thayer,*1,1 Chad Steele,† Carla M. Cuda,† Laurence Morel,† Jon D. Piganelli,* and Clayton E. Mathews*,†

Reactive oxygen species are used by the immune system to eliminate infections; however, they may also serve as signaling intermediates to coordinate the efforts of the innate and adaptive immune systems. In this study, we show that by eliminating macrophage and T cell superoxide production through the NADPH oxidase (NOX), T cell polarization was altered. After stimulation with immobilized anti-CD3 and anti-CD28 or priming recall, T cells from NOX-deficient mice exhibited a skewed Th17 phenotype, whereas NOX-intact cells produced cytokines indicative of a Th1 response. These findings were corroborated in vivo by studying two different autoimmune diseases mediated by Th17 or Th1 pathogenic T cell responses. NOX-deficient NOD mice were Th17 prone with a concomitant susceptibility to experimental allergic encephalomyelitis and significant protection against type 1 diabetes. These data validate the role of superoxide in shaping Th responses and as a signaling intermediate to modulate Th17 and Th1 T cell responses. The Journal of Immunology, 2010, 185: 5247–5258.

The absence of ROS in T cells has also been demonstrated to be associated with an increase in the severity of Ag-induced experimental allergic encephalomyelitis (EAE) and collagen-induced arthritis (11). In contrast, our previous genetic mapping studies using T1D-prone NOD with T1D-resistant ALR mice linked resistance against spontaneous autoimmune diabetes, reduced oxidative burst from neutrophils and macrophages, as well as elevated superoxide dismutase 1 activity to the Suppressor of peroxide production (Sasp) locus on chromosome 3 (12, 13). To study the impact of ablated NOX activity on the development of spontaneous autoimmune diabetes, in the absence of high superoxide dismutase 1 activity, we generated NOD mice with a spontaneous mutation in the neutrophil cytosolic factor 1 (Ncf1) gene. The Ncf1mut/mut mutation is a point mutation in exon 8 that results in an aberrant mRNA splicing event and terminal truncation of the p47phox subunit, preventing NOX assembly and superoxide synthesis (14).

In this study, we report a role for superoxide in modulating immune responses. NOX deficiency altered redox-dependent innate immune cytokine synthesis, observed as reductions in TNF-α, IL-1β, and IL-12 p70, whereas IL-23, a cytokine necessary for driving Th17 differentiation (15), was elevated. In addition, polyclonal or Ag-induced activated T cells from NOD.Ncf1mut/mut mice exhibited a decreased Th1 cytokine pattern, and instead demonstrated a cytokine profile reminiscent of a Th17 response. These immune polarizations were strongly correlated with the immune responses in the whole animal as NOX deficiency attenuated TID while promoting development of the prototypical Th17 disease, EAE. These data demonstrate the importance of superoxide in shaping immune responses.
Materials and Methods

Materials

NOD/ShiLtJ, ALR/LtJ, and NOD.B6-Ncf1m1J (NOD-Ncf1m1J) mice were bred and housed under specific pathogen-free conditions in the animal facilities of either Children’s Hospital of Pittsburgh or the University of Florida. B6.NOD-D17Mit21-D17Mit10 (B6-H2β2), B6.Cg-Ncf1m1J/J (B6-Ncf1m1J), and SILJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Female mice at 6–8 wk of age were used in all experiments except for the EAE induction, in which 6-wk-old males were used. All animal experiments were approved by the Institutional Animal Care and Use Committees of the Children’s Hospital of Pittsburgh and the University of Florida, and were in compliance with the laws of the United States of America.

Anti–IL-2 and anti–IFN-γ Ab pairs for ELISAs, as well as CD3, CD4, CD8, CD11b, Ly6g (Gr1), and CD11b fluorochrome-conjugated Abs were purchased from BD Biosciences (San Diego, CA). Hen egg lysozyme (HEL), anti-β-actin mAb, dichlorofluorescein diacetate (DCFDA), and 4-α-PMA were purchased from Sigma-Aldrich (St. Louis, MO). Dihydrorhodamine 123 (DHR123) was purchased from Invitrogen (Carlsbad, CA). Abs against p47phox, the gene product of Ncf1, and phospho-STAT3 were purchased from Cell Signaling Technology (Danvers, MA). Abs against STAT1, STAT4 and T-bet were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-STAT1 Abs was purchased from Zymed Laboratories (San Francisco, CA).

Construction of the B6-H2β2-Ncf1m1J and NOD.Ncf1m1J mouse strains

The spontaneous null allele Ncf1m1J (chromosome 5) (14) was congenically introgressed into the NOD genome to ablate NOX superoxide production by first generating F2 mice from outcrosses of B6-Ncf1m1J with B6-H2β2. The subsequent B6-H2β2-Ncf1m1J mice were outcrossed and backcrossed to NOD for 10 generations. To eliminate contaminating chromosomal segments, genotyping was performed by PCR amplification of 94 polymorphic microsatellite primers (Invitrogen) covering all 19 autosomes (Supplemental Table) for the first six generations, as described previously (16). By N6, mice were homozygous for NOD genome at all loci save those in tight linkage with Ncf1 on chromosome 5. From N6 until N10, genotyping was performed with markers on chromosomes 5 (Table I), allowing for mice with the smallest possible congenic segment to be bred. At N10, these marker-assisted or speed congenic mice were intercrossed to generate mice that were homozygous for the Ncf1m1J allele.

Genotyping for the Ncf1m1J allele

DNA were obtained from murine tail biopsies, as described previously (12). Ncf1 exon 8-specific PCR primers (forward, 5′-biotin-TAG AAA GGG AAA GCC AGA AAG AAT-3′, and reverse, 5′-ACG CTT TGA TGG TTA CAT AC GG T-3′) were used to distinguish single-nucleotide polymorphisms between the wild-type allele and a mutation in the splice site of exon 8, as previously described (11). DNA sequencing was performed using sequence-specific PCR (SS-PCR) 96MA, Pyrosequencing AB, Uppsala, Sweden. The pyrosequencing primer (5′-ACG CTT TGA TGG TTA CAT AC GGT-3′) was used for sequencing. Pyrosequencing data were quantified and background corrected using PSQ 96MA version 2.0.2 software (Pyrosequencing AB).

Flow cytometric analysis

Splenic leukocytes were harvested and washed twice in FACS buffer (1% BSA in PBS), counted, and resuspended in a final concentration of 2 × 107 cells/ml in FACS buffer. One million cells were stained with directly fluorochrome-conjugated Abs purchased from either eBiogenics (San Diego, CA) or BD Biosciences. The Abs (PE-labeled anti-Ly6g [Gr1], allophycocyanin-labeled anti-CD11b1, Pacific blue-labeled anti-CD4, allophycocyanin-labeled anti-CD25, PE-labeled anti-FoxP3, FITC-labeled anti-CD8, PE/Cy5-labeled anti-CD4, PE-labeled anti-CD202L, PerCP/Cy5.5-labeled anti-CD69, allophycocyanin-Cy7-labeled anti-CD44, FITC-labeled anti–IFN-γ, and PE-labeled anti–IL-17A) were used at the appropriate dilution (10 µl of each Ab), and fluorescence was measured using a LSRII (BD Biosciences). Results were analyzed with CellQuest software (BD Biosciences).

Measurement of intracellular ROS and flow cytometry

Neutrophil and macrophage superoxide production were assayed using FACS, as previously described (12). Briefly, bone marrow was isolated and purified using a Ficol gradient. Cells were labeled with PE-labeled anti-Ly6g (Gr1) and allophycocyanin-labeled anti-CD11b, and then loaded with DHR123 for 5 min at 37°C. Cells were subjected to flow cytometry prior to stimulation, and then at 5-min intervals after 98 nM PMA stimulation. The ROS-driven conversion of DHR123 to rhodamine was measured using a FACS Calibur. Ly6g and CD11b extracellular markers were used to discriminate neutrophils (Ly6g+, CD11b+) and macrophages (Ly6g−, CD11b+) from other bone marrow cells, and results were analyzed with CellQuest software.

Determination of anti–CD3–induced ROS generation in purified CD4+ T cells

ROS generation was measured with the oxidation-sensitive dyes DCFDA, as described previously (10), with the following modifications. Splenocytes from NOD or NOD-Ncf1m1J were homogenized into single cells to purify CD4+ T cells with a CD4+ T cell isolation kit (Miltenyi Biotec, Auburn, CA), before the manufacturer’s guidelines. Purified 106 CD4+ T cells were stained with 2 µM DCFDA (Sigma-Aldrich) at 37°C for 30 min, washed, and then stained with hamster anti-mouse CD3 Ab (BD Pharmingen, San Diego, CA) at 10 µg/ml for 25 min at 4°C. ROS generation was induced in 106 cells by anti-CD3 cross-linking using a One Touch Ultra (Lifescan, Mountain View, CA). Mice were considered diabetic if the blood glucose reading was >250 mg/dl (16.5 mM) on consecutive 2 d. Diabetic mice were euthanized by approved methods. To examine islet inflammation, a subset (n = 4) of nondiabetic females from each genotype at ages 7 and 22 wk was euthanized. Pancreata were fixed in 10% neutral buffered formalin, embedded in paraffin, stained with aldehyde fuchsin to identify granulated β cells, and counterstained with H&E. Islets were scored over a range from 0 (no lesions) to 4 (end-stage insulitis), and a mean insulitis score was calculated (17).

Isolation of mouse bone marrow-derived macrophages

Bone marrow-derived macrophages were cultured, as described previously (18), and plated on 6- and 24-well tissue culture plates and 100-mm petri dishes at concentrations of 4 × 105 cells/well, 106 cells/well, or 2.4 × 105 cells/dish, respectively. Macrophages were stimulated with 100 ng/ml LPS from Escherichia coli (055:B5; Sigma-Aldrich).

Plate-bound anti–CD3ε and anti–CD28 activation of purified CD4+ T cells and whole-cell lysates

Splenic CD4+ T cells were purified by negative selection with a CD4+ T cell isolation kit (Miltenyi Biotec) supplemented with biotinylated mouse anti-rat RT1B (OX-6) Ab. Naïve CD4+CD262L+ T cells were purified with a CD4+CD62L+ T cell isolation kit (Miltenyi Biotec) by following manufacturer’s protocol. A total of 5 × 10⁶ purified splenic T cells were used per reaction. Positive cells were determined by staining with hamster anti-mouse CD3 Ab (BD Pharmingen). For measurement of intracellular ROS generation, 5 × 10⁶ cells were stained with DHR123 as described previously. To examine islet inflammation, a subset (n = 4) of nondiabetic females from each genotype at ages 7 and 22 wk was euthanized. Pancreata were fixed in 10% neutral buffered formalin, embedded in paraffin, stained with aldehyde fuchsin to identify granulated β cells, and counterstained with H&E. Islets were scored over a range from 0 (no lesions) to 4 (end-stage insulitis), and a mean insulitis score was calculated (17).

Table 1. Chromosome 5 map of the NOD.B6-D5Mit30-D5Mit31 (NOD.Ncf1m1J) congenic mice that were produced

<table>
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Physical positions in Mb are from National Center for Biotechnology Information (NCBI) Ensembl Build 37, and corresponding cM positions are from the Mouse Genome Informatics database at The Jackson Laboratory. The total size of the congenic interval is 7.82 Mb (6 cM).

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FIGURE 1. Ncf1m1J mutation decreases the respiratory burst capacity of bone marrow macrophages, neutrophils, and splenic T cells without decreasing the percentage of cells. The percentage of bone marrow neutrophils (A) and macrophages (C) from C57BL/6.H2g7, C57BL/6, ALR, NOD, and NOD.Ncf1m1J mice was assessed by flow cytometry with gating for macrophage (Gr1^2 CD11b^+)- or neutrophil (Gr1^+ CD11b^+)-specific populations. The respiratory burst capacity of bone marrow neutrophils (B) and macrophages (D) was assessed by loading 5 \times 10^6 cells/ml with 0.99 M dihydrorhodamine and stimulated with 98 nM PMA for 40 min. The oxidation of dihydrorhodamine to rhodamine was assessed by flow cytometry with gating for macrophage (Gr1^2 CD11b^+)- or neutrophil (Gr1^+ CD11b^+)-specific populations. Whole-cell lysates of cell-sorted macrophages were used in an immunoblot analysis for p47phox and actin expression (E). T cell ROS production was measured by labeling purified CD4^+ T cells with DCFDA. Cells were activated by adding hamster anti-mouse CD3 and then cross-linked with rabbit anti-hamster IgG. Cells were analyzed at 0, 30, and 60 min. ROS generation was determined by the increase in DCFDA fluorescence upon anti-CD3 stimulation over unstimulated control (G). The percentage of splenic CD4^+ T cells was assessed by flow cytometry by gating for CD4^+ T cell-specific populations (CD3^+CD4^+CD8^-) (F). Results are representative of three independent experiments.
was stimulated with anti-CD3ε and anti-CD28 Ab at concentrations of 0.1 and 1 μg/ml, respectively, for 72 h (18). Supernatants were harvested for cytokine analysis and T cell proliferation, and whole-cell lysates were prepared, as described previously (18).

Cytokine measurements by ELISA and Luminex and Greiss assay

IL-2, IFN-γ, IL-12 p70, IL-17, TNF-α, and IL-1β were detected with DuoSet ELISA kits (R&D Systems, Minneapolis, MN) or with Ab pairs from BD Pharmingen. TGF-β was measured using a TGF-β Quantikine kit from R&D Systems. ELISA plates were read on a SpectraMax M2 microplate reader and analyzed using Softmax Pro v.5.0.1 (Molecular Devices Corp., Sunnyvale, CA). Additionally, cytokine levels in culture supernatants were quantified using the Bio-Plex multiplex suspension array system and a mouse cytokine/chemokine panel (Bio-Rad, Hercules, CA). The concentrations of each sample were determined using Bio-Plex Manager version 3.0 software. NO2− was measured by the Greiss assay, as described previously (18).

Quantitative RT-PCR

Bone marrow-derived macrophages were stimulated 4 h with LPS. RNA was isolated with TRIzol and cDNA prepared by SuperScript III (Invitrogen). SYBR Green reagent (Bio-Rad) was used for quantitative RT-PCR analyzed on a LightCycler 480 II (Roche, Basel, Switzerland) with forward and reverse primers specific for Xbp1, Hspa5 (BIP), Chop, and Actin (as adapted from Ref. 19).

Immunization of mice and Ag-recall and crisscross assays

Mice were injected with 100 μg HEL in CFA s.c. at the base of the tail. Seven days after immunization, mice were sacrificed and the inguinal and periaortic lymph node (LN) were harvested for in vitro Ag-recall assays, as described previously (20). LN single-cell suspensions were split into two groups with 25 μg HEL in complete DMEM, and supernatants were harvested for cytokine analysis at 48, 72, and 96 h. For Ag-recall crisscross assays, LN single-cell suspensions were split into two groups and used to purify T cells and APCs by negative selection. CD4+ T cells were purified by negative selection with a CD4+ T cell isolation kit (Miltenyi Biotec) supplemented with biotinylated mouse anti-rat RT1B (OX-6) Ab, according to the manufacturer’s protocol. APCs were purified by negative selection with anti-CD4 and anti-CD8 Abs conjugated to magnetic beads over a LS column (Miltenyi Biotec).

Intracellular cytokine staining

Intracellular IFN-γ and IL-17A were measured after polyclonal stimulation of naive CD4+CD62L− T cells. Stimulated cells were treated with Golgi-Stop for 5 h at 37°C in a 5% CO2 humid air incubator with the aid of the murine BD intracellular cytokine staining kit (BD Biosciences), according to the manufacturer’s protocol. After stimulation, cells were surface stained with PECy5-labeled anti-CD4, fixed in BD Cytofix/Cytoperm buffer, washed in BD Perm/Wash buffer, and then stained with FITC-labeled rat anti–IFN-γ (XMG1.2; BD Biosciences) and PE-labeled rat anti–IL-17A (TC11-18H10; BD Biosciences) and isotype controls, according to the manufacturer’s protocol. Cells were washed twice in BD Perm/Wash buffer and resuspended in FACS buffer, and stained cells were analyzed on a FACSCalibur. Results were analyzed with CellQuest software.

Western immunoblotting

Whole-cell lysates were separated on a 4–20% gradient SDS-PAGE gel and transferred onto 0.45-μm-charged polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C with Abs against STAT4, STAT1α, T-bet, STAT3, or β-actin and exposed to the appropriate secondary Ab conjugated to HRP (Jackson Immunoresearch Laboratories, West Grove, PA). Chemiluminescence was detected with ECL Plus (Amersham Biosciences, Piscataway, NJ). Image J software (National Institutes of Health) was used to generate densitometry data.

Induction of EAE

Eight-week-old SJL, NOD, and NOD-Ncf1m1J mice were treated with 100 μg myelin oligodendrocyte glycoprotein (MOG) peptide sequence 35–55 (MOG35–55) in CFA by s.c. injection in the base of the tail. Mice were also treated i.p. with 200 ng pertussis toxin on days 0 and 2. Mice were evaluated 3 d per week for symptoms following the standard 5-point scale: 0,
asymptomatic; 1, limp tail; 2, hind limb weakness/incomplete limb paralysis; 3, complete hind leg paralysis; 4, complete hind and partial front leg paralysis; and 5, moribund (21, 22).

Statistical analysis

Determination of the difference between mean values for each experimental group was assessed by Student t test, with \( p < 0.05 \) considered significant. All experiments were performed at least three separate times with data obtained in triplicate wells in each experiment.

Results

In both NOD and C57BL/6 (B6), Ncf1\textsuperscript{m1J} dampens respiratory burst in both neutrophils and macrophages

To test the role of superoxide in mediating immune function, a spontaneous null allele in Ncf1 on chromosome 5 (Ncf1\textsuperscript{m1J}), the p47\textsubscript{phox} subunit of NADPH oxidase complex (14), was congenically introduced into the NOD background. The size of the congenic interval in the mice used for the enclosed research was estimated to be 7.82-Mb–based genotyping with microsatellite markers in linkage with the Ncf1 locus (Table I). Mice with the Ncf1\textsuperscript{m1J} mutation had equal numbers of neutrophils (Fig. 1A) and macrophages (Fig. 1C) when compared with Ncf1-intact mice of the same strain, but NOD, NOD.Ncf1\textsuperscript{m1J}, and B6-H2\textsuperscript{g7} mice did exhibit reduced neutrophils when compared with B6 or ALR mice (Fig. 1A). Oxidation of DHR123 to rhodamine was significantly absent in both neutrophils (Fig. 1B) and macrophages (Fig. 1D) from NOD.Ncf1\textsuperscript{m1J} and C57BL/6.H2\textsuperscript{g7}.Ncf1\textsuperscript{m1J} mice. NOD- and C57BL/6.H2\textsuperscript{g7}-derived neutrophils and macrophages exhibited a normal oxidative burst. The inability of neutrophils and macrophages to generate a sufficient respiratory burst in the NOD.Ncf1\textsuperscript{m1J} mice was due to the absence of the p47\textsubscript{phox} protein. Immunoblot analysis of whole-cell lysates from purified macrophage and neutrophil (data not shown) cell populations demonstrated that the Ncf1 protein was absent in both cell types (Fig. 1E).

NOD.Ncf1\textsuperscript{m1J} T cells exhibit a reduced respiratory burst

T cells express a p47\textsubscript{phox}-containing NADPH oxidase, and polyclonal anti-CD3 stimulation elicits ROS production from primary T cells (10). Targeted deletion of Ncf1 eliminates T cell ROS production (10); therefore, we tested whether the Ncf1\textsuperscript{m1J} allele also inhibited anti-CD3–stimulated ROS production from CD4\textsuperscript{+} T cells in the NOD mouse. When purified CD4\textsuperscript{+} T cells from NOD mice were incubated with anti-CD3, there was a significant increase in ROS production by these CD4\textsuperscript{+} cells (Fig. 1G). In contrast, stimulation of NOD.Ncf1\textsuperscript{m1J} CD4\textsuperscript{+} T cells did not lead to an increase in oxidation of the fluorescent indicator (Fig. 1G, □). This lack of ROS production was not the result of low CD4\textsuperscript{+} T cell numbers, as no differences were observed when comparing NOD and NOD.Ncf1\textsuperscript{m1J} T cells (Fig. 1F).

FIGURE 3. Anti-CD3 and anti-CD28 stimulation of NOD.Ncf1\textsuperscript{m1J} T cells exhibits a decrease in Th1 cytokine profile concomitant with an increase in Th17 cytokine synthesis. Immunoblot analysis of p47\textsubscript{phox} expression in negatively selected and purified NOD and NOD.Ncf1\textsuperscript{m1J} T cells (A). Proliferation and cytokine synthesis of purified NOD and NOD.Ncf1\textsuperscript{m1J} T cells (2.5 × 10\textsuperscript{5} cells) after stimulation with polyclonal Abs for 72 h, as tested by cytokine-specific ELISAs and Luminex Bio-plex assay for cytokine synthesis (B–K). Proliferation was assessed by \textsuperscript{3}H-TdR incorporation. Results are representative of three independent experiments done in triplicate. n.m., Not measurable.
NOD.Ncf1m1J mice are protective against spontaneous diabetes

ROS synthesis is toxic to islet β cells and also responsible for pathological complications associated with T1D, but whether ROS production is also necessary for initiating diabetogenesis and an autoimmune effector T cell response is not known. To address these questions, spontaneous diabetes incidence of female NOD. Ncf1m1J mice in comparison with female NOD mice was determined. At 40 wk of age, 65% of female NOD.Ncf1m1J mice were euglycemic and diabetes free, whereas only 15% of the age-matched NOD females were euglycemic (Fig. 2A). Histological examination of pancreata from non-diabetic NOD mice at 7 and 22 wk demonstrated a significant increase in insulitis (Fig. 2B). Examination of pancreata from nondiabetic NOD mice at 7 and 22 wk revealed that the insulitis was not advanced with age in NOD. Ncf1m1J female mice (insulitis scores: 7 wk, 0.62 ± 0.54; 22 wk, 0.68 ± 0.62). Whereas the insulitis scores from 7-wk-old NOD and NOD.Ncf1m1J mice did not differ, the insulitis was significantly increased (p < 0.01) in 22-wk-old NOD compared with age-matched NOD.Ncf1m1J females. Representative histology is presented in Fig. 2C–F.

NOD.Ncf1m1J T cells exhibit a skewed cytokine response from Th1- to a Th17-like phenotype

Recent evidence has demonstrated that T cells express a phagocyte-type NADPH oxidase that is vital for TCR signaling upon CD3 and CD28 cross-linking (10); therefore, we hypothesized that NOX-deficient NOD mice would have decreased TCR signaling and subsequent adaptive immune effector function. Immunoblot analysis of whole-cell lysates from anti-CD3- and anti-CD28-stimulated NOD and NOD.Ncf1m1J T cells demonstrated that only NOD.Ncf1m1J T cells lacked p47phox expression (Fig. 3A) similar to bone marrow-derived macrophages (Fig. 1E). The Ncf1 mutation in NOD T cells also had a profound effect on cytokine synthesis as plate-bound anti-CD3 and anti-CD28 cross-linking of purified NOD.Ncf1m1J T cells exhibited a 3-fold decrease in proliferation, IL-2, and IFN-γ secretion (Fig. 3B–D) in comparison with NOD T cells. Interestingly, these polyclonal-stimulated T cells demonstrated a T cell response reminiscent of a Th17-like phenotype. NOD.Ncf1m1J T cells synthesized 2-fold decrease in TNF-α, 3-fold more IL-17, 40-fold increase in IL-23, 2-fold increase in IL-10, equivalent IL-6 levels, 2-fold decrease in IL-4, and a 2.5-fold decrease in GM-CSF levels compared with NOD T cells upon polyclonal stimulation (Fig. 3E–K). Additionally, intracellular cytokine staining of polyclonal stimulated naive CD4+CD62L+ T cells (Fig. 4) from NOD and NOD.Ncf1m1J mice was performed. No differences were observed in naive CD4+CD62L+CD69+CD44+ effector (CD4+CD62L+CD69+CD44+), and memory (CD4+CD62L+CD69+CD44+) splenic CD4+ T cell subsets from 8-wk-old, age-matched NOD and NOD. Ncf1m1J female mice by flow cytometry (Fig. 4A). Naive NOD-stimulated T cells exhibited a 1.5-fold increase in the percentage of CD4+IFN-γ+ cells (Fig. 4B), whereas NOD.Ncf1m1J T cells had a 5-fold increase in CD4+IL-17A+–expressing cells (Fig. 4C).

The decrease in Th1 cytokine synthesis (IFN-γ) from NOD. Ncf1m1J T cells was due to the absence of Th1-specific transcription factor expression such as T-bet, Stat4, and Stat1α (Fig. 5A, 5B). The absence of these key transcription factors in activated NOD.Ncf1m1J T cells was associated with the absence of Th1 cytokine synthesis and demonstrates an important role of superoxide in skewing Th responses. Activation of STAT3, which is necessary for Th17 lineage commitment (23, 24), was assessed by immunoblot analysis in NOD and NOD.Ncf1m1J T cells. Anti-CD3– and anti-CD28–stimulated NOD.Ncf1m1J T cells demonstrated an increase in phosphorylated STAT3 (Y705 and S727) in comparison with NOD T cells after stimulation for 48 and 72 h (Fig. 5C–E).

NOD.Ncf1m1J macrophages exhibit a decrease in proinflammatory cytokine and chemokine synthesis after innate immune activation

The generation of innate immune-derived proinflammatory cytokines is a redox-sensitive process that is highly dependent on the intra- and intercellular signaling capacities of ROS (20, 25–27) and necessary for the generation of an efficient Th1 adaptive immune effector response (20, 26–29). To determine whether the Ncf1m1J mutation in NOD.Ncf1m1J bone marrow-derived macrophages had an effect on skewing the NOD T cell response from a Th1- to a Th17-like phenotype, the innate immune response and the synthesis of proinflammatory cytokines from NOD.Ncf1m1J bone marrow-derived macrophages were analyzed after LPS stimulation. To determine whether the truncated form of p47phox inherently induced the unfolded protein response to affect macrophage activation, markers indicative of the unfolded protein response (BIP, CHOP, and XBP1 splicing) were evaluated by quantitative RT-PCR. No differences in expression were observed with control and LPS-stimulated NOD and NOD.Ncf1m1J macrophages (data not shown), eliminating endoplasmic reticulum stress as a mechanism for the heightened IL-23 production. NOD.Ncf1m1J macrophages demonstrated decreases of 3.5-fold in TNF-α, 3-fold in IL-1β, and 2-fold in IL-12 p70, but no difference in NO2–.
FIGURE 5. NOD.Ncf1m1/J T cells do not express Th1 lineage-specific transcription factors upon polyclonal stimulation. Immunoblot analysis of whole-cell lysates (20 μg protein) of negatively selected and purified NOD and NOD.Ncf1m1/J T cells after anti-CD3 and anti-CD28 stimulation for 72 h for T-bet, STAT4, and STAT1α. Actin was also probed to confirm equal protein loading on the gels (A). STAT3 activation was assessed by using phospho-specific STAT3 Abs (Y705 and S727) in an immunoblot analysis of anti-CD3– and anti-CD28–stimulated NOD and NOD.Ncf1m1/J T cells for 48 and 72 h. Actin was also probed to confirm equal protein loading on the gels (C). Cumulative data from three independent experiments employing Image J software (NIH) were used to determine the area under the curve values for T-bet, STAT4, or STAT1α to actin (B) or P-STAT3 (Y705) and P-STAT3 (S727) to actin (D, E) and as the change after NOD T cell samples were normalized to a value of 1. **p < 0.01 versus NOD T cells.
expression after LPS stimulation in comparison with NOD macrophages (Fig. 6A–D). In contrast, LPS-stimulated NOD.Ncf1−/− macrophages did exhibit increases in IL-12 p40 (3-fold), G-CSF (2-fold), and IL-6 (2-fold) cytokine synthesis as compared with NOD macrophages (Fig. 6E–G). Because IL-12 p40 levels in NOD. Ncf1−/− macrophages increased 3-fold (Fig. 6E), but IL-12 p70 levels decreased 2-fold (Fig. 6C) in comparison with NOD macrophages, we hypothesize that the increased IL-12 p40 subunit may heterodimerize with IL-12 p19 to generate IL-23. IL-23 synthesis in LPS-stimulated NOD and NOD.Ncf1−/− macrophages was examined at the protein level. NOD.Ncf1−/− macrophages exhibited a 2-fold increase in IL-23 synthesis by ELISA as compared with NOD macrophages (Fig. 6H).

Modulation of superoxide alters Th17 and Th1 cytokine responses after immunization

To determine whether a Th17 response in NOD.Ncf1−/− T cells was also occurring in response to nominal Ag immunization, NOD and NOD.Ncf1−/− mice were immunized with HEL and an Ag-recall assay was performed. The role and cell source of superoxide were assessed using a crisscross HEL Ag-recall assay with purified and negatively selected CD4+ T cell and APC LN cellular fractions from both HEL-immunized NOD and NOD.Ncf1−/− mice. As with the anti-CD3/anti-CD28 responses detailed above (Fig. 3B–K), in the recall portion of the assay when NOD T cells and APCs were mixed, they exhibited a Th1 cytokine response, whereas the mixture of NOD.Ncf1−/− T cells and APCs remained Th17 skewed (Fig. 7A–F). Interestingly, the development of HEL Ag-specific T cells in vivo with both intrinsic and extrinsic source of superoxide provided stringent commitment toward Th1 memory T cell responses that was unaltered when NOX-deficient APCs were used in the recall portion of the experiment. However, HEL Ag-specific NOD.Ncf1−/− T cells exhibited both Th1 and Th17 cytokine responses in the presence or absence of extrinsic superoxide, respectively. IFN-γ and IL-2 levels in HEL-specific NOD. Ncf1−/− T cells were restored to NOX-intact levels when NOD APCs provided an extrinsic source of superoxide (Fig. 7A, 7B). However, in the absence of an intrinsic source of NOX, NOD. Ncf1−/− T cells remained high producers of IL-17 and IL-10 (Fig. 7C, 7E). These data provide evidence that the presence and cellular source of superoxide are both key in the type of Th responses generated during immune responses.

NOD and NOD.Ncf1−/− mice do not differ in levels of T regulatory cells

Recently, links between Th17 cells and T regulatory (Treg) cells have been observed based on their requirement of TGF-β synthesis (30) and the established role of this cytokine in Treg maturation. Subsequently, experiments using combinations of IL-6, IL-21, and IL-23 with TGF-β established the concept that these cytokines influenced T cell progression toward a Th17 or Treg phenotype (31). Because NOD.Ncf1−/− mice respond to polyclonal and antigenic stimulation with a Th17-like cytokine profile (Figs. 3, 4, 6, 7), we further examined whether these mice also exhibited an increase in Treg cells. Flow cytometry for Treg cells, as defined by the cell-specific markers CD4+CD25+FoxP3+, was compared in NOD.Ncf1−/− and NOD mice. We did not observe a difference in absolute numbers of Treg cells (Fig. 8A) nor in the percentage of Treg cells relative to splenocytes (Fig. 8B) or CD4+ T cells (Fig. 1F) between NOD.Ncf1−/− and NOD mice.
NOD.Ncf1m1J mice exhibit enhanced sensitivity to MOG35–55 induced EAE

Th17 cells are essential mediators of autoimmune destruction in multiple sclerosis, enriched in the CNS infiltrates (32), and are highly pathogenic upon adoptive transfer (33). It is important to note that previous research has linked mutations in Ncf1 to enhanced RA and EAE susceptibility (11, 34), but associations with Ncf1 polymorphisms and enhanced Th17 responses have not been correlated. We therefore tested for differences in EAE induction between NOD and NOD.Ncf1m1J mice. T cells (2 × 105) and APCs (2 × 106) were combined in a crisscross fashion with 25 μg HEL and stimulated for 72 h. Supernatants were collected and cytokine synthesis was measured with cytokine-specific ELISAs and a Luminex Bio-plex cytokine panel (A−F). Results are representative of three independent experiments.

Discussion

In previous studies, we have demonstrated the importance of ROS in the pathogenesis of T1D (3–5, 12, 13, 16, 35–41). Whereas elevated ROS-dissipating enzymes protect against β cell destruction in vitro (42, 43), the transgenic overexpression of antioxidants in β cells via the rat insulin promoter has been less efficacious in protecting against spontaneous T1D development (44–46). Previous studies have demonstrated a role of systemic elevation of ROS dissipation (4, 5, 12, 13, 35, 41) in protection from T1D, yet the effect of altering immune cell ROS production in the context of T1D has not been extensively tested. To address this question, NOD mice containing a mutation in Ncf1 (NOD.Ncf1m1J) that prevents the proper assembly of the NOX complex, resulting in the absence of superoxide synthesis from stimulated neutrophils (Fig. 1B), macrophages (Fig. 1D), and T cells (Fig. 1G), were generated.

Inhibition of NOX activity had a positive impact on diabetes development. NOD.Ncf1m1J female mice exhibited a significant reduction (35% in NOD.Ncf1m1J versus 85% in NOD; p < 0.0003) and delay (p < 0.0001) in autoimmune diabetes development compared with female NOD mice. The delay in T1D was mirrored by a slower insulitis progression in NOD.Ncf1m1J pancreata. Insulitis scores for both NOD and NOD.Ncf1m1J were equal at 7 wk of age. A significant divergence (p < 0.01) was observed comparing NOX-deficient NOD mice at 22 wk of age with nondiabetic wild-type NOD females. This lack of advance in insulin damage signals a delay in the switch from peripheral to invasive insulitis. Therefore, NOX activity is not essential for T1D onset, but it is required for insulitis progression and T1D initiation in the majority of the cases of T1D.

Whereas we have observed reduced autoimmunity in NOD. Ncf1m1J mice, the Ncf1m1J allele has been characterized previously in murine models of rheumatoid arthritis (RA) and EAE (11). In addition, deactivating mutations in Ncf1 have been associated with RA in both patients as well as in a rat RA model (47, 48).

Table II.  NOD.Ncf1m1J mice exhibit enhanced susceptibility to EAE

<table>
<thead>
<tr>
<th>Strain</th>
<th>EAE Incidence</th>
<th>Mean Severity</th>
<th>First Day of Onset</th>
<th>Average Age of Onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJL</td>
<td>0/9</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>NOD</td>
<td>3/10</td>
<td>0.5</td>
<td>51</td>
<td>62 ± 10</td>
</tr>
<tr>
<td>NOD.Ncf1m1J</td>
<td>11/11b</td>
<td>1.8</td>
<td>9</td>
<td>23 ± 18</td>
</tr>
</tbody>
</table>

*EAE was induced as described in Materials and Methods.

bEAE incidence compared with NOD p < 0.0001.

*Mean severity compared with NOD p = 0.0018.
Likewise, NOD.Ncf1m1J mice also exhibited enhanced EAE sensitivity in contrast to the significant reduction in T1D observed in this mutant strain. There are differences between these diseases, with perhaps the most obvious being the invertebrate Th17 responses associated with both EAE (49, 50) and collagen-induced arthritis (51, 52), whereas data extant have positioned T1D as a Th1-mediated disease (53). Based on our results with the NOD. Ncf1m1J mouse, we hypothesized that the divergence in onset of autoimmunity in mice with the Ncf1m1J mutation results from alterations in the Th responses that are indicative of T1D (Th1) versus EAE and RA (Th17). The results contained within demonstrate that superoxide is a key mediator of this phenomenon.

In the absence of superoxide generation, the immune system may compensate for impaired neutrophil respiratory burst activity by deviating toward a Th17 phenotype (54). Neutrophils are an essential arm of innate immunity, and their main effector mechanism in response to infection is the initiation of a respiratory burst to generate superoxide. IL-17 has no direct chemotactic activity, but it can induce G-CSF, IL-6, and chemokine expression (55, 56) to recruit and activate circulating neutrophils (57). Loss of ROS-mediated inflammation may result in the immune system compensating for this defect by overexpressing signals necessary for neutrophil recruitment via IL-17R signaling. Recent evidence has demonstrated a role for IL-23 in mobilizing neutrophils when host defense requires superoxide production (54). Therefore, the overproduction of IL-23 in the NOD.Ncf1m1J mouse is likely a result of the absence of superoxide production. These findings provide evidence that the loss of superoxide synthesis will deviate a Th1-prone immune response toward a Th17 cytokine response.

It has been well characterized that NOD mice exhibit numerous T cell immunological defects such as a strong skewed Th1 cytokine response (58), altered secretion of IL-2 (59), and predisposition to heightened IFN-γ response compared with C57BL/6 (60). The enhanced Th1 cytokine response in NOD mice may be due to chronic redox signaling mediated by an inherent altered redox homeostatic state. The cytoplasmic and nuclear redox environment of NOD T cells may facilitate and enhance Th1 proinflammatory cytokine synthesis that is absent in C57BL/6 T cells. The role of NOX inactivity has conflicting interpretations on the inflammatory cytokine synthesis that is absent in C57BL/6 T cells. Polyclonal-stimulated CD4+ and CD8+ T cells from C57BL/6.gp91phox and C57BL/6.gg9phox mice demonstrate elevated IFN-γ levels and ERK1/2 phosphorylation in contrast to wild-type T cells. However, C57BL/6 T cells deficient in the GTP-binding protein Rac2, a coadaptor molecule essential for NOX activity (61, 62), exhibited altered T cell activation, as evidenced by a 2- to 3-fold reduction in IFN-γ synthesis, and decreased ERK1/2 and p38MAPK activation (63, 64). Based on these as well as our previous results, we propose that the introduction of the Ncf1m1J mutation in the NOD background lowers the chronic production of ROS, resetting redox homeostasis and signaling in this autoimmune prone strain.

We previously reported that in the absence of a redox-dependent proinflammatory third signal consisting of both ROS and proinflammatory cytokines (TNF-α, IL-1β, IL-12 p70), Ag-specific T cells fail to achieve optimal Th1 effector function (20). The NOD.Ncf1m1J mouse corroborates these observations due to their inherent inability to generate superoxide and the resulting altered cytokine profile. In our current study, we were able to further define and characterize the importance of proinflammatory third signal synthesis as it pertains to both Th1 and Th17 T cell adaptive immune maturation. Naive CD4+ T cells from NOD mice elicited a Th1 response synthesizing increased levels of IFN-γ after polyclonal stimulation. However, NOD.Ncf1m1J naive T cells had elevated IL-17, demonstrating a Th17 response when ROS production is lacking. Additionally, NOD mice immunized with HEL generated a memory effector Th1 cytokine response (IL-12 p70, IFN-γ), whereas NOD.Ncf1m1J mice generated a Th17 (IL-17) memory effector cytokine response upon secondary challenge with HEL. In NOX-intact NOD mice whereby an intrinsic (T cell) and extrinsic (APC) source of superoxide is available, Ag-specific Th1 T cells undergo efficient adaptive immune maturation and effector function. Upon secondary challenge, only a single source of NOX either intrinsically (T cell) or extrinsically (APC) will suffice to mediate an efficient Th1 cytokine response. The memory T cells from the NOD mouse remain committed in a Th1 phenotype; alternatively, the lack of extrinsic superoxide cannot reprogram these cells (Fig. 7C). Interestingly, the cytokine response of NOD.Ncf1m1J T cells from the HEL crisscross Ag-recall assay could be restored to a Th1 phenotype (IL-12 p70, IFN-γ) if an extrinsic source of superoxide (NOD APCs) was provided upon secondary challenge. Th17 cells have been noted for their ability to change phenotype. Highly polarized Th17 BDC-2.5 T cells expressing IL-12Rβ2 responded to IL-12 p70 and reverted to a Th1 phenotype after adoptive transfer in vivo (65). Further confirming the plasticity of Th17 T cells in T1D, Martin-Orozco et al. (66) also demonstrated that Th17-polarized BDC-2.5 T cells converted to IFN-γ–producing cells and mediate pancreatic β cell destruction. Th17 T cells may have a role in promoting inflammation, but it is the generation of IFN-γ and a Th1 effector response that is ultimately responsible for autoimmune-mediated β cell death in T1D.

The work presented in this study demonstrates the significance and importance of innate and adaptive immune sources of superoxide to mediate efficient Th1 T cell responses. Loss of intrinsic and extrinsic NOX elicited a novel and skewed Th17 lineage commitment program, while suppressing the expression of transcription factors necessary for Th1 T cell development. Alternately, the presence of superoxide appears to drive commitment to Th1. These in vitro responses were confirmed by enhanced susceptibility to EAE in superoxide-deficient NOD.Ncf1m1J mice, whereas NOX-intact NOD mice were polarized to Th1 and developed T1D. Recent reports have documented that Tbx21 and Gata3, genes for Th1 and Th2 lineage commitment, respectively, contain both active and repressive histone modifications in all effector T cell subsets that may explain the flexibility of T cells to change phenotypes (67). Whether oxidation of guanine nucleotides to 8-oxo-G in the promoter elements of Tbx21, STAT4, Rorc, and STAT3 can mediate gene silencing and/or expression similar to histone modifications warrants further study. Furthermore, characterizing the importance of ROS in T cell responses may provide a novel therapeutic target for the treatment of inflammatory-mediated diseases such as T1D, whereas enhancing ROS may skew immune responses that are at the root of multiple sclerosis or RA.

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Disclosures
The authors have no financial conflicts of interest.

References


**Supplemental Table.** Informative Microsatellite Markers Sets Used to Generate the NOD-\(Ncf1^{mlj}\) Strain.

| D1Mit430 | D5Mit287 | D12Mit79 |
| D1Mit18* | D6Mit384 | D13Mit274* |
| D1Mit132* | D6Mit322* | D13Mit250* |
| D1Mit495 | D6Mit100* | D13Mit256 |
| D1Mit159 | D6Mit52* | D13Mit74 |
| D1Mit206 | D6Mit198* | D13Mit260 |
| D1Mit155 | D6Mit374* | D14Mit206* |
| D2Mit362 | D7Mit178* | D14Mit222* |
| D2Mit297 | D7Mit117* | D14Mit259* |
| D2Mit100 | D7Mit213* | D14Mit262 |
| D2Mit395* | D7Mit101* | D14Mit72 |
| D2Mit287* | D7Mit101 | D14Mit95 |
| D2Mit528 | D7Mit155 | D15Mit229 |
| D2Mit148 | D8Mit190 | D15Mit71 |
| D3Mit151* | D8Mit144* | D15Mit159 |
| D3Mit175* | D8Mit211* | D15Mit161 |
| D3Mit254* | D8Mit88* | D16Mit98 |
| D3Mit292* | D9Mit252* | D16Mit43 |
| D3Mit256* | D9Mit48* | D16Mit222 |
| D3Mit128 | D9Mit355 | D17Mit100* |
| D3Mit19 | D9Mit120 | D17Mit16* |
| D4Mit193 | D10Mit213* | D17Mit176* |
| D4Mit286* | D10Mit55* | D17Mit93 |
| D4Mit17* | D10Mit259 | D18Mit111* |
| D4Mit9* | D10Mit230 | D18Mit177* |
| D4Mit308* | D10Mit233 | D18Mit51* |
| D4Mit251* | D11Mit296 | D18Mit4 |
| D4Mit180* | D11Mit177* | D19Mit96 |
| D5Mit69* | D11Mit179* | D19Mit19 |
| D5Mit61* | D11Mit333 | D19Mit55 |
| D5Mit352 | D11Mit48 |
| D5Mit309 | D12Mit285 |
| D5Mit101 | D12Mit214 |

* - Denotes a microsatellite primer pair that marks a genetic region previously defined to contribute to T1D in the NOD mouse.