Loss of NOX-Derived Superoxide Exacerbates Diabetogenic CD4 T-Cell Effector Responses in Type 1 Diabetes

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Reactive oxygen species (ROS) play prominent roles in numerous biological systems. While classically expressed by neutrophils and macrophages, CD4 T cells also express NADPH oxidase (NOX), the superoxide-generating multisubunit enzyme. Our laboratory recently demonstrated that superoxide-deficient nonobese diabetic (NOD, Ncf1m1J) mice exhibited a delay in type 1 diabetes (T1D) partially due to blunted IFN-γ synthesis by CD4 T cells. For further investigation of the roles of superoxide on CD4 T-cell diabetogenicity, the NOD.BDC-2.5.Ncf1m1J (BDC-2.5.Ncf1m1J) mouse strain was generated, possessing autoreactive CD4 T cells deficient in NOX-derived superoxide. Unlike NOD.Ncf1m1J, stimulated BDC-2.5.Ncf1m1J CD4 T cells and splenocytes displayed elevated synthesis of Th1 cytokines and chemokines. Superoxide-deficient BDC-2.5 mice developed spontaneous T1D, and CD4 T cells were more diabetogenic upon adoptive transfer into NOD.Rag recipients due to a skewing toward impaired Treg suppression. Exogenous superoxide blunted exacerbated Th1 cytokines and proinflammatory chemokines to approximately wild-type levels, concomitant with reduced IL-12Rβ2 signaling and P-STAT4 (Y693) activation. These results highlight the importance of NOX-derived superoxide in curbing autoactivity due, in part, to control of Treg function and as a redox-dependent checkpoint of effector T-cell responses. Ultimately, our studies reveal the complexities of free radicals in CD4 T-cell responses.
antiviral innate immune responses (17), dysregulated CD4 and CD8 T-cell responses (10,18,19), and an enhancement in alternatively activated M2 macrophages (19).

We sought to further examine the role of NOX-derived ROS in diabetogenic CD4 T-cell effector responses using the NOD.BDC-2.5 (BDC-2.5) mouse strain. The BDC-2.5 mouse is an invaluable tool for dissecting the role of the NOD.BDC-2.5 (BDC-2.5) mouse strain. The BDC-2.5 mice were purchased from R&D Systems. Fluorochrome-conjugated anti-CD19, -CD25, -CD44, and -CD69 antibodies were purchased from eBioscience, while biotin anti-mouse CD4, in addition to anti-F4/80 fluorochrome-conjugated and live/dead fluorochrome-conjugated antibodies, was purchased from Invitrogen. Anti–P-STAT4 (Y693) antibody was purchased from Cell Signaling, while anti-STAT4 antibody was obtained from Biolegend. The BDC-2.5 mimotope (EKAHRPIWARMDAKK) was synthesized by the University of Alabama at Birmingham peptide synthesis core facility. Xanthine oxidase and β-actin were obtained from Sigma-Aldrich, and r-hIL-2 was purchased from Peprotech.

**Generation of the BDC-2.5.Ncf1<sup>m<sub>L</sub></sup>L Mouse**

NOD.BDC-2.5.Ncf1<sup>m<sub>L</sub></sup>L (BDC-2.5.Ncf1<sup>m<sub>L</sub></sup>L) mice were generated by crossing BDC-2.5 (21) to NOD.Ncf1<sup>m<sub>L</sub></sup>L (10) mice followed by intercrossing of the F1 progeny. F2 progeny homozygous for the Ncf1<sup>m<sub>L</sub></sup>L mutation (10) and the BDC-2.5 transgene (21) were used to establish this strain. The inability to synthesize superoxide did not affect lymphocyte development and differentiation in BDC-2.5.Ncf1<sup>m<sub>L</sub></sup>L mice, as lymphocyte population percentages revealed equivalent expression of Vβ4, CD4 T cells (Supplementary Fig. 2A) within superoxide-sufficient and -deficient strains according to the gating strategy depicted in Supplementary Fig. 1. No differences were observed in the percentages of B cells (Supplementary Fig. 2B), macrophages (Supplementary Fig. 2C), dendritic cells (Supplementary Fig. 2D), and regulatory CD4 T cells (Supplementary Fig. 2E).

**Luminol Oxidation to Detect Superoxide Synthesis**

The redox-sensitive substrate luminol was used to detect superoxide synthesis (17). BDC-2.5 and BDC-2.5.Ncf1<sup>m<sub>L</sub></sup>L splenocytes and CD4 T cells were seeded onto 96-well polystyrene round-bottom plates with 200 μmol/L luminol (Sigma-Aldrich), 0.32 units/mL horseradish peroxidase (Sigma-Aldrich), 100 ng/mL phorbol 12-myristate 13-acetate (PMA), and 1 μg/mL ionomycin or 1 μmol/L BDC-2.5 mimotope (9). Luminescence was quantified using a SpectraMax L Luminescence microplate reader with readings every 2 min for 60 min.

**Immuno-Spin Trapping and Immunofluorescence**

Macromolecular-centered free radicals were detected upon stimulating splenocytes with 1 μmol/L BDC-2.5 mimotope in the presence of 1 mmol/L 5,5-dimethyl-1-pyrroline N-oxide (DMPO; Dojindo) in tissue culture–treated chamber slides. Cells were fixed in 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS, blocked with 5% BSA in PBS, and incubated with 20 μg/mL of chicken IgY anti-DMPO as previously described (25,26). DMPO adducts were detected with Alexa Fluor 488–conjugated goat anti-chicken IgY secondary antibody (1:500; Invitrogen). P-STAT4 (Y693) and STAT4 were detected with Alexa Fluor 647–conjugated goat anti-β-actin secondary antibody (1:500; Jackson ImmunoResearch), respectively. CD4 T cells were identified with Alexa Fluor 647–conjugated anti-β-actin antibody pairs, and fluorochrome-conjugated anti-IL-12Rβ2 antibody pairs were purchased from R&D Systems. Fluorochrome-conjugated anti-CD19, -CD25, -CD44, and -CD69 antibodies were purchased from eBioscience, while biotin anti-mouse CD4, in addition to anti-F4/80 fluorochrome-conjugated and live/dead fluorochrome-conjugated antibodies, was purchased from Invitrogen. Anti–P-STAT4 (Y693) antibody was purchased from Cell Signaling, while anti-STAT4 antibody was obtained from Biolegend. The BDC-2.5 mimotope (EKAHRPIWARMDAKK) was synthesized by the University of Alabama at Birmingham peptide synthesis core facility. Xanthine oxidase and β-actin were obtained from Sigma-Aldrich, and r-hIL-2 was purchased from Peprotech.

**RESEARCH DESIGN AND METHODS**

**Animals**

NOD.Cg-Ncf1<sup>m<sub>L</sub></sup>L/Mx (NOD.Ncf1<sup>m<sub>L</sub></sup>L), NOD.Cg-Tg (TcrαBDC2.5,TcrβBDC2.5)/DoiJ (BDC-2.5), and NOD.129S7 (B6)-Rag<sup>fl</sup>W<sub>1</sub>Bom/J (NOD.Rag) mice were bred and housed under pathogen-free conditions in the Research Support Building animal facility at the University of Alabama at Birmingham. BDC-2.5 mice were originally obtained from Kathryn Haskins at the National Jewish Hospital (Denver, CO), and NOD.Rag mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained on a light/dark (12/12 h) cycle at 23°C and received continuous access to standard laboratory chow and acidified water. Age- and sex-matched BDC-2.5 and NOD.Ncf1<sup>m<sub>L</sub></sup>L mice were used for all experiments and in accordance with the University of Alabama at Birmingham and University of Florida Institutional Animal Care and Use Committee.

**Materials**

Anti–γ-interferon (IFN-γ), − interleukin (IL)-2, − IL-10, and − IL-17A antibody pairs for ELISA; fluorochrome-conjugated anti-CD4, − CD8, − B220, − Vβ4, − Vβ5, − Vβ8, − CD11b, and − CD11c antibodies for fluorescence-activated cell sorter (FACS); and anti-CD3ε and anti-CD28 antibodies were purchased from BD Biosciences. IL-1β, CCLS, and tumor necrosis factor (TNF)-α DuoSet ELISA kits, CXCL10 antibody pairs, and fluorochrome-conjugated anti–IL-12Rβ2 antibody pairs were purchased from R&D Systems. Fluorochrome-conjugated anti-CD19, -CD25, -CD44, and -CD69 antibodies were purchased from eBioscience, while biotin anti-mouse CD4, in addition to anti-F4/80 fluorochrome-conjugated and live/dead fluorochrome-conjugated antibodies, was purchased from Invitrogen. Anti–P-STAT4 (Y693) antibody was purchased from Cell Signaling, while anti-STAT4 antibody was obtained from Biolegend. The BDC-2.5 mimotope (EKAHRPIWARMDAKK) was synthesized by the University of Alabama at Birmingham peptide synthesis core facility. Xanthine oxidase and β-actin were obtained from Sigma-Aldrich, and r-hIL-2 was purchased from Peprotech.
CD4 T-Cell Purification, Polyclonal Stimulation, Primary Recall Assays, Greiss Assay, ELISAs, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Assay

Splenocytes from BDC-2.5 and BDC-2.5.Ncf1m1J mice were cultured in FACS buffer (1% BSA in PBS) with appropriately diluted fluorochrome-conjugated antibodies and isotype controls (BD Biosciences, eBioscience, and R&D) (Supplementary Figs. 6 and 10) (17). One thousand cell events were collected with the FACSCalibur, and data were analyzed with FlowJo, version 9.6.1, software (Tree Star Incorporated).

CD4 T-Cell Purification, Polyclonal Stimulation, Primary Recall Assays, Greiss Assay, ELISAs, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Assay

Splenocytes from BDC-2.5 and BDC-2.5.Ncf1m1J mice were purified by negative selection according to the manufacturer’s protocol using the EasySep CD4 T-cell enrichment kit (STEMCELL Technologies). CD4 T-cell purity was routinely assessed by flow cytometry and found to be >90% (data not shown). T cells were stimulated with plate-bound anti-CD3ε (0.1 μg/mL) and anti-CD28 (1 μg/mL), and exogenous superoxide was added via 1 μM/mL xanthine oxidase (XO) (17). BDC-2.5 and BDC-2.5.Ncf1m1J splenocytes were incubated with 1 μmol/L BDC-2.5 mimotope or 104 dispersed NOD.Rag islet cells in the presence or absence of 1 μM/mL XO (9,15). Greiss assay and ELISAs were read on a Synergy2 microplate reader with Gen5, version 1.10, software (BioTek) as previously described (17). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to assess T-cell proliferation was performed according to the manufacturer’s protocol (Sigma-Aldrich).

Adoptive Transfer of Type 1 Diabetes and In Vivo Suppression Assay

Adoptive transfer of purified CD4 T cells into NOD.Rag recipients was performed as described previously (10,27). BDC-2.5 and BDC-2.5.Ncf1m1J Tregs were purified via negative selection, stained with fluorescein isothiocyanate–labeled anti–glucocorticoid-induced tumor necrosis factor receptor–related protein and phycoerythrin–labeled anti–CD25. The CD25/GITR double-positive Treg population was sorted with a BD FACSaria III (BD Biosciences). The percentage of Foxp3+ Treg cells isolated after sorting was similar between BDC-2.5 and BDC-2.5.Ncf1m1J (Supplementary Fig. 8). Polyclonally stimulated BDC-2.5 CD4 T cells were used as effector cells. Stimulated-BDC-2.5 CD4 effector T cells (105) were transferred intraperitoneally, either alone or cotransferred with BDC-2.5 or BDC-2.5. Ncf1m1J Tregs (2 × 108), into NOD.Rag mice and monitored for T1D as described.

Statistical Analysis

Data were analyzed via GraphPad Prism, version 5.0, statistical software. The difference between mean values for each experimental group was assessed using the two-tailed Student t test with P < 0.05 considered significant.

RESULTS

BDC-2.5.Ncf1m1J CD4 T Cells and Splenocytes Exhibited a Dampened Respiratory Burst

For exploration of the role of NOX-derived superoxide on diabetogenic CD4 T-cell effector responses, the BDC-2.5. Ncf1m1J mouse strain was generated, and luminol oxidation by BDC-2.5 and BDC-2.5.Ncf1m1J splenocytes was used to corroborate the deficiency in NOX-derived superoxide (Fig. 1A). Stimulated BDC-2.5.Ncf1m1J splenocytes displayed a 21-fold reduction in chemiluminescence compared with BDC-2.5 splenocytes with luminol and horseradish peroxidase (HRP). To validate blunted free radical synthesis within the CD4 T-cell compartment, we stimulated BDC-2.5 and BDC-2.5.Ncf1m1J CD4 T cells with PMA/ionomycin in the presence of luminol and HRP (Fig. 1B). Primary human and mouse CD4 T cells have been shown to express a functional phagocyte type NOX, and T-cell receptor (TCR) stimulation elicits rapid production of superoxide and hydrogen peroxide (28). Similar to splenocytes, luminol oxidation by stimulated BDC-2.5. Ncf1m1J CD4 T cells was decreased 1.4-fold compared with BDC-2.5 CD4 T cells, confirming the blunted respiratory burst capacity from our previous studies using CD4 or CD8 T lymphocytes (10,18).

BDC-2.5.Ncf1m1J CD4 T Cells Displayed Blunted Cellular Self-Oxidation

For further demonstration that NOX enzymatic activity was curtailed in the absence of NOX-derived superoxide, immuno-spin trapping using the free radical spin trap DMPO was performed to quantify the self-oxidation of BDC-2.5 and BDC-2.5.Ncf1m1J splenocytes (Fig. 2). The formation of free radicals in macromolecules of the cells results in the formation of stable DMPO adducts that were imaged and quantified. The BDC-2.5 mimotope elicited an increase in DMPO-adduct formation by BDC-2.5 CD4 T cells and MHC-II (OX-6) cells; however, BDC-2.5-Ncf1m1J splenocytes displayed a severe reduction in DMPO adducts within CD4 and MHC-II compartments compared with BDC-2.5 (Fig. 2A). In the absence of DMPO addition to antigen-stimulated BDC-2.5 and BDC-2.5.Ncf1m1J splenocytes, immunofluorescence detection of free radical adducts was absent (Supplementary Fig. 3). The diminution in DMPO-adduct synthesis by BDC-2.5.Ncf1m1J CD4- and MHC-II–expressing cells was significant upon quantification, further corroborating reduced respiratory burst activity by...
NOX-deficient BDC-2.5 CD4 T cells upon stimulation with their cognate antigen (Fig. 2B).

**BDC-2.5.Ncf1m1J Splenocytes and CD4 T Cells Exhibited Enhanced Synthesis of NO2 and Proinflammatory Cytokines and Chemokines**

For investigation of the role of NOX-derived superoxide in diabetogenic CD4 T cells, BDC-2.5 and BDC-2.5.Ncf1m1J splenocytes were stimulated with dispersed NOD.Rag islet cells and the BDC-2.5 mimotope. Contrary to results from our laboratory and others implicating ROS as a proinflammatory-derived third signal essential for effective adaptive immunity (8–10,14), BDC-2.5.Ncf1m1J splenocytes displayed significant elevations in nitrite (NO2−) (2.7-fold), IFN-γ (4.3- and 2.0-fold), TNF-α (4- and 1.7-fold), IL-1β...
(1.5- and 2.5-fold), IL-17A (3.0- and 2.0-fold), and IL-2 (2- and 1.5-fold) upon islet and mimotope stimulation, respectively, compared with BDC-2.5 (Fig. 3A–F). Besides an influx in pathogenic proinflammatory cytokines that contribute to β-cell demise and T1D autoreactivity in T1D, BDC-2.5. Ncf1m1J splenocytes displayed enhanced synthesis of proinflammatory chemokines implicated in T1D pathogenesis, including CCL5 (chemokine [C-C] motif ligand 5) (3- and 2.0-fold) and CXCL10 (chemokine [C-X-C motif] ligand 10) (332- and 2.0-fold) upon incubation with dispersed islet cells or mimotope, respectively (Fig. 3G–H) (29–31). In addition to splenocytes, purified BDC-2.5. Ncf1m1J CD4 T cells exhibited significant elevations in nitrite (3.8-fold), IFN-γ (2.8-fold), TNF-α (twofold), IL-1β (4.4-fold), IL-17A (4.5-fold), IL-2 (1.5-fold), CCL5 (2.9-fold), and CXCL10 (twofold) (Supplementary Fig. 4A–H). Thus, despite possessing deficiencies in NOX-derived superoxide, BDC-2.5. Ncf1m1J splenocytes and CD4 T cells displayed

**Figure 3**—BDC-2.5. Ncf1m1J splenocytes displayed an upregulated proinflammatory effector response. Supernatants from BDC-2.5 and BDC-2.5. Ncf1m1J splenocytes stimulated with dispersed NOD.Rag islets or 1 μmol/L BDC-2.5 mimotope for 48, 72, and 96 h were assayed for synthesis of nitrite (96 h (A)), IFN-γ (72 h (B)), TNF-α (72 h (C)), IL-1β (96 h (D)), IL-17A (48 h (E)), IL-2 (72 h (F)), CCL5 (72 h (G)), and CXCL10 (72 h (H)). Data shown represent average of 3 experiments performed in triplicate. Ag, antigen; ND, not detected. ***P < 0.0001; **P < 0.01; *P < 0.05.
elevated levels of proinflammatory mediators implicated in T1D pathogenesis.

**BDC-2.5.Ncf1m1J CD4 T Cells Exhibited Elevated Activation Markers**

For further demonstration that BDC-2.5.Ncf1m1J CD4 T cells display an exacerbated effector response in contrast to BDC-2.5, expression of the T-cell activation markers CD69, CD25, and CD44 was assessed by flow cytometry after antigenic stimulation (Fig. 4). Superoxide-deficient BDC-2.5 CD4 T cells exhibited an increase in surface expression of CD69 (early activation marker) (Fig. 4A), a 1.6-fold elevation in percentage of CD44 (Fig. 4C), a cell-surface glycoprotein that marks effector memory CD4 T cells (32). Pooled data from activated BDC-2.5.Ncf1m1J CD4 T cells further demonstrated a significant (P < 0.05) increase in CD69 (A), CD25 (B), and CD44 (C) T-cell activation markers in contrast to BDC-2.5 (Supplementary Fig. 7). In agreement with increased proinflammatory cytokine and chemokine synthesis by stimulated BDC-2.5.Ncf1m1J T cells (Fig. 3 and Supplementary Fig. 4), the inherent absence of NOX-derived superoxide synthesis elicited an elevated T-cell activation phenotype.

**BDC-2.5.Ncf1m1J Mice Developed Spontaneous T1D and Enhanced Diabetogenicity Upon Adoptive Transfer With Purified BDC-2.5.Ncf1m1J CD4 T Cells**

For further demonstration that loss of NOX-derived superoxide can augment CD4 T-cell effector responses, spontaneous T1D incidence was compared between BDC-2.5 and BDC-2.5.Ncf1m1J mice. While BDC-2.5 mice do not spontaneously develop autoimmune diabetes, 20% of BDC-2.5.Ncf1m1J female mice were hyperglycemic at 30 weeks of age (Fig. 5A). For examination of the influence of NOX-derived superoxide on CD4 T-cell diabetogenicity, adoptive transfers of purified, preactivated BDC-2.5 and BDC-2.5.Ncf1m1J CD4 T cells into NOD.Rag recipients were performed (Fig. 5B). Because of the enhanced synthesis of proinflammatory cytokines and chemokines by BDC-2.5.Ncf1m1J splenocytes (Fig. 3) and CD4 T cells (Supplementary Fig. 4), we hypothesized that adoptive transfer of superoxide-deficient CD4 T cells would elicit enhanced diabetogenicity compared with BDC-2.5 (21,33). Indeed, transfer of BDC-2.5.Ncf1m1J CD4 T cells was more diabetogenic. Only 26% of BDC-2.5.Ncf1m1J-transferred NOD.Rag (n = 45) recipients remained diabetes free at 11 days posttransfer. In contrast, 56% of BDC-2.5-transferred NOD.Rag mice (n = 24) (Fig. 5B) did not develop T1D. However, at 31 days posttransfer, no significant difference in diabetogenicity was observed among the groups of NOD.Rag mice receiving BDC-2.5 or BDC-2.5.Ncf1m1J CD4 T cells (Fig. 5B).

For determination of whether the observed enhanced diabetogenicity could be due to an intrinsic defect in Treg suppressive function, splenic BDC-2.5 and BDC-2.5.Ncf1m1J Treg cells were purified and used in an in vivo suppression assay with effector CD4 T cells isolated from BDC-2.5 mice (Fig. 5C). Coadoptive transfers into NOD.Rag recipients demonstrated that, as expected (34), BDC-2.5 Treg cells were functional and capable of significantly delaying T1D. The majority of the NOD.Rag mice transferred with BDC-2.5 Treg cells remained diabetes free (7 of 10 at 30 days posttransfer). However, BDC-2.5.Ncf1m1J Treg cells were compromised in their ability to delay T1D. This in vivo suppression assay resulted in only 33% of the NOD.Rag recipients remaining diabetes free (n = 9) at 30 days postcotransfer (Fig. 5C) even though the percentage of Foxp3+ CD4+ CD25+ GITR+ T cells was similar from purified BDC-2.5 and BDC-2.5.Ncf1m1J splenic Treg cells (Supplementary Fig. 8).

**Figure 4**—BDC-2.5.Ncf1m1J CD4 T cells exhibited increased expression of T-cell activation markers. Flow cytometric analysis of CD69 (A), CD25 (B), and CD44 (C) by live BDC-2.5 and BDC-2.5.Ncf1m1J CD4 T cells upon mimotope stimulation for 24 h. Lymphocytes were gated using forward-scatter and side-scatter profiles, while live cells were gated via side-scatter profiles using a fixable live/dead stain (Invitrogen). FACS plots represent data from 3 independent experiments.
Addition of an Extrinsic Source of Superoxide

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Elevated Proinflammatory Cytokine and Chemokine Synthesis by BDC-2.5.Ncf1m1J—Was Attenuated by the Addition of an Extrinsic Source of Superoxide

For determination of whether elevated proinflammatory cytokine and chemokine synthesis was directly linked to inefficiencies in NOX-derived superoxide, XO was added to BDC-2.5 and BDC-2.5. Ncf1m1J splenocytes stimulated with the BDC-2.5 mimotope (Fig. 6A–E) or to purified, splenic BDC-2.5 and BDC-2.5.Ncf1m1J CD4 T cells stimulated with α-CD3ε and α-CD28 (Fig. 6F–H). At 24 h poststimulation with the BDC-2.5 mimotope, BDC-2.5.Ncf1m1J splenocytes exhibited a 2.3-fold elevation in IFN-γ synthesis that was blunted by this superoxide donor. XO-mediated superoxide production reduced IFN-γ synthesis levels similar to BDC-2.5 CD4 T cells (Fig. 6A). The enhanced levels of TNF-α by stimulated BDC-2.5.Ncf1m1J splenocytes were also reduced to wild-type levels with XO addition (Fig. 6B). Synthesis of IL-17A was threefold enhanced with mimotope stimulation, but XO addition only slightly attenuated IL-17A production by BDC-2.5.Ncf1m1J in comparison with BDC-2.5 (Fig. 6C). Further demonstrating the selective decreases in cytokine and chemokine responses by XO addition to stimulated BDC-2.5.Ncf1m1J splenocytes, synthesis of the T-cell growth factor IL-2 remained unchanged with this superoxide donor (Fig. 6D). CCL5 synthesis was regulated by redox status, as XO induced profound reductions in CCL5 by both stimulated BDC-2.5 and BDC-2.5.Ncf1m1J splenocytes (Fig. 6E). Similar results were obtained with the chemokine CXCL10, with levels below the limit of detection after XO addition (data not shown). In addition to an elevation in effector cytokine and chemokine synthesis, antigen-stimulated BDC-2.5.Ncf1m1J splenocytes also displayed a twofold increase in T-cell proliferation as demonstrated by formazan release in an MTT assay, in contrast to BDC-2.5 splenocytes (Supplementary Fig. 9). Interestingly, unlike effector cytokine synthesis, addition of XO did not decrease T-cell proliferation of mimotope-stimulated BDC-2.5 and BDC-2.5.Ncf1m1J splenocytes.

For ascertainment of whether exacerbations in IFN-γ by purified BDC-2.5.Ncf1m1J CD4 T cells were similarly attributed to defects in NOX-derived superoxide, XO was added to α-CD3ε and α-CD28—stimulated BDC-2.5 and BDC-2.5. Ncf1m1J CD4 T cells (Fig. 6F–H). As anticipated, polyclonal stimulation of BDC-2.5.Ncf1m1J CD4 T cells elicited a two-fold elevation in IFN-γ, but exogenous XO addition dampened IFN-γ production by 1.4-fold to approximately BDC-2.5 levels (Fig. 6F). Similar to BDC-2.5.Ncf1m1J splenocytes, modulation of redox status did not affect Th17 cytokine responses with BDC-2.5.Ncf1m1J CD4 T cells, as production of IL-17A was unchanged with XO addition (Fig. 6G). Interestingly, XO addition enhanced IL-2 synthesis with BDC-2.5 and BDC-2.5.Ncf1m1J CD4 T cells (Fig. 6H). XO did not negatively impact the viability of BDC-2.5 or BDC-2.5.Ncf1m1J splenocytes or CD4 T cells, as determined by an MTT assay (data not shown).

BDC-2.5.Ncf1m1J Splenocytes Displayed Increased IL-12Rβ2 and P-STAT4 (Y693) Redox-Dependent Expression Upon Mimotope Stimulation Compared with BDC-2.5

For determination of the redox-dependent mechanism of elevated Th1 cytokine responses by BDC-2.5.Ncf1m1J (Fig. 3B; Supplementary Fig. 4), expression of IL-12Rβ2, a Th1 signal transducer, (Fig. 7 and Supplementary Fig. 10), and

Elevated Proinflammatory Cytokine and Chemokine Synthesis by BDC-2.5.Ncf1m1J—Was Attenuated by the Addition of an Extrinsic Source of Superoxide

For determination of whether elevated proinflammatory cytokine and chemokine synthesis was directly linked to inefficiencies in NOX-derived superoxide, the superoxide-generating enzyme XO was added to BDC-2.5 and BDC-2.5. Ncf1m1J splenocytes stimulated with the BDC-2.5 mimotope (Fig. 6A–E) or to purified, splenic BDC-2.5 and BDC-2.5.Ncf1m1J CD4 T cells stimulated with α-CD3ε and α-CD28 (Fig. 6F–H). At 24 h poststimulation with the BDC-2.5 mimotope, BDC-2.5.Ncf1m1J splenocytes exhibited a 2.3-fold elevation in IFN-γ synthesis that was blunted by this superoxide donor. XO-mediated superoxide production reduced IFN-γ synthesis levels similar to BDC-2.5 CD4 T cells (Fig. 6A). The enhanced levels of TNF-α by stimulated BDC-2.5.Ncf1m1J splenocytes were also reduced to wild-type levels with XO addition (Fig. 6B). Synthesis of IL-17A was threefold enhanced with mimotope stimulation, but XO addition only slightly attenuated IL-17A production by BDC-2.5.Ncf1m1J in comparison with BDC-2.5 (Fig. 6C). Further demonstrating the selective decreases in cytokine and chemokine responses by XO addition to stimulated BDC-2.5.Ncf1m1J splenocytes, synthesis of the T-cell growth factor IL-2 remained unchanged with this superoxide donor (Fig. 6D). CCL5 synthesis was regulated by redox status, as XO induced profound reductions in CCL5 by both stimulated BDC-2.5 and BDC-2.5.Ncf1m1J splenocytes (Fig. 6E). Similar results were obtained with the chemokine CXCL10, with levels below the limit of detection after XO addition (data not shown). In addition to an elevation in effector cytokine and chemokine synthesis, antigen-stimulated BDC-2.5.Ncf1m1J splenocytes also displayed a twofold increase in T-cell proliferation as demonstrated by formazan release in an MTT assay, in contrast to BDC-2.5 splenocytes (Supplementary Fig. 9). Interestingly, unlike effector cytokine synthesis, addition of XO did not decrease T-cell proliferation of mimotope-stimulated BDC-2.5 and BDC-2.5.Ncf1m1J splenocytes.

For ascertainment of whether exacerbations in IFN-γ by purified BDC-2.5.Ncf1m1J CD4 T cells were similarly attributed to defects in NOX-derived superoxide, XO was added to α-CD3ε and α-CD28—stimulated BDC-2.5 and BDC-2.5. Ncf1m1J CD4 T cells (Fig. 6F–H). As anticipated, polyclonal stimulation of BDC-2.5.Ncf1m1J CD4 T cells elicited a two-fold elevation in IFN-γ, but exogenous XO addition dampened IFN-γ production by 1.4-fold to approximately BDC-2.5 levels (Fig. 6F). Similar to BDC-2.5.Ncf1m1J splenocytes, modulation of redox status did not affect Th17 cytokine responses with BDC-2.5.Ncf1m1J CD4 T cells, as production of IL-17A was unchanged with XO addition (Fig. 6G). Interestingly, XO addition enhanced IL-2 synthesis with BDC-2.5 and BDC-2.5.Ncf1m1J CD4 T cells (Fig. 6H). XO did not negatively impact the viability of BDC-2.5 or BDC-2.5.Ncf1m1J splenocytes or CD4 T cells, as determined by an MTT assay (data not shown).

BDC-2.5.Ncf1m1J Splenocytes Displayed Increased IL-12Rβ2 and P-STAT4 (Y693) Redox-Dependent Expression Upon Mimotope Stimulation Compared with BDC-2.5

For determination of the redox-dependent mechanism of elevated Th1 cytokine responses by BDC-2.5.Ncf1m1J (Fig. 3B; Supplementary Fig. 4), expression of IL-12Rβ2, a Th1 signal transducer, (Fig. 7 and Supplementary Fig. 10), and
the phosphorylation status of STAT4, a transcription factor essential for Th1 responses (Fig. 8 and Supplementary Fig. 11), were examined via flow cytometry and immunofluorescence, respectively. Mimotope stimulation elicited a 1.5-fold increase in IL-12Rβ2+, CD4+ T cells by superoxide-deficient splenocytes and a 1.35-fold elevation in gMFI (geometric mean fluorescence intensity) (Fig. 7). Upon addition of xanthine oxidase, IL-12Rβ2 expression was attenuated by mimotope-stimulated superoxide-deficient CD4 T cells (Fig. 7). Similar to IL-12Rβ2, P-STAT4 (Y693), an essential Th1 transcription factor, was significantly enhanced by BDC-2.5.Ncf1m1J (Fig. 8 and Supplementary Fig. 11).
With exogenous superoxide addition via XO, P-STAT4 (Y693) was severely diminished to levels similar to NOX-intact BDC-2.5 cells (Fig. 8). Thus, in addition to functioning as a proinflammatory-derived third signal, ROS can attenuate Th1 proinflammatory cytokine responses by lessening IL-12Rβ2 signaling and STAT4 activation.

**DISCUSSION**

In T1D, ROS directly destroy β-cells and induce proinflammatory cytokines that further propagate β-cell destruction by maturing autoreactive adaptive immune effector responses (35,36). Reports by our laboratory and others have demonstrated that systemic ablation of innate immune-derived ROS results in antigen-specific hyporesponsiveness (8,15). We previously demonstrated that NOD mice defective in NOX-derived superoxide via the spontaneous mutation Ncf1m1J were protected against T1D onset due to skewed CD4 T-cell responses (10), reduced CD8 T-cell cytotoxic capacity (18), lessened antiviral proinflammatory responses (17,27), and deviation to an alternatively-activated M2 macrophage phenotype (27). To further define the role of ROS in diabetogenic CD4 T-cell responses, we generated the BDC-2.5.Ncf1m1J mouse strain, possessing autoreactive and superoxide-deficient...

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**Figure 7**—Superoxide donors dampened the expression of IL-12Rβ2. Flow cytometric analysis of IL-12Rβ2 by live BDC-2.5 and BDC-2.5. Ncf1m1J CD4 T cells upon mimotope stimulation with or without addition of XO for 72 h. Lymphocytes were gated using forward-scatter and side-scatter profiles, while live cells were gated via side-scatter profiles using a fixable live/dead stain (Invitrogen). IL-12Rβ2 flow cytometry is representative of 2 independent experiments.
Figure 8—BDC-2.5.Ncf1m1J splenocytes exhibited enhanced P-STAT4 (Y693) activation that was curtailed with exogenous superoxide. Immunofluorescence identification of CD4 T cells (Alexa Fluor 647), STAT4 (Cy3), and P-STAT4 (Y693) (Alexa Fluor 488) by BDC-2.5 and BDC-2.5.Ncf1m1J splenocytes stimulated with 1 μmol/L BDC-2.5 mimotope with or without 1 mU/mL XO for 24 h (A). The fluorescence intensity of P-STAT4 (Y693) by immune cells was quantitated with ImageJ software and normalized to STAT4 (B). P-STAT4 (Y693) immunofluorescence is representative of 3 independent experiments with the following total number of counted cells per group: BDC-2.5 no antigen, n = 278; BDC-2.5.Ncf1m1J no antigen, n = 570; BDC-2.5 + mimotope, n = 652; BDC-2.5.Ncf1m1J + mimotope, n = 497; BDC-2.5 + XO, n = 366; BDC-2.5.Ncf1m1J + XO, n = 375; BDC-2.5 + mimotope + XO, n = 670; BDC-2.5.Ncf1m1J + mimotope + XO, n = 641). Images were magnified at 40× and digitally enlarged. ns, not significant. *P < 0.05.
CD4 T cells. Because of the unique T1D resistance displayed by NOD.Ncf1\textsuperscript{m1J} mice, we hypothesized that BDC-2.5.Ncf1\textsuperscript{m1J} CD4 T cells would exhibit decreased effector cytokine synthesis and diabetogenicity. Surprisingly, unlike the reduced Th1 cytokine profile displayed by NOD. Ncf1\textsuperscript{m1J}, activated BDC-2.5.Ncf1\textsuperscript{m1J} splenocytes and CD4 T cells displayed elevated synthesis of proinflammatory chemokines, Th1 and Th17 cytokines; enhanced diabetogenicity; and reduced Treg suppressive function.

Although initially apparently contradictory, these results are supported by other lines of evidence in additional autoimmune diseases. Deficiencies in NOX-derived superoxide have been shown to enhance autoimmunity in animal models of autoimmune arthritis (37,38), multiple sclerosis (10,37), Crohn disease (39), systemic lupus erythematosus (40), and psoriasis (41). With the aid of the NOD.BDC-2.5. Ncf1\textsuperscript{m1J} high-affinity TCR transgenic murine model, we can now include NOX-derived superoxide as a novel immunoregulatory molecule of diabetogenic CD4 T cell responses and pancreatic β-cell destruction in T1D. In addition to enhanced proinflammatory cytokine and chemokine synthesis, we report that BDC-2.5.Ncf1\textsuperscript{m1J} CD4 T cells were more diabetogenic during spontaneous and adoptive transfer of T1D, due to trending reductions in Treg-mediated immunosuppression. Our data corroborate a previous report of defective immunoregulation by Tregs possessing the Ncf1\textsuperscript{m1J} mutation (42). Additionally, rodents bearing a spontaneous mutation in the Ncf1\textsuperscript{m1J} gene were more susceptible to autoimmune arthritis (37), and administration of the ROS donor phytol curbed arthritogenic T-cell responses (43). Similarly, we demonstrated that T1D-resistant NOD.Ncf1\textsuperscript{m1J} mice exhibited enhanced sensitivity to myelin oligodendrocyte glycoprotein (MOG\textsubscript{35-55})-induced experimental autoimmune encephalomyelitis (10). Analogous to murine models, chronic granulomatous disease patients, harboring mutations in the phagocyte NOX complex, possessed an elevated type I interferon gene signature, increased synthesis of proinflammatory cytokines (i.e., IL-17A) and chemokines (40), macrophages that were profoundly less efficient in inducing Tregs compared with healthy controls (44), and an elevated risk of developing autoimmunity (45–47). These results provide evidence that NOX-derived superoxide can negatively regulate effector T-cell and B-cell responses in various autoimmune diseases.

Recent studies by our laboratory demonstrated that NOD.Ncf1\textsuperscript{m1J} CD4 T cells were equally pathogenic as NOD CD4 T cells, as cotransfers of purified NOD.Ncf1\textsuperscript{m1J} CD4 T cells with NOD CD8 T cells resulted in rapid T1D-onset kinetics (18). These results provide evidence that NOX-derived superoxide is involved in peripheral tolerance and elicits a redox-dependent signaling cue to halt diabetogenic CD4 T cells. ROS synthesis negatively impacted IL-12Rβ2 signaling and STAT4 activation, providing mechanistic insight to the process whereby ROS can blunt CD4 T-cell activation. Our results provide evidence that moderate increases in oxidative stress may be beneficial as an essential checkpoint to curtail T cell–mediated diseases dominated by Th1 cytokine responses, such as T1D.

Here, we demonstrate that NOX-deficient CD4 T cells bearing a high-affinity autoreactive T-cell receptor exhibited elevated IFN-γ production. This is in conflict with our previous observation that IFN-γ was blunted in the absence of NOX-derived superoxide by polyclonal NOD.Ncf1\textsuperscript{m1J} CD4 T cells (10). This difference in Th1 responses could be attributed to inherent disparities in T-cell phenotype. NOD CD4 T-cell responses are polyclonal, characterized by distinct avidities to different autoantigens, while BDC-2.5 CD4 T cells are inherently skewed to a Th1-like phenotype and recognize a single autoantigen, chromogranin A (21,22). NOD mice spontaneously develop T1D, whereas BDC-2.5 mice exhibit a low incidence due to immunosuppressive Treg cells (34). Enhanced Th1 cytokine responses have not only been observed within BDC-2.5.Ncf1\textsuperscript{m1J} CD4 T cells; our preliminary data also demonstrate that BDC-6.9, another diabetogenic CD4 T cell clone that recognizes islet amyloid polypeptide (IAPP) (48), exhibits an enhanced Th1 and Th17 cytokine response in the absence of NOX-derived superoxide (L.E. Padgett, N.N. Morgan, and H.M. Tse, unpublished data). These monoclonal TCR transgenic murine models of T1D have enabled us to uncover the importance of ROS in T-cell effector inhibition, and thus, similar to rheumatoid arthritis (RA) (37,38), multiple sclerosis (10,37), Crohn disease, psoriasis (41), and systemic lupus erythematosus (40), increasing the production of free radicals by CD4 T cells may be efficacious in T1D.

The disparate in vitro Th1 responses by NOD.Ncf1\textsuperscript{m1J} and BDC-2.5.Ncf1\textsuperscript{m1J} could additionally be attributed to differences in inherent levels of ROS and sensitivity of immune cells to redox-dependent signaling. Illustrative of the inherent dissimilarity in redox status, BDC-2.5.Ncf1\textsuperscript{m1J} CD4 T cells displayed elevated nitrite, but no difference was observed in NOD and NOD. Ncf1\textsuperscript{m1J} CD4 T cells (10). A profound mechanism by which ROS influence T-cell effector responses at the immunological synapse is by reversibly oxidizing thiol residues located within TCR adaptor molecules. TCR adaptor molecules are regulated by redox status and, more importantly, T-bet, the master transcription factor involved in Th1 cytokine responses, is also influenced by ROS synthesis (10). Future studies will dissect differences in redox-dependent signaling by exploring TCR adaptor molecule oxidation status within NOD.Ncf1\textsuperscript{m1J} and BDC-2.5.Ncf1\textsuperscript{m1J} CD4 T cells, as unraveling these may reveal novel targets for T1D protection and prevention.

By using T1D-resistant NOD.Ncf1\textsuperscript{m1J} mice lacking the ability to generate NOX-derived superoxide (10), our laboratory has defined an influential role for free radicals on the maturation of autoimmune responses in T1D. We have mechanistically determined that the induction of innate immune signaling pathways and differentiation of islet-resident and islet-infiltrating proinflammatory
M1 macrophages are dependent on NOX-derived superoxide during spontaneous T1D (10,17,18,27). The absence of superoxide can skew macrophage responses toward an alternatively activated M2 phenotype and elicit β-cell protection. However, the role of NOX-derived superoxide on T cells is more complex. Free radicals may have distinct effects on T-cell subsets, in addition to expansion, contraction, and memory formation. CD8 T cells from NOD.Ncf1m1J mice are impaired and unable to transfer T1D (10,18). These observations would suggest that CD8 T cells are highly dependent on NOX-derived superoxide as a signaling mediator for cytotoxic lymphocyte maturation. CD4 T cells from NOD.Ncf1m1J mice also require superoxide as a third signal to efficiently generate Th1 cytokine-producing cells (10), but within a subset of high-affinity autoreactive T cells (BDC-2.5, BDC-6.9), NOX-derived superoxide may also be necessary as a checkpoint to quell T-cell effector responses, facilitate T-cell contraction, and/or restore peripheral tolerance in T1D. Our studies further define the synergism of redox biology in autoimmune dysregulation in T1D and may lead to the development of novel immunotherapies to delay disease progression.

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