A Metalloporphyrin-Based Superoxide Dismutase Mimic Inhibits Adoptive Transfer of Autoimmune Diabetes by a Diabetogenic T-Cell Clone

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We present here the first report of a metalloporphyrin-based antioxidant that can prevent or delay the onset of autoimmune diabetes. Type 1 diabetes is an autoimmune process whereby T-cells recognize pancreatic β-cell antigens and initiate a leukocyte infiltrate that produces proinflammatory cytokines and reactive oxygen species (ROS), ultimately leading to β-cell destruction. Because islet β-cells have a reduced capacity to scavenge free radicals, they are very sensitive to ROS action. Metalloporphyrin-based superoxide dismutase (SOD) mimics scavenge ROS and protect cells from oxidative stress and apoptosis. To investigate the effect of SOD mimics and the role of oxidative stress in the development of autoimmune diabetes in vivo, we used a diabetogenic T-cell clone, BDC-2.5, to induce rapid onset of diabetes in young nonobese diabetic-severe combined immunodeficient mice (NOD.scid). Disease was significantly delayed or prevented altogether by treatment of recipient mice with an SOD mimic, AEOL-10113, before transfer of the BDC-2.5 clone. To investigate the mechanisms of protection, in vitro assays for T-cell proliferation and γ-interferon (IFN-γ) production were carried out using the T-cell clone BDC-2.5. We found that the SOD mimic significantly inhibited antigen-presenting cell–dependent T-cell proliferation and IFN-γ production in vitro. In addition, pretreatment of lipopolysaccharide (LPS)-stimulated peritoneal macrophages with SOD mimic inhibited the LPS-dependent increase in TNF-α as well as the NADPH oxidase–dependent release of superoxide. Finally, this compound protected NIT-1 insulinoma cells from interleukin-1β and alloxan cytotoxicity in vitro. *Diabetes* 51:347–355, 2002

Type 1 diabetes is caused by the autoimmune destruction of insulin-producing pancreatic β-cells. A large body of evidence supports the concept that the antigen-specific T-cell–mediated infiltration of inflammatory cells to the pancreas leads to the generation of reactive oxygen species (ROS) (superoxide [$O_2^{\cdot-}$], hydroxyl radical [·OH], nitric oxide [NO·], peroxynitrite [ONOO$^-$]) and proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and γ-interferon (IFN-γ) (1–4). Synergistic interaction between ROS and these cytokines results in the ultimate destruction of pancreatic β-cells.

Locally produced ROS are involved in the effector mechanisms of β-cell destruction (1–3,5–7). In vitro, T-cell and macrophage cytokines such as IFN-γ, IL-1β, and TNF-α induce the production of ROS by β-cells. In addition, ROS either given exogenously or elicited in β-cells by cytokines lead to β-cell destruction (8). This destruction appears to ultimately be caused by apoptotic and/or necrotic mechanisms (9–14). β-Cells engineered to overexpress antioxidant proteins have been shown to be resistant to ROS and NO· (15–21). Furthermore, stable expression of Mn superoxide dismutase (SOD) in insulinoma cells prevented IL-1β–induced cytotoxicity and reduced NO· production (22). Finally, others have shown that transgenic mice with β-cell–targeted overexpression of copper, zinc SOD, or thioredoxin are resistant to autoimmune and streptozotocin-induced diabetes (23–25).

We used a pharmacological approach to protect β-cells from the T-cell–mediated ROS and cytokine destruction associated with autoimmune diabetes by using a synthetic metalloporphyrin-based SOD mimic, AEOL-10113. The SOD mimics are designed with a redox-active metal center that catalyzes the dismutation of $O_2^{\cdot-}$ in a manner similar to the active metal sites of the mammalian Cu-, Zn- or Mn-containing SODs (26–31). The Mn porphyrins have a broad antioxidant specificity, which includes scavenging $O_2^{\cdot-}$ (32), H_2O_2 (31,33), ONOO$^-$ (34), NO· (35), and lipid peroxyl radicals (36). SOD mimics have recently been found to rescue vascular contractility in endotoxic shock (37), protect neuronal cells from excitotoxic cell death (38) and apoptosis (39), inhibit lipid peroxidation (36,40), block hydrogen peroxide–induced mitochondrial DNA damage (41), and partially rescue a lethal phenotype in a Mn-SOD knockout mouse (42). The ability of the SOD

mimics to scavenge a broad range of ROS allows for their use in inflammatory diseases.

For rapid induction of hyperglycemia and β-cell destruction, we used a diabetogenic T-cell clone in an adoptive transfer system. BDC-2.5 is an islet antigen–specific CD4^{+} Th1 T-cell clone (43,44). In vivo, it rapidly and reproducibly transfers diabetes into young (<2 weeks old) NOD or NOD.*scid* recipients (45,46). The adoptive transfer of BDC-2.5 into susceptible recipients provides an ideal model for studying the protective effects of metalloporphyrin-based SOD mimics on T-cell–mediated inflammation. In this article, we show that the SOD mimic can prevent diabetes in young NOD.*scid* recipients after adoptive transfer of BDC-2.5. We also show that the mimic has immunomodulatory function and prevents the cytotoxic action of inflammatory cytokines or pro-oxidant diabetogenic agents in islet β-cells in vitro.

RESEARCH DESIGN AND METHODS

Mice. NOD.*scid* breeding pairs were obtained either from the Jackson Laboratory (Bar Harbor, ME) or the breeding colony at the Barbara Davis Center. NOD, NOD.*scid*, and BDC-2.5-TCR-Tg/NOD (2.5 TCR Tg/NOD) mice were bred and housed under specific pathogen-free conditions in the Center for Laboratory Animal Care at the University of Colorado Health Sciences Center.

Expansion cultures of BDC-2.5. Expansion cultures for in vivo transfers were produced by culturing $3–6 \times 10^6$ T-cells from 4-day restimulation cultures (43,44) in 60 ml complete medium (CM) and 14 units/ml IL-2. CM is Dulbecco's modified Eagle's medium supplemented with 44 mmol/l sodium bicarbonate, 0.55 mmol/l L-arginine, 0.27 mmol/l L-asparagine, 1.5 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 50 mg/l gentamicin sulfate, 50 μmol/l mercapto-ethanol, 10 mmol/l HEPES, and 10% FCS. Cells were cultured in 75-cm² flasks for 4 days at 37°C and 10% CO_2. T-cells were harvested, washed three times, resuspended in Hanks' balanced salt solution (HBSS), and injected into young (<15 days of age) NOD.*scid* recipients.

Metalloporphyrin SOD mimic (AEOL-10113). The SOD mimic Mn(III) tetrakis(*N*-ethylpyridinium-2-yl)porphyrin (AEOL-10113) was a gift from Incara Pharmaceuticals. Stock solutions of 600 μg/ml in sterile HBSS for in vivo use or 680 μmol/l in sterile CM for in vitro experiments were prepared.

Adoptive transfer of BDC-2.5 T-cell clones. Experimental mice were young NOD.*scid* mice 3–14 days of age. The recipient mice were given one intraperitoneal injection with BDC-2.5 (1×10^7 cells) 1 day after the administration of either the SOD mimic or HBSS as a control. The mimic or HBSS was administered every other day, for a total of five treatments. Urine glucose was monitored daily, and blood glucose measurements were taken when animals became diabetic. Overt diabetes was defined as a positive urine glucose (>1%), followed by a positive blood glucose test of >250 mg/dl (14 mmol/l). Recipients were killed when blood glucose readings were 320 mg/dl (18 mmol/l) or higher. At death, the pancreases were removed for histological analysis.

Histology. At death, pancreases were removed and placed in formalin for at least 24 h. Pancreases were subsequently embedded in paraffin, sectioned, and stained with hematoxylin and eosin to detect mononuclear cell infiltration or with aldehyde fuchsin to detect insulin.

Preparation of purified CD4^{+} T-cells from 2.5 TCR-Tg/NOD mice. The 2.5 TCR-Tg/NOD mice were injected intraperitoneally with either 10 mg/kg SOD mimic or HBSS every day for 7 days. At day 8, animals were killed, and the spleens were removed for isolation of CD4^{+} T-cells by immunomagnetic-positive selection using the MACs magnetic cell separation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. The purified T-cells were then plated in 96-well round-bottom plates and precoated with 50 μl of a 1 μmol/l solution of BDC-2.5 peptide mimotope, HRPI-RM, as an antigen. Antigen-presenting cells (APCs), treated with either the SOD mimic or HBSS, were added to the T-cells in a crisscross fashion. The assay plates were incubated for 4 days and then pulsed with 1 μCi titrated thymidine (^{3}H-TdR) for 6 h before harvesting.

T-cell and macrophage functional assays. IFN-γ production by BDC-2.5 was assessed by sandwich enzyme-linked immunosorbent assay (ELISA) analysis of responder T-cells stimulated with α-CD3 and α-CD28, concanavalin A (ConA), or islet cell antigen. For α-CD3/α-CD28 stimulation, 96-well round-bottom plates were precoated with 0.125 μg/ml α-CD3 and 1 μg/ml α-CD28 for 1 h at 37°C. After washing the plates with sterile HBSS and

blocking with CM at 37°C for 1 h, the blocking solution was removed, and the BDC-2.5 T-cell clone (2×10^4 cells) was added to the wells in the presence or absence of the SOD mimic at concentrations of 17 and 34 μmol/l. The negative control was BDC-2.5 alone without α-CD3 and α-CD28. For ConA stimulation, BDC-2.5 T-cells were plated at 2×10^4 cells/well in 96-well flat-bottom plates with or without 5×10^5 irradiated syngeneic spleen cells as APC and ConA (2.5 μg/ml final concentration), in the presence or absence of the SOD mimic at concentrations of 17 and 34 μmol/l. Cultures were incubated at 37°C for 24 h before the supernatants were harvested and assayed by sandwich ELISA for IFN-γ production. For antigen-specific recall assays, BDC-2.5 T-cells were cultured in 96-well flat-bottom plates at a density of 2×10^4 cells/well, with 5,000 islet cells as antigen and 2.5×10^4 APCs, in the presence or absence of 17 and 34 μmol/l SOD mimic. Cultures were incubated at 37°C for 48 h before the supernatants were harvested and assayed for IFN-γ. For macrophage assays, peritoneal macrophages (PCs) were harvested from unprimed NOD mice by lavage, washed two times in sterile HBSS, and adjusted to 5×10^5 cells/well in a 24-well plate in CM with *Escherichia coli* lipopolysaccharide (LPS) (055:B5) at 200 ng/ml in the presence or absence of 17 or 34 μmol/l SOD mimic. Cultures were incubated at 37°C for 48 h before the supernatants were harvested and assayed by specific sandwich ELISA for TNF-α production, following the manufacturer's protocol (R&D Systems, Minneapolis, MN). The remaining cells were collected by trypsinization and washed three times in sterile PBS and 4% FCS.

Respiratory burst of PCs. PCs, harvested as described above, were washed two times in sterile HBSS and then plated (5×10^5 cells/well) in 24-well plates in CM medium with *E. coli* LPS (055:B5) at 200 ng/ml in the presence or absence of the SOD mimic at 34 or 3.4 μmol/l. Cultures were incubated at 37°C for 48 h. Cells were trypsinized and then washed to remove the trypsin and subsequently transferred to microfuge tubes. Phorbol myristate acetate (PMA) was added to a final concentration of 50 ng/ml. After incubation at 37°C for 20 min, superoxide production was assessed spectrophotometrically by ferricytochrome c reduction using $\epsilon = 20,000 \ mol \cdot l^{-1} \cdot cm^{-1}$, monitoring the reduction over a period of 10 min.

Determination of β-cell apoptosis. In vitro apoptosis studies were conducted using the β-cell adenoma line NIT-1 (47). Tumor cells were propagated in 75-cm² flasks at 37°C in CM. Cell lines were refed with new medium every other day and were grown to confluence in the 75-cm² tissue culture flasks, at which time they were harvested using nonenzymatic Cell Dissociation Buffer (Life Technologies, Grand Island, NY) and transferred to the appropriate culture dishes either for expansion or for the experiments described. Alloxan monohydrate (Sigma, St. Louis, MO) was prepared fresh as a 0.5 mol/l stock solution in PBS adjusted to pH 2 with hydrochloric acid. IL-1β was purchased from R&D Systems. NIT-1 cells were grown to confluence in 12-well tissue culture dishes. Media were removed and replaced with PBS alone or PBS containing 34 μmol/l mimic. All solutions were supplemented with 4% FCS. After a 1-h incubation, 10 mmol/l alloxan was added to the appropriate wells, and cells were incubated for an additional 2 h. For cytokine cytotoxicity assays, NIT-1 cells were grown to 80% confluence in 12-well plastic tissue culture dishes. Growth media were removed and replaced with 500 μl/well of either media alone or media + 34 μmol/l mimic. After a 1-h incubation, 500 μl/well of media alone or 20 ng/ml IL-1 (10 ng/ml final concentration) ± 34 μmol/l SOD mimic was added. Cells were incubated an additional 48 h and processed. Alloxan- or cytokine-treated NIT-1 cells were harvested by brief trypsinization (200 μl/well of a 12-well dish), followed by addition of 50 μl FCS to inhibit trypsin. Cells were transferred to a microcentrifuge tube and centrifuged for 5 min at 200g. Supernatants were aspirated carefully, leaving ~25 μl to allow resuspension of the cell pellets by gentle shaking of the tube. After addition of 1.3 μl dye mix (100 μg/ml acridine orange + 100 μg/ml of ethidium bromide in PBS), 10 μl cell suspension was transferred to a clean microscope slide, and a coverslip was placed on the suspension. Cells were scored for morphological evidence of apoptosis as described (48,49), using a fluorescence microscope with an excitation of 450–490 nm. Briefly, assessment of apoptotic versus necrotic cells was determined by visualization of the cell for both the color and the state of the nucleus, using a mixture of the DNA intercalating dyes acridine orange and ethidium bromide. Acridine orange penetrates the plasma membrane of living cells and stains their nuclei bright green, whereas ethidium bromide can only be taken up by cells for which membrane integrity is compromised. The criteria we used to score the cells after treatment were as follows: 100 cells (minimum) were scored into one of four categories: live nonapoptotic (green nuclei, normal distribution of chromatin), live apoptotic (green nuclei, condensed chromatin), dead non-apoptotic (necrotic) (orange nuclei, normal distribution of chromatin), and dead apoptotic (orange nuclei, condensed chromatin).

Statistical analysis. Statistical significance within experiments was determined using JMP analysis software (SAS Institute, Cary, NC). Survival analysis was done using the product-limit (Kaplan-Meier) method. The end point of the

experiment was defined as diabetes. Data on animals that did not become diabetic by the end of the experiment were censored. The *P* values shown were determined by the log-rank test. All other statistical analysis was done by one-way ANOVA (Wilcoxon's/Kruskal-Wallis rank-sum test). *P* values ≤0.05 were considered significant.

RESULTS

In vivo treatment of young NOD.*scid* mice with the SOD mimic prevents adoptive transfer of T-cell–mediated diabetes. SOD mimic was delivered paraenterally to NOD.*scid* recipients, and 24 h later, mice were adoptively transferred with the diabetogenic T-cell clone BDC-2.5. The SOD mimic or HBSS was then given every other day for a total of five treatments. Treatment with the SOD mimic significantly delayed onset of diabetes ($P <$ 0.0002) (Fig. 1*A*), with 50% of the treated mice still normoglycemic after 28 days, at which time all animals were sacrificed for histological examination. Pancreatic tissue from positive control animals (BDC-2.5, no SOD mimic) showed a disseminated infiltrate resembling pancreatitis, and the pancreatic architecture was almost absent (Fig. 1*B*, a). In contrast, the SOD mimic–treated animals showed an intact pancreatic architecture with few or no infiltrating mononuclear cells (Fig. 1*B*, b and c), as well as healthy and well-granulated islets (Fig. 1*B*, d). These data clearly demonstrate that the SOD mimic is inhibiting the infiltration by BDC-2.5 T-cells and mononuclear cells to the pancreas. Remarkably, in these experiments, the animals were still protected on day 21, even though the SOD mimic was stopped on day 9, suggesting that this compound prevents priming and subsequent activation of the APC, the T-cell, or both. Longer administration of the SOD mimic may prove to be even more protective.

IFN-γ production by BDC-2.5 is inhibited by the SOD mimic in vitro: indirect effect on the APC, leading to inhibition of T-cell priming. In vivo, BDC-2.5 must be primed by its antigen via presentation by APCs to become activated and produce IFN-γ. Therefore, the SOD mimic could directly inhibit T-cell activation or the interaction between the APC and the T-cell or both. To elucidate the mechanism of inhibition of disease transfer, we studied priming of BDC-2.5 in vitro in the presence or absence of APC. To determine whether the SOD mimic has a direct effect on IFN-γ production by the T-cell, we cultured BDC-2.5 with plate-bound α-CD3 and α-CD28. This type of activation substitutes for signals 1 and 2 of T-cell activation (50–53), thus removing the contribution of the APC. Fig. 2*A* shows that α-CD3 and α-CD28 stimulation resulted in no significant difference in IFN-γ production by the BDC-2.5 clone, whether or not the SOD mimic was present. These results demonstrate that when plate-bound antibodies substitute for signals 1 and 2, the SOD mimic has no direct effect on the ability of BDC-2.5 to be stimulated to effector function and produce IFN-γ. Although primed T-cells can directly respond to ConA, optimal ConA-induced T-cell cytokine production requires the participation of accessory cells (e.g., macrophages) (54–60). To determine whether the SOD mimic could inhibit APC-mediated ConA stimulation of T-cells, BDC-2.5 cells were incubated with ConA and APC in the presence or absence of the SOD mimic. Figure 2*B* shows that 34 or 17 μmol/l SOD mimic inhibited IFN-γ production by 47 or

30%, respectively. The levels of IFN-γ produced in the presence of the SOD mimic were similar to levels seen when BDC-2.5 was incubated with ConA alone. These results suggest that the SOD mimic inhibits the ability of the APC to optimally stimulate ConA-dependent T-cell activation and IFN-γ production. To further study the SOD mimic's effect on APC–T-cell interactions, we measured IFN-γ production in the presence of macrophages as APC and islet cells as a source of antigen. Figure 2*C* shows that when this more physiological in vitro assay was done, the ability of BDC-2.5 to make IFN-γ was reduced: the 17 μmol/l concentration of SOD mimic was inhibited by 46% ($P < 0.05$), whereas the 34 μmol/l concentration was inhibited by 66% ($P < 0.05$).

In vivo treatment of 2.5 TCR Tg/NOD mice with the SOD mimic affects T-cell proliferation by inhibiting APC function. To determine whether the SOD mimic can influence T-cell priming in vivo, 2.5 TCR-Tg/NOD mice, which carry the rearranged TCR genes of the BDC-2.5 T-cell clone (61), were treated with either the SOD mimic (10 mg/kg) or HBSS each day for 7 days. The T-cells and APCs were purified from SOD mimic–treated and control mice and cultured in a crisscross proliferation assay using a peptide mimotope HRPI-RM that acts as a stimulating antigen for the 2.5 TCR-Tg cells (H. Kikutani, and K.H., unpublished data). Figure 3 demonstrates that APC from SOD mimic–treated mice showed a reduced ability to support T-cell proliferation whether they are presenting the peptide to SOD mimic–treated or untreated T-cells. Notably, when control APCs were used as presenters, the proliferative response in SOD mimic–treated T-cells approached the level achieved with control APCs and T-cells. These data demonstrate that in vivo SOD mimic treatment inhibits the response in TCR-Tg mice primed to a specific self-peptide and suggest that using the SOD mimic in combination with candidate autoantigens may provide a form of antigen-specific tolerance.

LPS-induced respiratory burst and cytokine production by PC is inhibited by the SOD mimic. Macrophages are activated in the two-stage reactions of priming and triggering (62). To assess the inhibitory effect of the SOD mimic on this process, we cultured PC with LPS in the presence or absence of the mimic. The supernatants were collected, and the PC were washed and triggered with PMA to measure NADPH oxidase–mediated respiratory burst and superoxide production. Figure 4*A* shows that 3.4 μmol/l SOD mimic results in a 75% ($P < 0.02$) reduction in superoxide production, and increasing the concentration of SOD mimic to 34 μmol/l did not significantly further decrease superoxide production. Moreover, Fig. 4*B* shows that TNF-α production by LPS-primed PC was inhibited 34% by 17 μmol/l mimic ($P < 0.02$), whereas 34 μmol/l mimic resulted in a 51% inhibition ($P < 0.02$). These data clearly demonstrate that preincubation of LPS-primed macrophages with SOD mimic inhibited TNF-α production and may have reduced the activation of NADPH oxidase. It should be noted that the SOD mimic had been washed off before the assay and therefore was not present in the extracellular space where superoxide generation is measured. Therefore, a decrease in superoxide production was not merely due to the SOD mimic scavenging the extracellular superoxide but may also have
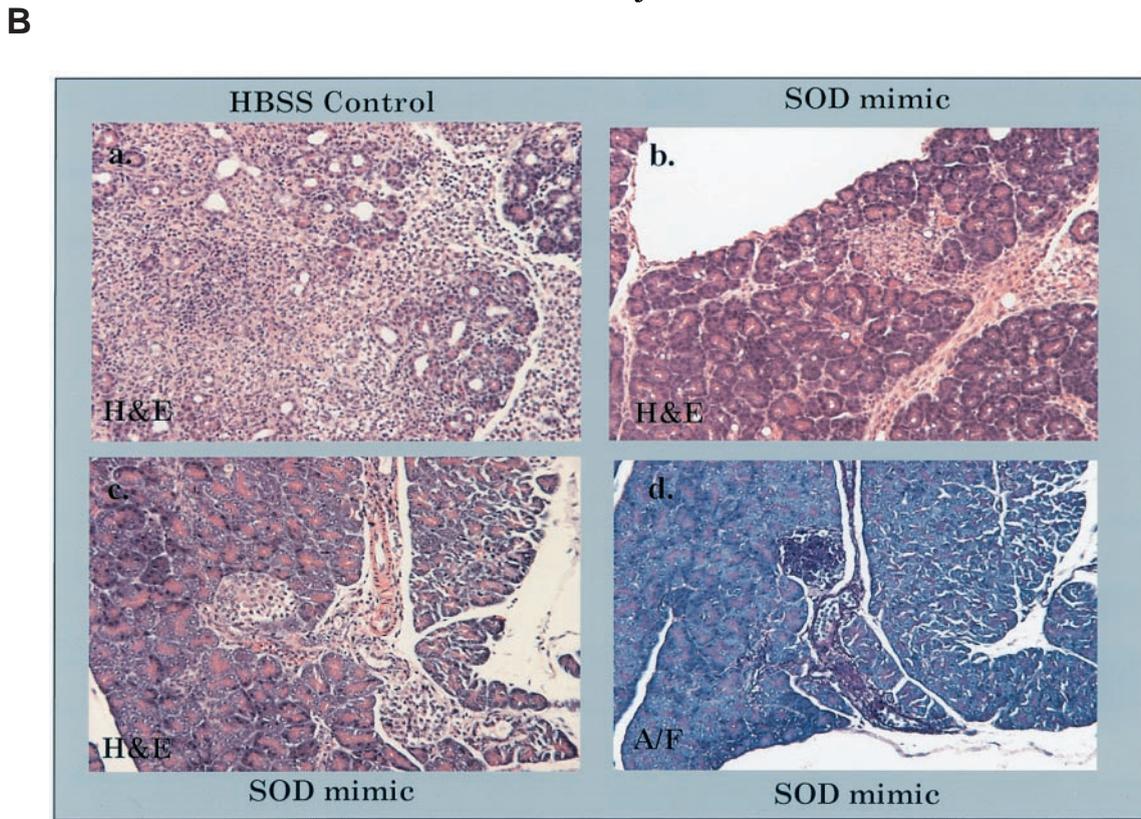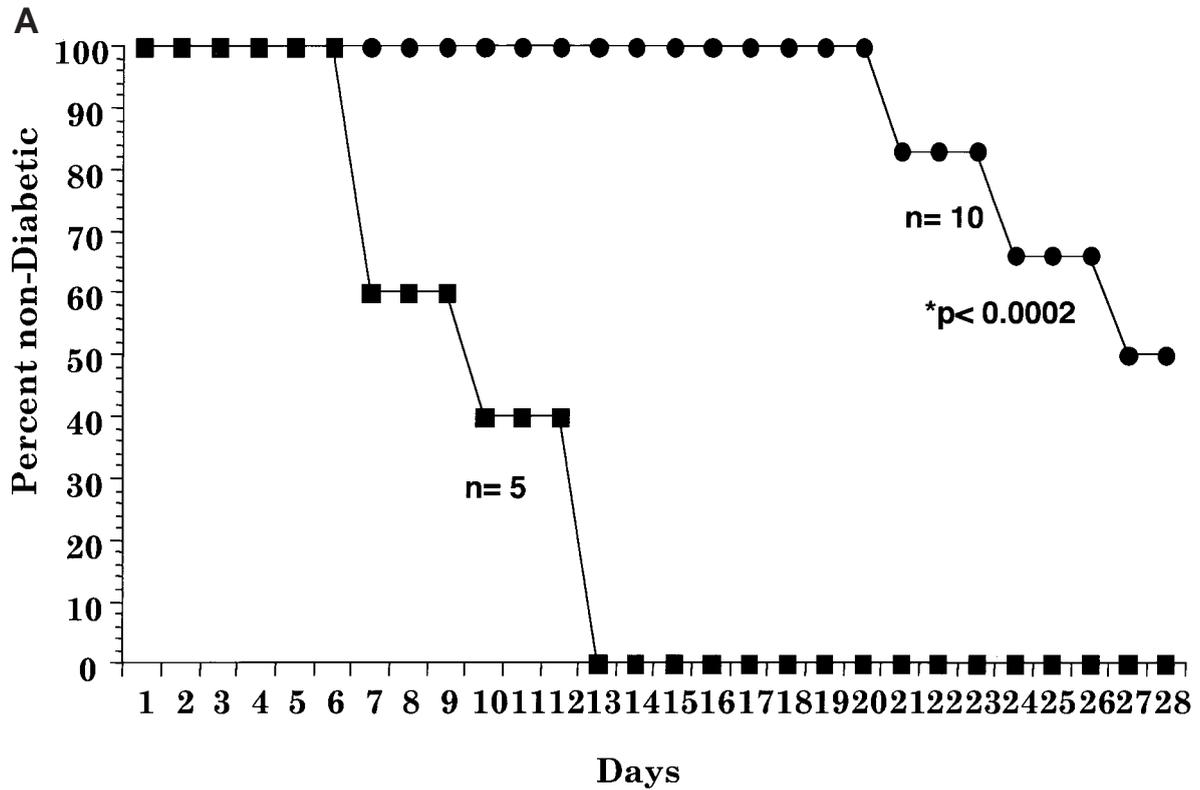
FIG. 1. SOD mimic administration delays or prevents T-cell–mediated diabetes in young NOD.*scid* recipients after diabetogenic T-cell clone BDC-2.5 transfer. *A*: NOD.*scid* mice 9–14 days of age were injected intraperitoneally 1 day before adoptive transfer of 1×10^7 BDC-2.5 T-cell clones with 10 mg/kg of the SOD mimic (●) (*n* = 10) or HBSS control (■) (*n* = 5). The SOD mimic was then given every other day for a total of 5 days. The data represented in *A* are the combination of three separate experiments. *B*: Representative pancreatic histology from young NOD.*scid* mice treated with SOD mimic or control after adoptive transfer of the T-cell clone BDC-2.5. *a*: Hematoxylin and eosin (H&E) staining of a heavily infiltrated pancreas from the positive control, a young NOD.*scid* mouse after adoptive transfer of BDC-2.5. *b* and *c*: H&E staining of pancreas from young NOD.*scid* mice treated with SOD mimic (10 mg/kg) after adoptive transfer of BDC-2.5. *d*: Aldehyde-fuchsin (A/F) staining of pancreas from SOD mimic–treated NOD.*scid* mouse.
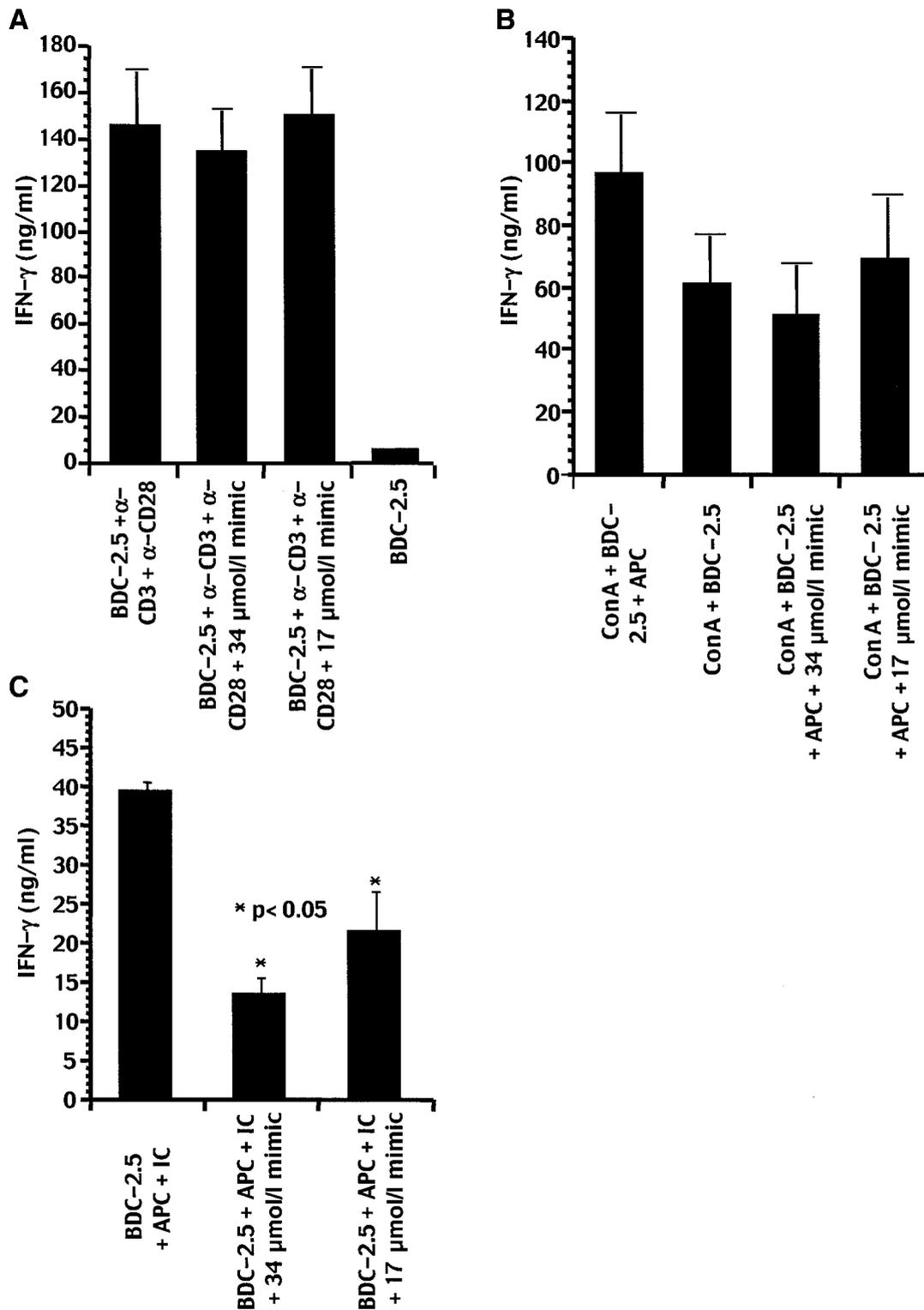
FIG. 2. Production of IFN-γ by BDC-2.5–treated cells with SOD mimic in vitro using three types of T-cell stimulation. *A*: The 96-well round-bottom plates were precoated with 0.125 µg/ml α-CD3 and 1 µg/ml α-CD28 for 1 h at 37°C. The plates were washed twice with sterile HBSS and then blocked with CM at 37°C for 1 h. Blocking solution was removed, and 2×10^4 BDC-2.5 T-cell clones were added to the wells in the presence or absence of the SOD mimic at concentrations of 34 and 17 µmol/l; the negative control was BDC-2.5 without α-CD3 and α-CD28. Cultures were incubated at 37°C for 48 h before the supernatants were harvested and assayed by sandwich ELISA for IFN-γ production. Data are the mean and SE of three separate experiments; *P* values are shown for conditions where statistical significance was noted. *B*: BDC-2.5 T-cells were plated at 2×10^4 cells/well in 96-well flat-bottom plates with or without 5×10^5 irradiated syngeneic spleen cells as APC and ConA (2.5 µg/ml final concentration) in the presence or absence of the SOD mimic at concentrations of 34 and 17 µmol/l. Cultures were incubated at 37°C for 24 h before the supernatants were harvested and assayed by sandwich ELISA for IFN-γ production. Data are the mean and SE of three separate experiments. *C*: BDC-2.5 T-cell clones were cultured in 96-well flat-bottom plates at a density of 2×10^4 cells/well, with 5,000 islet cells as antigen and 2.5×10^4 APC in the presence or absence of SOD mimic at 34 and 17 µmol/l. Cultures were incubated at 37°C for 48 h before the supernatants were harvested and assayed by sandwich ELISA for IFN-γ production. Data are the mean and SE of three separate experiments (*P* < 0.05). IC, islet cells.

resulted in a reduction in oxidase-dependent superoxide. The fact that superoxide production by activated macrophages (Fig. 4A) is inhibited by 3.4 μmol/l SOD mimic, whereas inhibition of TNF-α or IFN-γ production requires a higher SOD mimic concentration (Figs. 4B and 2C), suggests that the oxidant concentration necessary to activate the NADPH oxidase of macrophages is lower than the oxidant concentration necessary to activate the signal transduction pathways required for cytokine production. These results point to the fascinating prospect that biological responses to oxidants are not just "all-or-none," but instead are specific to the pathway involved.

SOD mimic–treated NIT-1 insulinoma cells are protected from alloxan- and cytokine-mediated cytotoxicity. Both alloxan and pro-inflammatory cytokines have been shown to be cytotoxic to β-cells in vitro. In this series of experiments, we sought to determine whether the SOD mimic could protect islet cells from alloxan- and cytokine-mediated cytotoxicity using the well-established NIT-1 insulinoma cell line. Figure 5A shows that incubation of NIT-1 cells with 10 mmol/l alloxan induces 50% apoptosis compared with 5% for control untreated or control plus SOD mimic. However, NIT-1 cells exposed to alloxan and treated with the SOD mimic show 70% viability ($P < 0.02$).

Figure 5B demonstrates the protective effect of the SOD mimic on NIT-1 cells exposed to IL-1β in culture. The addition of 10 ng/ml IL-1β was cytotoxic to NIT-1 cells ($\sim$50% of the cells were apoptotic) compared with control or control plus SOD mimic. A clear protective effect was seen when NIT-1 cells exposed to IL-1β were treated with SOD mimic ($P < 0.02$). The SOD mimic's protective effect is consistent with other reports of antioxidant proteins conferring resistance to immunological damage in insulinoma cells (15–21).

DISCUSSION

Type 1 diabetes is characterized by the T-cell–mediated infiltration of inflammatory cells into the pancreatic islet, which in turn leads to the generation of ROS and the liberation of proinflammatory cytokines that cause the destruction of the β-cells. We have shown that a SOD mimic protects β-cells from the ROS and cytokine-mediated damage. Our results demonstrate that the SOD mimic not only protects against disease transfer by a diabetogenic T-cell clone in young NOD.*scid* mice by indirectly influencing the ability of the clone to become activated (Figs. 1A; 2B, c; and 3) but also by a direct effect in which islet cell susceptibility to immunological damage is reduced (Fig. 5A and B). Furthermore, the absence of inflammatory cells suggests that the nonspecific infiltrate characteristic of insulitis is inhibited by the SOD mimic. These data suggest the intriguing possibility that ROS have an immunomodulatory effect, and adjustments in the oxidant/antioxidant balance may be used therapeutically to treat a variety of autoimmune diseases.

The in vivo protection achieved with treatment of young NOD.*scid* mice with the SOD mimic may be due to the inability of the APCs to obtain an optimal priming signal in the presence of the drug, inhibiting APC activation, cytokine production, and possibly NADPH oxidase activation (Fig. 4A and B). It has recently been reported that numerous signaling pathways involved in inflammation, apopto-
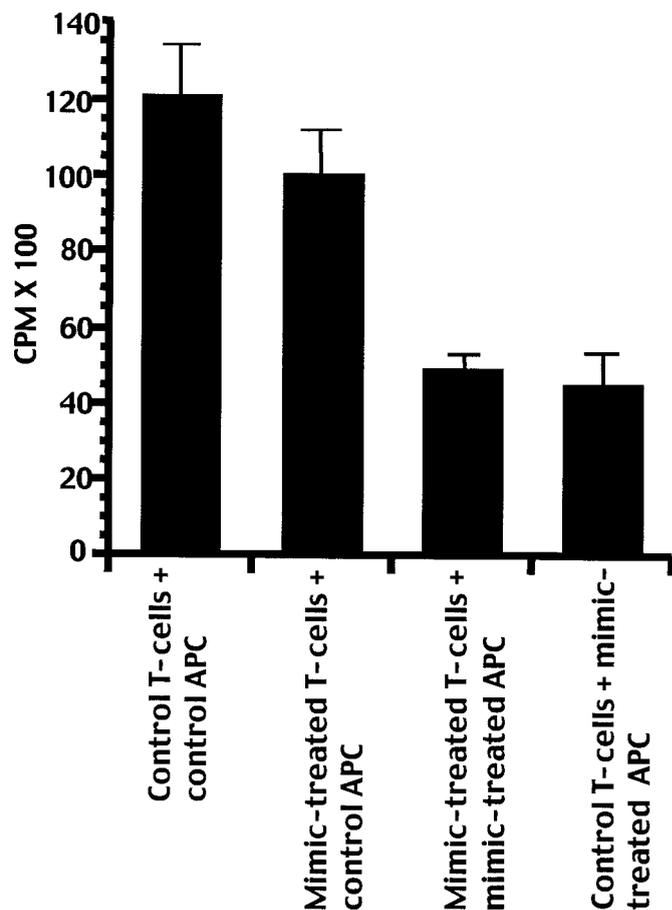

FIG. 3. In vivo treatment of 2.5 TCR Tg/NOD mice with the SOD mimic. The 2.5 TCR-Tg/NOD mice were treated for 7 days with 10 mg/ml SOD mimic or HBSS. Spleen cells were harvested from the animals on day 8, and the T-cells were purified from SOD mimic or control mice and plated (6×10^4 cells/well) with APCs (3×10^5 cell/well) from either SOD mimic or control mice in a crisscross fashion. The cultures were pulsed with 1 μmol/l of HRPI-RM peptide, and on day 4 of culture, the plate was pulsed with 1 μCi ^{3}H-TdR for 6 h before harvest. Values are the mean and SE of triplicate wells. Data are representative of duplicate experiments. CPM, counts per minute.

sis, and differentiation are activated after ROS and reactive nitrogen intermediate (RNI) induction, such as the mitogen-activated protein kinase (MAPK) pathway and the protein kinase B/Akt pathway (63,64). It has also been reported that MAPK pathways are important in the regulation of leukocyte function and the polymorphonuclear leukocyte (PMN) oxidative burst (65,66). Finally, it has been shown that there is a biphasic concentration-dependent regulation of the PMN oxidant burst by NO·-derived ONOO$^-$ (67). Therefore, it is tempting to speculate that the ability of the SOD mimic to scavenge ROS and RNI species may allow for the regulation of ROS- and RNI-dependent signaling pathways, such as oxidase-dependent superoxide production. The lack of APC priming leads to failure in activating the T-cell clone, ultimately blunting and in some instances completely stopping disease progression (Fig. 1A). This lack of APC priming is further illustrated by the fact that APC from in vivo SOD mimic–treated 2.5 TCR-Tg mice exhibited a reduced ability to support transgenic T-cell proliferation, whether they were presenting peptide to T-cells from SOD mimic–treated or untreated mice. However, when control APCs were used as presenters, the
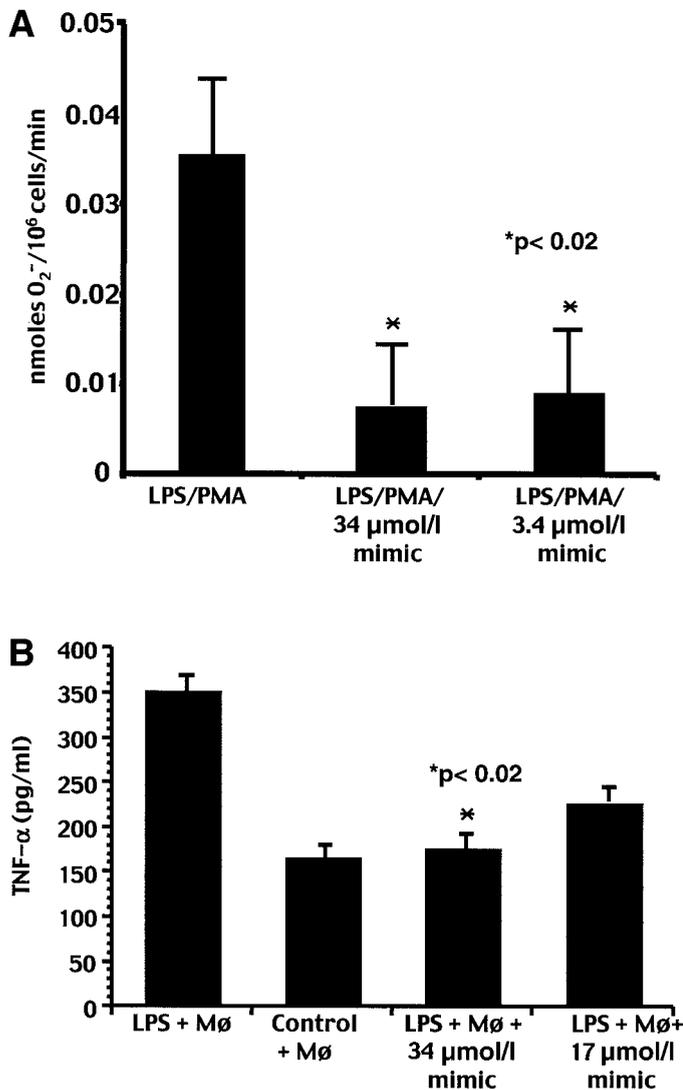
FIG. 4. LPS-induced respiratory burst and cytokine production by PCs. A: PCs were harvested from unprimed NOD mice and plated (5×10^5 cells/well) in 24-well plates in CM with *E. coli* LPS (055:B5) at 200 ng/ml in the presence or absence of the SOD mimic at 34 or 3.4 μmol/l final concentration. Cultures were incubated at 37°C for 48 h; the cells were trypsinized, washed to remove the trypsin, and subsequently transferred to microfuge tubes. PMA was added to a final concentration of 50 ng/ml. After incubation at 37°C for 20 min, superoxide production was assessed spectrophotometrically by ferricytochrome c reduction using $\epsilon = 20,000 \text{ mol} \cdot l^{-1} \cdot cm^{-1}$. The reduction was monitored over a period of 10 min. Data are mean and SD of duplicate experiments using triplicate wells per treatment. B: Peritoneal macrophages were harvested from unprimed NOD mice by washing the cavity of each animal with 7 ml HBSS. The cells were then washed two times in sterile HBSS and adjusted to 5×10^5 cells/well in a 24-well plate in CM with *E. coli* LPS (05:B5) at 200 ng/ml in the presence or absence of 34 or 17 μmol/l SOD mimic. Cultures were incubated at 37°C for 48 h before the supernatants were harvested and assayed by specific sandwich ELISA for TNF-α. The data are the mean and SE of three separate experiments ($P < 0.02$). Mø, macrophage.

status of the T-cell and leads to T-cell activation (68). Endogenous oxygen radicals modulate protein tyrosine phosphorylation and activation of JNK-1 and nuclear factor-κB (NF-κB) activation (69). In addition, IL-2 gene expression and NF-κB activation through engagement of CD28 requires reactive oxygen production by 5-lipoxygenase (70).

The in vitro T-cell clone system has allowed us to investigate the role of antioxidants in immunomodulating T-cell activation, whereas experiments with the 2.5 TCR-Tg/NOD mice allowed us to investigate the role of antioxidants in immunomodulating T-cell priming in vivo. The in
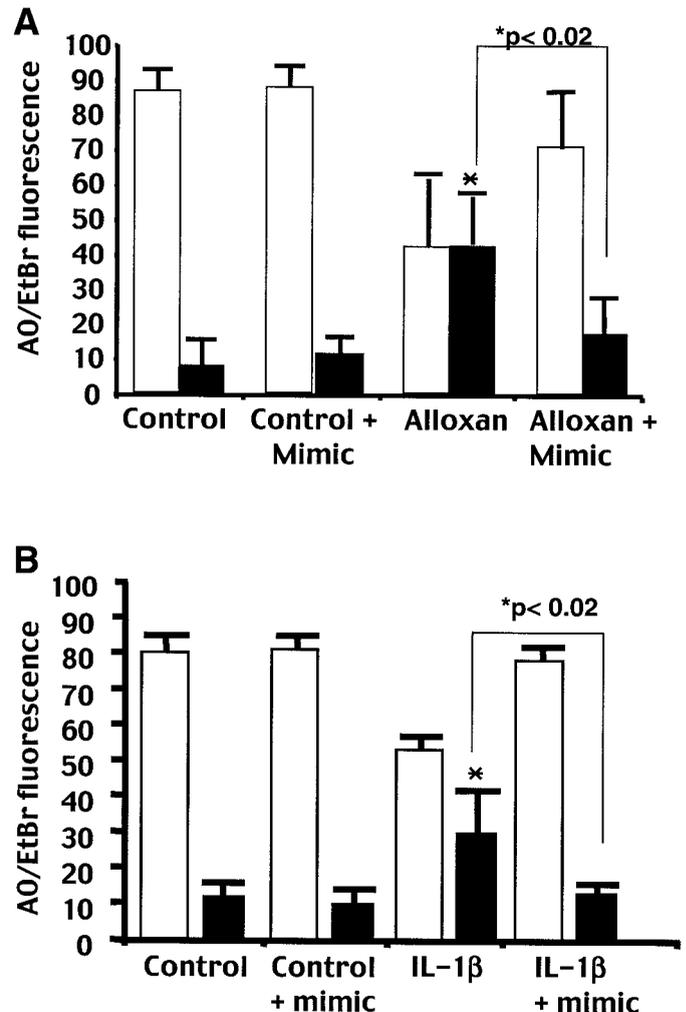

FIG. 5. Alloxan and cytokine cytotoxicity of SOD mimic–treated NIT-1 cells. A: NIT-1 cells were grown to confluence in 12-well tissue culture dishes. Media were removed and replaced with PBS alone or PBS containing 34 μmol/l SOD mimic. All solutions were supplemented with 4% FCS. After a 1-h incubation, 10 mmol/l alloxan was added to the appropriate wells, and cells were incubated for an additional 2 h. Cells were washed, collected by trypsinization, and processed for viability via ethidium bromide/acridine orange fluorescence live ($\square$) and live apoptotic ($\blacksquare$). Data are mean and SD of duplicate experiments using triplicate wells per treatment. NIT-1 cells were grown to 80% confluence in 12-well plastic tissue culture dishes. Growth media were removed and replaced with 500 μl/well of either media alone or media + 34 μmol/l SOD mimic. After a 1-h incubation, 500 μl/well of media alone or 20 ng/ml (10 ng/ml final concentration) IL-1 ± 34 μmol/l SOD mimic were added. Cells were incubated an additional 48 h and assessed for viability via ethidium bromide/acridine orange fluorescence live ($\square$) and live apoptotic ($\blacksquare$). Data are mean and SD of duplicate experiments using triplicate wells per treatment ($P < 0.02$). AO, acridine orange.

proliferative response in SOD mimic–treated T-cells approached the level achieved with control APCs and T-cells, demonstrating that the SOD mimic has the same inhibitory effect on APC priming in vivo that was observed in vitro. The inhibition of APC priming (Fig. 4A and B), which prevented optimal T-cell activation, suggests a model whereby T-cell activation depends on an initial change in APC redox status, which in turn allows for a lowering of signal thresholds to the T-cell. This result alters the redox

vivo results are of particular interest because they suggest that the SOD mimic can be used to inhibit ongoing T-cell responses to self-reactive antigens. Furthermore, the data suggest that with respect to the effects of ROS, there is a qualitative difference between signaling events occurring during cross-linking the T-cell receptor with α-CD3 and α-CD28 versus the more physiological T-cell stimulation via APC-mediated antigen presentation. Harhaj and Sun (71) reported that free-radical generation and cytokine production by primed APCs are critical for early T-cell signaling events. Although the T-cell clone BDC-2.5 can normally be detected in the pancreas after adoptive transfer (72), APCs in the pancreas of SOD mimic–treated recipients may be unable to activate the clone to produce IFN-γ and, consequently, the T-cells leave the pancreas and move to a secondary lymphoid organ such as the spleen. In fact, we detected BDC-2.5 in the spleen of SOD mimic–treated recipient NOD.*scid* mice after transfer (data not shown).

The results presented in this article are exciting because they are consistent with previous reports in which vector-mediated or transgenic overexpression of antioxidants was found to protect β-cells. Despite initial enthusiasm, the application of gene transfer in the clinical setting has been problematic, and to date there is little evidence of therapeutic benefit (73). Furthermore, because of the numerous limitations associated with enzyme therapies, including instability in solution, limited cellular accessibility, immunogenicity, short half-lives, cost of production, and proteolytic digestion (74), metalloporphyrin SOD mimics are an ideal alternative where inflammation plays a major role in disease pathogenesis. This is the first study demonstrating that a metalloporphyrin-based SOD mimic has a protective effect on the progression of T-cell–mediated autoimmune diabetes—results that support the model of free radical generation as a pathogenic mechanism of type 1 diabetes. Finally, understanding how the SOD mimic modulates the free radical–dependent signaling events between the APC and the T-cell will allow for the dissection of precise molecular targets and lead to the design of more specific pharmacological reagents.

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