Modulation of Redox Balance Leaves Murine Diabetogenic TH1 T Cells “LAG-3-ing” Behind

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Preventing activation of diabetogenic T cells is critical for delaying type 1 diabetes onset. The inhibitory molecule lymphocyte activation gene 3 (LAG-3) and metalloprotease tumor necrosis factor-α converting enzyme (TACE) work together to regulate TH1 responses. The aim of this study was to determine if regulating redox using a catalytic antioxidant (CA) could modulate TACE-mediated LAG-3 shedding to impede diabetogenic T-cell activation and progression to disease. A combination of in vitro experiments and in vivo analyses using NOD mouse strains was conducted to test the effect of redox modulation on LAG-3 shedding, TACE enzymatic function, and disease onset. Systemic treatment of NOD mice significantly delayed type 1 diabetes onset. Disease prevention correlated with decreased activation, proliferation, and effector function of diabetogenic T cells; reduced insulin-specific T-cell frequency; and enhanced LAG-3+ cells. Redox modulation also affected TACE activation, diminishing LAG-3 cleavage. Furthermore, disease progression was monitored by measuring serum soluble LAG-3, which decreased in CA-treated mice. Therefore, affecting redox balance by CA treatment reduces the activation of diabetogenic T cells and impedes type 1 diabetes onset via decreasing T-cell effector function and LAG-3 cleavage. Moreover, soluble LAG-3 can serve as an early T-cell-specific biomarker for type 1 diabetes onset and immunomodulation. Diabetes 61:1760–1768, 2012

In addition to direct cell-mediated killing of β-cells in type 1 diabetes, soluble inflammatory mediators, including cytokines and reactive oxygen species (ROS), often precede the later stages of fulminant β-cell destruction. Regulation of local and systemic redox state affects activation and proliferation of a variety of immune cells and protects tissues/cells from innate and cell-mediated damage (1). On the basis of previous studies showing the importance of ROS in chronic inflammation, our laboratory has used a catalytic antioxidant (CA) to modulate both innate and adaptive immunity in type 1 diabetes. CA is a manganese metallnoporphyrin—Mn(III) meso tetraakis (N-alkylypyridinium-2-yl) porphyrin, MnTE-2-PyP5+—that catalyzes superoxide dismutation, mimicking superoxide dismutase activity (2). CA also scavenges a broad range of ROS, including superoxide, hydrogen peroxide, peroxynitrite, and lipid peroxyl radicals (2–4). CA activity regulates proinflammatory immune processes by decreasing tumor necrosis factor (TNF-α), interleukin-1β, and ROS synthesis from activated antigen-presenting cells (APCs) (5), likely by inhibition of nuclear factor-κB (NF-κB)—dependent gene transcription and efficient innate immune activation (4). CA also induces CD4+ T-cell antigen–specific hyporesponsiveness (5) and decreases the cytolytic activity of CD8+ T cells (6), delaying islet allograft rejection (7). In the context of type 1 diabetes, diabetogenic BDC-2.5 T-cell clones exhibit impaired diabetes transfer in CA-treated NOD.scid recipient mice (8).

Our previously published work shows that TNF-α secretion is reduced in CA-treated macrophages (5). A disintegrin and metalloproteinase-17, or TNF-α converting enzyme (TACE), is a metalloprotease responsible for cleaving pro–TNF-α from the cell surface. Many metalloproteases, such as TACE, are redox-dependent enzymes, initially formed as latent zymogens that become active upon oxidation of specific Cys residues in their disintegrin/Cys-rich region (9–12). We hypothesize that CA treatment may not only scavenge ROS, decrease proinflammatory cytokine production, and inhibit NF-κB activation but also inhibit TACE, altering the cleavage kinetics of T-cell surface proteins. Support for this hypothesis derives from studies showing that TACE is responsible for the shedding of key transmembrane proteins, such as Notch, epithelial growth factor receptor ligands, CD44, CD62L, and CD223 (lymphocyte activation gene 3 [LAG-3]), making it an essential enzyme in normal immune function (13–18).

LAG-3 is a negative regulator of immune cell activation expressed on activated CD4+ and CD8+ T cells and plasmacytoid dendritic cells (19,20). Upon T-cell receptor (TCR) binding with major histocompatibility complex class II, LAG-3 levels increase on the surface of T cells, resulting in attenuated TCR-dependent T-cell activation and eventual clonal exhaustion (21), possibly by physical competition for major histocompatibility complex interaction (22). LAG-3<sup>−/−</sup> mice have increased T-cell proliferation and interferon (IFN)-γ cytokine production (21), and antibody-mediated LAG-3 blockade results in enhanced CD69 expression and T-cell differentiation (23). Recent studies (24,25) report that LAG-3<sup>−/−</sup> NOD mice demonstrate accelerated spontaneous diabetes, further indicating a potential immunoregulatory function of LAG-3. Soluble LAG-3 (sLAG-3) is a surrogate measure of TACE activity (9,16) and an additional marker of T-cell activation (26,27). Indeed, serum levels of sLAG-3 are considered biomarkers of T-cell activation in breast cancer (26). Therefore, in the context of type 1 diabetes, sLAG-3 could serve as a surrogate marker of autoreactive T-cell activation as well as a predictive biomarker of diabetes progression from preclinical to clinical disease.

In this study, we demonstrate the effects of CA treatment on the TACE redox state, coupled with LAG-3 expression...
and T-cell activation, to promote autoreactive T-cell hyporesponsiveness and reduce type 1 diabetes onset.

**RESULTS**

**CA treatment delays spontaneous diabetes.** CA treatment disrupts innate immune-mediated proinflammatory signals (5) and delays islet allograft rejection (7), prompting us to determine the effects of its long-term administration on type 1 diabetes onset. NOD females (aged 4 weeks) implanted with CA pellets demonstrated delayed diabetes onset compared with control mice (P < 0.0001). Furthermore, stopping CA pellet implantation at 29 weeks afforded protection against diabetes until 40 weeks of age (Fig. 1), suggesting that redox modulation imparts inhibition of autoreactive processes and delays end-organ autoimmunity.

**Redox modulation decreases TH1 effector function.** TH1-like T cells play a key role in mediating type 1 diabetes (30,31). To mechanistically determine how modulation of the redox state affects diabetogenic CD4+ TH1 adaptive immune effector responses, BDC-2.5.TCR.Tg splenocytes were stimulated plus or minus M in the presence of CA or 200 μmol/L TAPI-1 antibody as described (29). Western blot analysis, data are representative of at least three independent experiments and fold change of expression is calculated as indicated. Data are mean ± SEM. Survival analysis was done using the product-limit (Kaplan-Meier) method with the end point defined as disease onset. Data on animals that did not develop type 1 diabetes were censored. The P values were determined by log-rank test.

**FIG. 1.** Spontaneous diabetes is reduced upon systemic CA treatment. NOD females (n = 7) were implanted with a 14-day sustain-release CA pellet (2.1 mg/kg/day) biweekly, and control NOD mice (n = 14) were left untreated. Pellet implantation was stopped at 29 weeks of age. Diabetes was monitored by blood glucose, with two consecutive readings of >300 mg/dL indicating overt disease.
T-cell proliferation ($P < 0.05$ at 96 h) (Fig. 2B). In conjunction with previous results (5), redox modulation significantly lowered IFN-$\gamma$ production ($P < 0.05$) as well as reduced Tbet protein expression (Fig. 2C and D). These data indicate that CA diminishes T-cell activation and TH1 effector function, likely contributing to the diabetes protection observed above (Fig. 1).

CA treatment limits antigen-specific T-cell frequency. To determine if CA treatment affected the frequency of antigen-specific TH1 cells in vivo, we immunized NOD mice (aged 6–8 weeks) with a known autoantigen, insulin, and used inguinal LN cells on day 6 for primary intracellular IFN-$\gamma$ detection and recall ELISPOT assay (Fig. 3). IFN-$\gamma$-expressing CD4$^+$ T cells were reduced after CA treatment compared with control animals ($P < 0.005$) (Fig. 3A). Furthermore, LN cells from CA-treated animals displayed decreased IFN-$\gamma$-secreting cells compared with control animals ($P < 0.05$), with a significant reduction in insulin-specific effector function after recall stimulation (Fig. 3B). The frequency of antigen-specific cells in control animals was ~1 in 19,000, whereas in CA-treated animals, the frequency diminished to 1 in 46,000. These results suggest that redox modulation disrupts insulin-specific T cells, which may lead to delays in autoimmune-mediated $\beta$-cell destruction and type 1 diabetes onset.

CD4$^+$LAG-3$^+$ T-cell frequency is enhanced after CA treatment. LAG-3 is important in negatively regulating T-cell responses and, thus, may play a role in mediating decreased T-cell activation after CA administration (21,32,33). We first measured LAG-3$^+$ T-cell frequency after M plus or minus CA stimulation of BDC-2.5.TCR.Tg splenocytes in vitro. M plus CA treatment resulted in a higher frequency of LAG-3$^+$ T cells compared with samples stimulated with M alone (Fig. 4A). The mean fluorescence intensity of LAG-3 did not differ between groups (data not shown). Because LAG-3 is not constitutively expressed (34,35), unstimulated cells plus or minus CA treatment expectedly demonstrated low LAG-3$^+$ T-cell frequencies. Upon quantification of the in vitro results, the fold change in LAG-3$^+$ T-cell frequency reached significance at 24 and 48 h ($P < 0.05$) post-stimulation (Fig. 4B), indicating kinetically delayed T cells and decreased TH1 activation.

We next looked in vivo for LAG-3 kinetics after insulin immunization and CA administration of NOD mice (aged 6–8 weeks). As shown by others (16,36–39) and similar to our in vitro results (Fig. 4A and B), control-treated animals
exhibited a lower peak of LAG-3+ cells by day 3 post-immunization in comparison with CA-treated mice; however, redox modulation demonstrated an enhanced trend toward LAG-3+ cells at day 3 postimmunization (Fig. 4C). No difference in LAG-3 mean fluorescence intensity was seen between the groups (data not shown). These data demonstrate CA treatment can affect LAG-3+CD4+ T-cell frequency in vivo, albeit not to significance, suggesting slight obstruction of T-cell activation after autologous immunization in the presence of redox modulation.

LAG-3 shedding is reduced upon CA treatment. In addition to surface levels, sLAG-3 was also analyzed after M stimulation of BDC-2.5.TCR.Tg splenocytes. LAG-3 contains four extracellular domains (D1–D4), a C-peptide region, a transmembrane domain (16), and a cytoplasmic tail (27). Within the immunological synapse, an antigen-mediated respiratory burst activates TACE by oxidizing Cys522 and Cys600 to release the TACE prodomain (10,12). Active TACE then cleaves the 70-kDa full-length LAG-3 within the C-peptide, shedding D1 through D4 domains, a 5-kDa fragment (16). sLAG-3 shed into the serum can be measured as a marker of T-cell activation (26). Immunoprecipitation of BDC-2.5.TCR.Tg splenocyte culture supernatants demonstrated reduced sLAG-3 after CA treatment compared with control samples (Fig. 5A), illustrating decreased LAG-3 cleavage upon redox modulation. It is notable that sLAG-3 was undetectable in the no antigen sample, which did not undergo antigenic stimulation. From these results, we positulate that CA can reduce LAG-3 shedding by modulating TACE enzymatic function.

CA exposure reduces TACE levels and enzymatic activity. We next wanted to test the dependence, as a result of T-cell activation, of redox-mediated TACE modifications on LAG-3 cleavage. M plus CA decreased sLAG-3 levels in comparison with M alone (P < 0.05) as detected by ELISA (Fig. 5B) and consistent with Fig. 5A. To attribute the reduction of LAG-3 shedding to CA-regulated TACE, we compared M versus M plus CA versus M plus TAPI, a known TACE inhibitor. Both CA and TAPI reduced the amount of detectable sLAG-3 to a similar extent (P < 0.05) in comparison with M alone. Furthermore, a comparison between M plus TAPI and M plus CA and TAPI demonstrated no significant difference in reducing sLAG-3 levels. These data suggest that CA treatment is likely inhibiting TACE-dependent LAG-3 cleavage.

We also wanted to determine whether CA treatment specifically decreased TACE enzymatic activity. Using BDC-2.5.TCR.Tg splenocytes in an in vitro TACE-specific fluorogenic assay, enzymatic activity in CA-treated cells was significantly decreased compared with M-stimulated cells (P < 0.005) (Fig. 6A). As a positive control, TAPI-treated cells also demonstrated a significant reduction in TACE activity (P < 0.0005). To delineate whether the difference in enzymatic activity corresponded with decreased protein levels of TACE, we performed Western blots for the TACE prodomain and active isoforms. TACE is formed as a latent/inactive enzyme containing a disulfide linkage, whereby oxidation of the bond promotes autocatalytic cleavage of the prodomain (20 kDa) from the active subunit (80 kDa) (10,12). Under CA exposure, TACE prodomain was reduced compared with control samples (Fig. 6B), indicating less oxidation of the critical Cys switch and likely resulting in decreased enzymatic activity. Membrane lysates also exhibited diminished levels of active TACE after CA treatment (Fig. 6C). It is interesting that immature TACE was also reduced upon redox modulation, suggesting less overall activation-induced expression of TACE (10,40).

CA treatment prevents diabetes transfer in correlation with reduced sLAG-3 serum levels. Lastly, we monitored LAG-3 in conjunction with diabetes progression upon adoptive transfer of disease. Measurement of sLAG-3 has been used as an index of breast cancer prognosis, with greater levels corresponding to better antitumor cytotoxic T-cell responses and patient survival (26). Underlying these observations is an increase in T-cell activation and, therefore, we propose that diabetogenic T-cell activation can be indirectly ascertained by serum sLAG-3. To assess this possibility, NOD.scid mice (aged 10 weeks) were adoptively transferred with BDC-2.5.TCR.Tg splenocytes and treated daily with CA. Control animals all developed diabetes by day 15 post-transfer, whereas CA-treated mice remained disease free until the end of the study at day 28 (P < 0.0001) (Fig. 7A). Furthermore, serum levels of sLAG-3 steadily increased in control animals over time, yet sLAG-3 from CA-treated mice was significantly lower at days 12 and 16 posttransfer (P < 0.05) (Fig. 7B). Splenocytes were isolated either at

**FIG. 3. CA treatment reduces insulin-specific T-cell effector function and frequency. NOD mice were treated with CA or HBSS daily. Mice were immunized with insulin in CFA. A: Inguinal LNs were removed at day 6 postimmunization and surface stained for CD4+ cells as well as intracellularly stained for IFN-γ for flow cytometric analysis. Cells were gated on CD4+ cells (n = 3 independent experiments with two mice per group). **P < 0.005. B: Inguinal LNs were isolated on day 6 and stimulated with insulin in a recall IFN-γ ELISPOT. Two days after stimulation, ELISPOT plates were developed, and spots were counted using the Zeiss KS Elispot Imaging system. Frequency = 2.5 × 106/average number of spots per treatment. Graph shows the average of three independent experiments performed in triplicate. *P < 0.05. MFI, mean fluorescence intensity.**
diabetes onset (day 16) or at the end of the experiment (day 28) and stained for LAG-3. CA-treated animals had a higher frequency of LAG-3+CD4+ T cells versus control animals (*P < 0.05) (Fig. 7C). In addition, in vivo CA treatment decreased active TACE protein levels compared with control animals (Fig. 7D). sLAG-3, therefore, serves as a biomarker of type 1 diabetes progression and correlates with enhanced LAG-3+ T cells, decreased active TACE levels, and inhibition of disease after CA treatment.

**DISCUSSION**

Because CA treatment directly affects innate immune cells and proinflammatory third signal synthesis (5) as well as NF-kB and NF-κB–dependent gene transcription (4), we sought to understand how modulating redox balance could influence activation and function of diabetogenic TH1 cells. In particular, we hypothesized that CA administration would decrease TACE-dependent LAG-3 shedding, leading to autoreactive T-cell hyporesponsiveness and reduced type 1 diabetes.

In this study, long-term modulation of the redox state resulted in significantly delayed type 1 diabetes onset, illustrating the importance of ROS in promoting autoreactive immune responses. However, stopping the CA treatment at 29 weeks does not seem to afford absolute enduring protection against disease onset. This may be due to blood clearance of the modulator as a result of troughing of the CA level below the effective concentration and, consequently, loss of therapeutic efficacy. Under this circumstance, CA treatment alone may require chronic administration to inhibit diabetes onset; however, CA administration in combination with an antigen-specific therapeutic approach targeting self-reactive T cells might afford long-lasting protection by inducing T-cell–specific ignorance. Nonetheless, CA treatment has marked effects on early T-cell responses, resulting in a delay in diabetes onset.

BDC-2.5.TCR.Tg T cells demonstrated decreased activation, proliferation, and effector function upon CA treatment, which correlated with enhanced LAG-3+CD4+ T cells in vitro and a trend toward significant increases in vivo.
Absence of T-cell activation coupled with greater LAG-3+CD4+ T-cell frequency indicates two possible consequences of redox modulation: 1) less activation/progression to effector function of antigen-specific autoreactive T cells and/or 2) obstruction of LAG-3 shedding. It is notable that T cells from insulin-immunized mice exhibited reduced TH1 effector responses (decreased IFN-γ synthesis and a lower frequency of antigen-specific T cells), suggesting that regulation of LAG-3 may be responsible for this phenomenon. With recent reports showing accelerated diabetes in LAG-3−/− NOD mice (24,25), our redox modulation results may be reflective of T-cell ignorance (41,42). Diabetogenic T cells in NOD mice already have an advantage of efficiently expanding from a greatly reduced precursor pool (43). If CA delays or prevents the autoantigen-specific T-cell pool necessary for reaching the threshold at which a break in tolerance to self-antigen occurs, disease onset should be reduced.

FIG. 5. sLAG-3 protein is decreased after CA treatment. A: Supernatants from BDC-2.5.TCR.Tg splenocytes left untreated or stimulated with M ± CA for 48 h were concentrated using Amicon Ultra Centrifugal Filters at a 30-kDa (K) cutoff. The >30-kDa portion was then immunoprecipitated with anti-LAG-3 antibody, separated on an SDS-PAGE gel, and probed for LAG-3 by Western blot. Data representative of three independent experiments. B: BDC-2.5.TCR.Tg splenocytes were left untreated or stimulated with M ± CA or TAPI-1 for 72 h. Supernatants were collected and used in sLAG-3 ELISAs. Graph shows the average of four independent experiments performed in triplicate. *P < 0.05. Ag, antigen; N.D., none detected.

FIG. 6. Redox modulation diminishes active TACE levels and enzymatic function. A: BDC-2.5.TCR.Tg splenocytes were stimulated with M ± CA ± TAPI for 24 h and supplemented with TACE-specific fluorogenic substrate. Fluorescence was measured at 6 h post substrate addition. The fold change in activity was calculated by stimulated/unstimulated vs. stimulated + CA/unstimulated vs. stimulated + TAPI/unstimulated cells. Graph shows the average of three independent experiments performed in triplicate. **P < 0.005, ***P < 0.0005. B and C: BDC-2.5.TCR.Tg splenocytes were stimulated with M ± CA for 72 h and probed for TACE by Western blot. Whole-cell lysates were used in B. Actin was probed as a loading control. Membrane lysates were used in C. Data are representative of three independent experiments.
LAG-3 abates T-cell responses and is regulated through cleavage via redox-dependent TACE (16,21–23,32,38). CA was able to affect TACE activation and enzymatic activity, thereby leading to decreased sLAG-3. Immature TACE levels were also reduced after treatment. Because oxidants activate signaling kinases and GTPases to drive the expression of metalloproteases (10,40), scavenging of oxidants by CA (2–4) may inhibit proper signal transduction and cause decreased expression of immature TACE. Another metalloprotease, a disintegrin and metalloproteinase-10, unlike TACE, is responsible for constitutive LAG-3 shedding (16) and may also be partially inhibited by CA treatment (9–11,15). However, our experiments using TAPI blocked LAG-3 shedding by both metalloproteases, ruling out enzyme-specific differences (44). Both TAPI and CA demonstrated similar reductions in sLAG-3 versus M alone, supporting the notion that CA can directly modify TACE-dependent cleavage.

In addition to LAG-3, TACE, and IFN-γ, we have previously identified NF-κB as another target of redox modulation (4). Of note, redox-dependent NF-κB is a predicted transcription factor responsible for LAG-3 expression (SABiosciences’ Text Mining Application). Therefore, it is tempting to speculate that new LAG-3 protein synthesis is retarded as a result of CA-mediated NF-κB inhibition and, hence, contributes to the reduction in sLAG-3 observed. Furthermore, it may be possible that redox-modulated cells are functioning as suppressive T cells that retain their surface expression of LAG-3 (39,45,46). Collectively, our results also propose that an overall delay in T-cell activation kinetics may be the main cause of these anomalies. Impaired TACE activity, via LAG-3, offers new insights into a novel mechanism of autoreactive T-cell activation thus far unknown, and redox-dependent modifications together can contribute and feed forward to prevent diabetogenic immune responses.

sLAG-3 shedding coincides with in vivo staphylococcal enterotoxin B-mediated T-cell activation (27) as well as T-cell activation in breast cancer screens (26). At present, there is a paucity of serum biomarkers that measure T-cell activation before overt diabetes (47), and little is known about the endogenous role of LAG-3 in autoimmune settings. Therefore, we wanted to determine whether we could monitor diabetes progression via serum sLAG-3. Redox modulation prevented type 1 diabetes onset in the rapid adoptive transfer model, similar to previous reports (8), but more importantly, sLAG-3 directly correlated with T-cell activation and autoimmunity. Serum sLAG-3 was enhanced in control animals before and upon disease onset, suggesting greater activation of diabetogenic T cells. sLAG-3, therefore, may serve as a T-cell-specific diagnostic marker for initiation of β-cell destruction, and along with surface LAG-3 expression, may additionally function as surrogates of immunomodulation. Therapies that use prophylactic drugs as well as attempts at reversal of autoimmunity may result in the modification of adaptive immune responses.
and measurement of sLAG-3 would allow for determination of treatment efficacy on autoreactive T cells in a noninvasive manner.

Taken together, our data suggest that redox modulation arrests LAG-3 shedding by impeding expression kinetics and decreasing TACE activity. Through redox manipulation, LAG-3 surface expression is maintained at levels adequate to attenuate TCR-mediated TH1 cell activation and effector function. This would be beneficial for maintaining diabetogenic effector cells in a quiescent state or in preventing their activation entirely. In concert with the recently reported mechanisms of direct and indirect actions of CA on immune cells and diabetes progression (1,4–8), the identification of LAG-3–mediated immunoregulation adds another layer to the control of autoimmunity. Our discovery also supports the use of sLAG-3 as a novel surrogate marker of type 1 diabetes progression in preclinical situations and possibly as a means to monitor the effectiveness of T-cell–directed immunotherapy.

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