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CD4$^+$ T cells epigenetically modified by oxidative stress cause lupus-like autoimmunity in mice

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ABSTRACT

Lupus develops when genetically predisposed people encounter environmental agents such as UV light, silica, infections and cigarette smoke that cause oxidative stress, but how oxidative damage modifies the immune system to cause lupus flares is unknown. We previously showed that oxidizing agents decreased ERK pathway signaling in human T cells, decreased DNA methyltransferase 1 and caused demethylation and overexpression of genes similar to those from patients with active lupus. The current study tested whether oxidant-treated T cells can induce lupus in mice. We adoptively transferred CD4$^+$ T cells treated in vitro with oxidants hydrogen peroxide or nitric oxide or the demethylating agent 5-azacytidine into syngeneic mice and studied the development and severity of lupus in the recipients. Disease severity was assessed by measuring anti-dsDNA antibodies, proteinuria, hematuria and by histopathology of kidney tissues. The effect of the oxidants on expression of CD40L, CD70, KirL1 and DNMT1 genes and CD40L protein in the treated CD4$^+$ T cells was assessed by Q-RT-PCR and flow cytometry. H_2O_2 and $ONOO^-$ decreased Dnmt1 expression in CD4$^+$ T cells and caused the upregulation of genes known to be suppressed by DNA methylation in patients with lupus and animal models of SLE. Adoptive transfer of oxidant-treated CD4$^+$ T cells into syngeneic recipients resulted in the induction of anti-dsDNA antibody and glomerulonephritis. The results show that oxidative stress may contribute to lupus disease by inhibiting ERK pathway signaling in T cells leading to DNA demethylation, upregulation of immune genes and autoreactivity.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic relapsing autoimmune disease that primarily affects women. SLE develops and flares when genetically predisposed people encounter certain environmental agents. The genes predisposing to lupus are being identified, and epidemiologic evidence indicates that environmental agents which cause oxidative stress, such as infections, sun exposure, silica and smoking, are associated with lupus onset and flares [1]. However, how environmentally induced reactive oxygen species interact with the immune system to trigger lupus flares remains unclear.

Our group reported that CD4$^+$ T cells epigenetically altered with DNA methylation inhibitors like 5-azacytidine (5-azaC), procainamide (Pca) or hydralazine (Hyd) cause lupus-like autoimmunity in animal models [2], and that similar epigenetically modified T cells are found in lupus patients during disease flares [1]. We traced the cause of the epigenetic defect to PKCδ inactivation, which prevents upregulation of DNA methyltransferase 1 (Dnmt1) during mitosis to copy methylation patterns [3]. Others reported that serum proteins are nitrated in patients with active lupus, caused by superoxide (O_2^-) combining with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), a highly reactive molecule that nitrates proteins and other molecules [4]. Our group subsequently found that PKCδ is similarly nitrated in T cells from patients with active lupus, and that nitrated PKCδ is unable to transmit signals that upregulate Dnmt1 to copy DNA methylation patterns in dividing T cells, causing demethylation and overexpression of genes normally suppressed by DNA methylation [5]. We extended these observations by

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demonstrating that treating human $CD4^+$ T cells with the oxidizing agents H_2O_2 or $ONOO^-$ inhibits PKCδ activation, thereby decreasing ERK pathway signaling, decreasing Dnmt1 levels and causing demethylation and overexpression of CD70 and Kir genes similar to T cells from patients with active lupus [6]. However, whether the epigenetically modified T cells are sufficient to cause lupus-like autoimmunity was unknown.

We have now tested if female murine $CD4^+$ T cells treated with H_2O_2 or $ONOO^-$ also overexpress methylation sensitive genes, and if the treated cells cause lupus-like autoimmunity in mice. The results demonstrate that the oxidized T cells overexpress the X-linked gene CD40L, one copy of which is silenced by DNA methylation in female human and mouse T cells [7,8], and Kir genes, normally expressed by NK cells but silenced by DNA methylation in human and mouse T cells [9]. The results also demonstrate that adoptive transfer of the treated cells into syngeneic recipients causes anti-DNA antibodies and an immune complex glomerulonephritis, similar to T cells treated 5-azaC, Pca or Hyd. Together these studies support the contention that environmentally-induced oxidative stress may trigger lupus flares by epigenetically altering T cells through effects on T cell signaling and Dnmt1 expression.

2. Materials and methods

2.1. Drugs and reagents

5-azaC and H_2O_2 were purchased from Sigma–Aldrich (St. Louis, MO), and $ONOO^-$ from Calbiochem (San Diego, CA).

2.2. Mice

Female SJL mice were purchased from Jackson Laboratories, housed in filter-protected cages, and provided with standard irradiated rodent diet 5053 (Lab Diet; PMI Nutrition International) and water ad libitum. Urinary protein and hemoglobin were measured using Chemstrip 7 dipsticks (Roche, Madison, WI). The mice were maintained in a specific pathogen–free facility by the Unit for Laboratory Animal Medicine at the University of Michigan in accordance with the National Institutes of Health and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International Guidelines. All procedures were approved by the University of Michigan Institutional Animal Care and Use Committee.

2.3. T cell isolation, culture, treatment and injection

Splenocytes, thymocytes and lymph node cells were isolated from female SJL mice, pooled and stimulated in vitro with 5 μg/ml concanavalin A in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 1000 U Penicillin/1 mg streptomycin (Fisher Scientific, Pittsburgh, PA) for 18–24 h at 37 °C, 5% CO_2/balanced air incubator. $CD4^+$ T cells were then isolated using magnetic beads (Miltenyi, Auburn, CA), and treated with 5 μM 5-azaC, 20 μM H_2O_2 or 20 μM $ONOO^-$ for 3 days in 6 well plates as previously described [2,6,10]. The cells were then washed and 5×10^6 viable cells injected into the tail vein of each female SJL recipients, beginning when the mice were 12 weeks of age and continuing every 2 weeks for a total of 7 injections. Cells analyzed for gene expression by PCR were additionally treated with 50 ng/ml phorbol myristate acetate (PMA) and 500 ng/ml ionomycin during their final 6 h of culture. Blood and urine samples were obtained every other week. Two weeks after the last injection the mice were sacrificed, $CD4^+$ T cells isolated for further study and kidneys removed for histologic analysis.

2.4. Flow cytometric analysis

Spleen cells were washed twice in Standard Buffer (PBS containing 1% horse serum and 1 mg/ml sodium azide) at 4 °C. All incubations were performed on ice. Non-specific binding was blocked by incubating the cells 1 h on ice in Standard Buffer containing 10% horse serum. The cells were then stained in the dark for 1 h with PE-Cy5-rat anti-mouse CD4 (BD Pharmingen, Fullerton, CA) together with PE-hamster anti-mouse CD154 (CD40L) or matching IgG controls washed, then fixed in 2% paraformaldehyde and stored in the dark at 4 °C. The cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) as previously described [8].

2.5. RT-PCR

Total RNA and DNA were simultaneously isolated from bead-purified $CD4^+$ T cells using a Qiagen RNAEasy Mini Kit (Qiagen, Valencia, CA). RNA was quantified using a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE). One microgram of total RNA per sample was used to synthesize cDNA using a Transcriptor First Strand cDNA Synthesis Kit and anchored oligo$(dT)_{18}$ primers (Roche, Indianapolis, IN) according to the manufacturer's instructions. Primers for murine CD70, CD40L, KirL1, β-actin, and Dnmt1 were obtained from Integrated DNA Technologies (Coralville, IA) and used in RT-PCR to measure mRNA gene expression as previous described [8].

2.6. Anti-DNA antibody ELISA

Mouse IgG anti-dsDNA antibodies were measured by ELISA as previously described [11]. Briefly, Costar (Corning, NY) 96 well flat bottom microtiter plates were coated overnight at 4 °C with 10 μg/ml dsDNA in PBS, pH 7.2. Various dilutions of mouse sera or murine monoclonal IgG anti-dsDNA antibody (Millipore, Billerica, MA) standard were added in PBS and incubated overnight at 4 °C. Bound anti-dsDNA antibodies were detected using HRP-goat anti-mouse IgG-Fc-specific (Bethyl Labs, Montgomery, TX) antibodies and One Step Ultra TMB substrate (Thermo, Rockford, IL) and measured at 450 nm [11].

2.7. Histologic analyses

Kidneys were fixed in 10% neutral buffered formalin, routinely processed and paraffin embedded. Five micron sections were deparaffinized, hydrated and rinsed for 5 min in tap water and stained with hematoxylin and eosin or treated with citrate antigen retrieval buffer according to the manufacturer's instructions (Vector laboratories, Burlingame, CA). Endogenous peroxidase was blocked by treating the tissue sections with 0.3% hydrogen peroxide in methanol for 30 min at ambient temperature followed by a 5 min wash in PBS. The tissues were then incubated with 2.5% horse serum/PBS for 20 min and IgG detected using the IMPRESS Detection system and NOVA-Red© substrate (Vector Laboratories, Inc). The tissue was counter stained with hematoxylin according to the manufacturer's instructions, mounted with Immu-Mount (Thermo Fisher) and coverslipped.

2.8. Statistical analyses

The significance of differences between means was assessed using Student's *t*-Test or ANOVA. The linear mixed model was used to assess the effect of different treatments on antibody production relative to injections of Hanks balanced salt solution (HBSS) over time, and the Chi square test to assess differences in proteinuria