Increased Expression of Cathepsins and Obesity-Induced Proinflammatory Cytokines in Lacrimal Glands of Male NOD Mouse

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PURPOSE. Lacrimal glands (LGs) of male NOD mice, a model of Sjögren's syndrome (SjS), exhibit immune cell infiltration and lipid deposition. The mechanism of SjS was further investigated by characterizing gene expression profiles of NOD mouse LGs in comparison with those of healthy control mice. Differentially expressed genes were further investigated at the protein level to correlate changes in location and abundance with development of disease.

METHODS. Microarray followed by real-time RT-PCR was conducted to compare the gene expression in 12-week-old male NOD mouse LG relative to that in matched BALB/c mouse LG. Immunofluorescence and Western blot analyses were used to localize and quantify proteins of interest. Enzymatic assays measured catalytic activity of cathepsins.

RESULTS. Cathepsin H (Ctsh), S (Ctss), and Z (Ctsz) and proinflammatory factors, including tumor necrosis factor (Tnf), interleukin 6 (Il6), and interleukin 1 beta (Il1b), were upregulated at the mRNA level. Increased cathepsin S immunofluorescence was detected in lysosomes and secretory vesicle-like organelles in LG acinar cells and CD68-positive infiltrating macrophages in NOD mouse LG. Cathepsin S (CATS) and cathepsin H (CATH) activities were significantly higher in NOD mouse LG lysate than in control lysates, and CATS was also significantly elevated in NOD mouse tears.

CONCLUSIONS. Expression of CATS and CATH increases in parallel with proinflammatory cytokines during the development of autoimmune inflammatory disease in the NOD mouse disease model. Tear CATS may represent a biomarker for diagnosis of dacryoadenitis in SjS. (*Invest Ophthalmol Vis Sci.* 2010; 51:5019–5029) DOI:10.1167/iovs.09-4523

Sjögren's syndrome (SjS) is a chronic autoimmune inflammatory disease characterized by lymphocytic infiltration and destruction of lacrimal glands (LGs) and salivary glands (SGs). The male nonobese diabetic (NOD) mouse is a well-established animal model in which to evaluate the processes of the dacryoadenitis and sialoadenitis characteristic of the human disease. This mouse strain spontaneously develops insulin-dependent diabetes mellitus (IDDM) as well as SjS-like disease.^{1–3} Dacryoadenitis, which is more severe than sialoadenitis in this model, is fully manifested by 12 to 20 weeks.^{1,4} The NOD severe combined immunodeficiency (SCID) mouse strain is an immune-incompetent NOD mouse Prkdc congenic strain that can be compared to the NOD mouse, to distinguish events associated with inflammation from events characteristic of the strain that are independent of T- and B-cell-mediated inflammatory responses.⁵ The NOD SCID strain is significantly depleted in functional T, B, and natural killer (NK) cells and is free of exocrine tissue destruction.⁶

The early pathologic events associated with dacryoadenitis in the NOD mouse and other disease models include the development of functional quiescence (e.g., inability of acinar cells to secrete tear proteins from preformed secretory vesicles) in regions of the LGs, with otherwise normal-appearing and intact acinar cells, the infiltration of inflammatory cells, and formation of foci in the periductal regions and then in widespread foci throughout the LGs and the damage of extracellular matrix and acinar cells by factors released from these infiltrating immune cells. Over time, the healthy acinar cell mass in the LGs is replaced by lymphocytic foci and regions of necrotic and apoptotic cell debris.

We have recently reported that lipid droplets, primarily containing cholesterol esters, start accumulating in the cytoplasm of acinar cells in the LGs of the male NOD mouse between 5 and 6 weeks and that this event correlates directly with the immune cell infiltration detected within the same course.^{4,7} Changes in apolipoprotein expression and sorting may partially underlie this particular change in NOD mouse LGs.⁴ The observation of significant lipid deposition in NOD mouse LGs is consistent with findings in SjS patients that progressive lipid deposition occurs in both the SGs and the LGs.^{8,9} We considered the possibility that accumulation of lipids triggers or potentiates the subsequent dacryoadenitis and

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damage of the LGs. The lipid accumulation characteristic of the male NOD mouse LG was also observed in the male NOD SCID mouse LG,⁴ indicating that it is genetically determined, not inflammation induced.

The commonly accepted view of obesity, a consequence of lipid metabolic malfunction with excessive storage of lipids in adipocytes, defines this disease as a chronic inflammatory disorder associated with induction of a group of proinflammatory factors, including TNF α , IL-6, IL-1 β , and cathepsin S (CATS) in human visceral adipose tissues.¹⁰ Another cytokine, IL-15, has been reported as a negative regulator of adipose tissue mass in humans and rats, besides its regulation of T and NK cells.^{11,12} More detailed analysis has defined the major source of proinflammatory factors as macrophages,¹³ and the major source of CATS as adipose cells.¹⁰ It has been demonstrated that this condition promotes the development and progression of atherosclerosis.¹⁴ In addition, studies in recent years have unraveled deleterious roles of CATS and other family members in diseases such as cancer, viral infection, osteoporosis, and arthritis.^{15,16} Furthermore, T lymphocytes and autoantibodies have been detected that are directed against lipids or lipid adducts in multiple sclerosis.^{17–22} On the other hand, inactivation of CATS results in diminished collagen-induced arthritis, and application of CATS inhibitor prevents autoantigen presentation and autoimmunity in mouse models.^{23,24}

Based on these observations and the knowledge acquired from previous studies, we hypothesized that key factors contributing to disease development and progress in the LGs of the NOD mouse may be induced or upregulated in response to an imbalance in acinar cell lipid homeostasis. These factors would be proinflammatory and could be produced in either epithelial or nonepithelial cells. Our results showed that several gene products related to lipid accumulation in obesity—cathepsins and cytokines—were also collectively upregulated in diseased LGs from NOD and NOD SCID mice.

METHODS

Animals and Animal Procedures

NOD and BALB/c mouse colonies were bred in the University of Southern California Vivarium from breeding pairs purchased from Taconic Farms, Inc. (Hudson, NY) and Charles River Laboratories, Inc. (Wilmington, MA). NOD SCID mice were purchased from Taconic Farms, Inc. Animals were treated and killed in accordance with policies approved by the University of Southern California Institutional Animal Care and Use Committee which adheres to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. LG pairs were removed from the mice, after euthanatization by intraperitoneal injection with 55 mg ketamine and 14 mg xylazine per kilogram of body weight, followed by cervical dislocation. After removal, LGs were snap frozen and stored in liquid nitrogen for RNA preparation, fixed immediately with 4% paraformaldehyde and 4% sucrose in PBS for cryonic tissue processing, or homogenized for lysate preparation.

Reagents and Supplies

An RNA isolation kit was obtained from 5 Prime, Inc. (Gaithersburg, MD; VersaGene RNA Tissue Kit, originally from Gentra Systems, now sold as the PerfectPure RNA Tissue Kit 2302410). All materials and reagents for microarray were purchased from Applied Biosystems (ABI, Foster City, CA) through the Vanderbilt Microarray Shared Resources (Vanderbilt University, Nashville, TN). All the following materials and reagents for RT and real-time PCR were purchased directly from Applied Biosystems: the high capacity cDNA RT kit (cat. no. 4368814), universal PCR master mix for real-time PCR (cat. no. 4324018; TaqMan), optical 384-well reaction plates (cat. no. 4309849, MicroAmp; ABI) and optical adhesive films (cat. no. 4311971, MicroAmp; ABI), and gene expression microarrays (groups 1 and 2; TaqMan). Group 1 included those for genes *Il1b*

(Mm00434228_m1), *Il6* (Mm00446190_m1), *Il10* (Mm00439616_m1), *Il12a* (Mm00434165_m1), *Il15* (Mm00434210_m1), *Tnf* (Mm00443258_m1), *Irfng* (Mm00801778_m1), *Ctsb* (Mm00514455_m1), *Ctss* (Mm00457902_m1), and *Ctsz* (Mm00517697_m1). Group 2 included those for genes serving as internal controls, including *Sdha* (Mm01352357_m1). Mm followed by eight digits represents the company assay ID for a gene expression assay (TaqMan; ABI) corresponding to a specific mRNA locus of a gene.

For immunofluorescence microscopy, goat anti-mouse CATH polyclonal antibody (sc-6497) and goat anti-mouse CATS polyclonal antibody (sc-6505) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rat anti-mouse CD68 monoclonal antibody (ab53444) and rat anti-mouse Lamp2 monoclonal antibody (ab13524) were purchased from Abcam USA. (Cambridge, MA). Carbamylcholine (CCH) for stimulation in tear collection was purchased from Sigma-Aldrich (St. Louis, MO). For Western blot analysis, rat anti-mouse CATH monoclonal antibody (MAB1013) was purchased from R&D Systems, Inc. (Minneapolis, MN), and goat anti-mouse CATS polyclonal antibody (sc-6505) used for blotting was as sourced for immunofluorescence.

Flow cytometry (FACS; BD Biosciences, San Jose, CA) analysis was performed on inflammatory cells that were isolated from LGs of 12-week-old BALB/c, NOD, and NOD SCID mice ($n = 5$ mice, pooled), as described elsewhere.²⁵ Inflammatory cell subsets were dual labeled with the following antibodies at 1:200 dilution: FITC-conjugated CD49b/pan-NK, Cy5-conjugated CD11b (Mac-1) and PE-conjugated Gr1, PE-conjugated B220, FITC-conjugated CD19, and FITC-conjugated CD4 and PE-conjugated CD8. All antibodies were purchased from BD Biosciences/PharMingen and were used according to the manufacturer's protocol. Nonspecific isotype-matched Cy5-, PE-, and FITC-conjugated IgGs served as control antibodies, also at a 1:200 dilution. Staining with 7-amino-actinomycin (BD PharMingen) at a 1:50 dilution was performed to exclude dead cells according to the manufacturer's instructions. Cells were gated properly, and a total of 10,000 events were acquired for each sample. All analyses were performed with a flow cytometer (BD LSR2; BD Biosciences). The results were exported and analyzed (FlowJo 8.3.3 software; Tree Star, Inc., Ashland, OR).

Preparation of Total RNA

RNA was isolated at room temperature (VersaGene or PerfectPure RNA Tissue Kit; 5 Prime, Inc.). Snap-frozen LG pairs were removed from the liquid nitrogen and quickly homogenized on ice with a tissue homogenizer (Polytron; Heidolph Brinkmann, Elk Grove, IL). The lysate was processed according to the manufacturer's instructions. Three LG RNA samples were pooled from three 12-week-old mice in equal amounts for microarray analysis and real-time RT-PCR. Each RNA sample was prepared for real-time RT-PCR from three to four pairs of pooled LGs when 4-week-old mice were used. All the purified RNA samples were stored at -80°C .

Gene Expression Microarray Analysis

Triplicates of a mouse microarray (Mouse Genome Survey Microarray, AB1700 version 1.0.1; no. 4382672; ABI) were used for each group of mice. Each chip was printed with approximately 33,000 60-mer oligos as probes, representing a complete annotated and curated set of approximately 32,000 mouse genes from the public databases and a commercial database (Celera, Alameda, CA). The microarray analysis and the sequential data normalization were conducted by the Vanderbilt Microarray Shared Resource. Before microarray analysis, the purity and integrity of the RNAs were confirmed by measurement on a bioanalyzer (Agilent, Palo Alto, CA), according to the manufacturer's instructions and as described elsewhere.⁴ All hybridization reagents, hybridization controls, wash reagents, and chemiluminescent reagents were provided in a kit (Chemiluminescence Detection Kit 4342142; ABI), and the manufacturer's protocol was used for hybridization, as described elsewhere.⁴ The array was immediately imaged (model 1700 Chemiluminescent Microarray Analyzer; ABI). The images were assessed for QA/QC, and a primary analysis was then completed (1700

Expression Array System Software; ver. 1.1.1; ABI). The raw data were normalized using the ABI quantile-based method and filtered according to the average scores of flags with the analyzer and associated software.

Reverse Transcription (RT) and Real-Time Polymerase Chain Reaction (PCR)

RT and real-time PCR reaction steps were performed with ABI reaction kits and reagents, according to the manufacturer's protocols and according to published procedures.⁴ The PCR reaction with the gene expression assay (TaqMan; ABI) for the house-keeping gene *Sdha* (succinate dehydrogenase complex, subunit A), was run as an internal control. The recorded data were analyzed by using the $\Delta\Delta C_t$ calculating function in the software (SDS 2.1; ABI). The change ratio for a specific mRNA was obtained by calculations as $\Delta C_t = C_t (\text{mRNA}) - C_t (\text{housekeeping gene mRNA})$, $\Delta C_t (\text{NOD}) - \Delta C_t (\text{BALB/c}) = \Delta\Delta C_t$, and change ratio (NOD/BALB/c) = $2^{\Delta\Delta C_t}$. The real-time PCR quantification results of each studied gene were confirmed three to nine times with different pooled RNA samples prepared from three to four mice. Sample sets were evaluated for statistical significance with a Student's *t*-test.

Confocal Fluorescence Microscopy

After removal, the LGs were incubated in PBS containing 4% paraformaldehyde and 4% sucrose at room temperature for 2 to 3 hours and then transferred to PBS containing 30% sucrose at 4°C overnight. They were embedded in OCT and snap frozen in liquid nitrogen. The blocks were stored at -80°C before sectioning into 5-μm-thick sections. For dual labeling of CATS (or CATH) and CD68, the sections were permeabilized with 0.1% Triton X-100 for 15 to 30 minutes and then 1% SDS for 5 minutes. For dual labeling of CATS and Lamp2, the sections were permeabilized with 0.05% saponin for 5 to 10 minutes. All primary antibodies were used at a 1:100 dilution and secondary antibodies at a 1:200 dilution. After permeabilizations, the slides were blocked with 1% BSA at room temperature for 30 minutes, and then incubated sequentially with primary antibodies in 1% BSA at 37°C for 1 hour, followed with fluorophore-labeled secondary antibodies in 1% BSA along with fluorophore-labeled phalloidin (where appropriate) continually for 1 hour. Finally, the slides were incubated with DAPI for 5 minutes, rinsed with water, and mounted with water-soluble anti-fade mounting medium (Invitrogen, Carlsbad, CA). The slides were washed with PBS two to three times between the treatments. The samples were imaged with a confocal multiphoton imaging system (LSM 510 Meta NLO; Carl Zeiss, Jena, Germany).

Western Blot Analysis with LG Lysate

Pooled LGs freshly removed from two to three mice or stored at -80°C were homogenized with a motor-driven homogenizer in RIPA buffer containing protease inhibitor cocktail in a tissue-buffer ratio of 1:5 (wt/vol). The following protease inhibitors were purchased from Sigma-Aldrich and used in cocktail with individual concentrations as follows: pepstatin A (1 μg/mL), *N*-tosyl-L-phenylalanine chloromethyl ketone (10 μg/mL), leupeptin (1 μg/mL), *N*-α-*p*-tosyl-L[SCAP]-lysine chloromethyl ketone (10 μg/mL), *N*-α-*p*-tosyl-L-arginine methyl ester (10 μg/mL), and phenylmethylsulfonyl fluoride (174 μg/mL). The homogenate was clarified by centrifugation in a centrifuge (model 5415C Max RCF 9271; Eppendorf, Fremont, CA) at 10,000 rpm at 4°C for 10 minutes. The supernatant was collected and stored at -80°C. Mouse macrophage Raw264.7 cells were lysed in the same buffer and prepared in parallel as a positive control for cathepsin detection. LG lysate containing 100 μg of total proteins and Raw264.7 cell lysate containing 30 μg of total proteins were loaded into individual wells and resolved by SDS-PAGE on 10% to 12% gels. The proteins were transferred and the membranes blotted with anti-mouse CATS or anti-mouse CATH antibodies at 1:500 dilution followed with an infrared dye-conjugated secondary antibody at 1:2000 dilution (IRDye 800; LI-COR, Lincoln, NE). Membranes were then scanned (Odyssey Infrared Imaging System; LI-COR).

Measurement of Cathepsin Catalytic Activities in Tear and LG Lysate

For tear collection, mice were anesthetized as described, and tear fluid was collected from paired male NOD and BALB/c mice at 12 weeks of age. The mice were placed resting on their sides under a dissection microscope (model SMZ-140; Motic, Xiamen, China). The LG was exposed by a small incision along an axis defined by the outer junction of the eyelid and the ear, and the connective tissue capsule enclosing the gland was opened and removed from the upper surface of the gland, to which a layer of fine cellulose mesh (Kimwipe; Fisher Scientific, Pittsburgh, PA) cut in the shape of the gland was applied. The ocular surface was washed (AK-Rinse Eye Irrigating Solution; Akorn, Abita Spring, LA). The LG was stimulated by adding the agonist, CCH (3 μL, 50 μM), topically to the gland, and tear fluid was collected by carefully applying a 2-μL microcapillary pipette (Microcaps; Drummond, Broomall, PA) at the medial canthus for 5 minutes. Each LG was stimulated with CCH three times, resulting in a total collection time of 15 minutes per LG. The capillary pipettes were emptied into sterile vials by the aspirator supplied by the manufacturer. The tears collected from both eyes of the same mouse were pooled and immediately analyzed.

For preparation of LG lysates, freshly collected LG pairs from each mouse, either after stimulation with topical CCH or without stimulation, were homogenized with a (Polytron tissue homogenizer; Heidolph Brinkmann) on ice in enzyme-specific cell lysis buffer (1 mg tissue/5 μL buffer) provided in the CATS (K144-100), CATH (K145-100), or CATD (K143-100) activity assay kit. The homogenate was clarified by centrifugation at 10,000g at 4°C for 10 minutes. The same number of NOD and BALB/c mice was processed in parallel each time. The resulting lysates were analyzed immediately after preparation.

Catalytic activities of cathepsins in LG lysate and tear fluid samples were determined with the assay kits described herein, according to the manufacturer's instructions. Briefly, the collected tear fluid of whole volume from each mouse or 10 μg of LG lysate was diluted to constitute the reaction mixture of 100 μL containing the substrate in the presence or absence of enzyme-specific inhibitor. CATS and CATH inhibitors were provided in the kits. The CATD inhibitor pepstatin A (P5318) was purchased from Sigma-Aldrich. The reactions were incubated at 37°C for 1, 2, or 18 hours. The quantity of the resulting fluorescent products was measured in a microplate spectrofluorometer (Spectromax Gemini EM; Molecular Devices, Sunnyvale CA) with 400-/505-nm excitation/emission filters for CATS and 328/460-nm excitation/emission filters for CATD. The enzymatic activities for tear samples were calibrated to fluorescent units per microliter of tear fluid.

The activities of CATH, CATS, or CATD measured from individual samples from mouse strains were compared for evaluation of statistical significance by Student's *t*-test. Standard error of the mean (SEM) was used in all cases to describe variations between the sample values in a group.

RESULTS

Gene Expression Profiles of Cathepsin Family Members and Proinflammatory Factors in LG of NOD and BALB/c Mice

Immune cell infiltration accompanied by severe extracellular matrix degradation is a prominent feature of LG in male NOD mouse aged 12 to 18 weeks. Cathepsin S has been suggested to be a contributory factor in the development of autoimmune dacryoadenitis in thymectomized NFS/sld mice, another SJS model.²⁴ Cathepsin B and S have also been implicated as the proteases responsible for extracellular matrix degradation in prostate cancer.²⁶ To investigate the possibility that cathepsin family members contribute to the destruction of the extracellular matrix and the other pathologic changes associated with immune cell infiltration, we analyzed gene expression profiles to determine possible alterations in their expression in male NOD mouse LGs. The results from the microarray analysis are presented in Table 1.

TABLE 1. Differentially Expressed Cathepsin Family Members in LGs of NOD Mice versus Those of BALB/c Mice, Characterized by Microarray Analysis

Gene	NCBI Accession	Change Ratio*	P	Change in NOD
<i>Ctsb</i>	NM_007798	1.0	0.3330	No change
<i>Ctsc</i>	NM_009982	1.2	0.0624	No change
<i>Ctsd</i>	NM_009983	1.0	0.3311	No change
<i>Ctsf</i>	NM_019861	0.7	0.0006	No change
<i>Ctsb</i>	NM_007801	2.1	0.0015	Increase
<i>Ctsk</i>	NM_007802	0.4	0.1128	No change
<i>Ctsl</i>	NM_009984	0.9	0.4897	No change
<i>Ctso</i>	NM_177662	1.4	0.1111	No change
<i>Ctsr</i>	NM_020284	6.9	0.0015	Increase
<i>Ctss</i>	NM_021281	4.4	1.7E-07	Increase
<i>Ctsw</i>	NM_009985	3.1	0.0230	Increase
<i>Ctsz</i>	NM_022325	1.8	0.0029	No change

Gene symbols are for individual cathepsin family members.

* The NOD/BALB ratio comparing the hybridization signal of NOD mouse LG to the signal from BALB/c mouse LG. Changes >2-fold with $P < 0.05$ are shown in bold. Significant changes in gene expression that were >2-fold are shown in italic.

mRNAs of CATH (Ctsh), -R (Ctsr), -S (Ctss) and -W (Ctsw) were significantly elevated (i.e., at least twofold) in the LGs of NOD mice compared with their presence in LGs of the BALB/c control animals. Ctsz was significantly elevated as well, but to a lesser degree, whereas Ctsf was significantly decreased.

We also probed levels of cytokines and their receptors at the mRNA level in the LG of NOD mice relative to the BALB/c control mice. Since our working hypothesis proposed that abnormalities in lipid metabolism in acinar cells contribute to the onset or development of dacryoadenitis through the action of macrophages migrating and propagating in the LG of NOD mice, we focused on cytokines that had been characterized as being expressed in macrophages and associated with obesity or other lipid-associated inflammatory diseases. We do not mean to imply that other cytokines are not expressed in the NOD mouse LG, but these cytokines were our chosen focus. Only a subset of these cytokines and receptors were detected by microarray as shown in Table 2. The mRNA levels of interferon- γ (*Ifng*),

TABLE 2. Increased mRNA Levels of Cytokines and Proinflammatory Factors in LG of NOD versus BALB/c Mice Characterized by Microarray Analysis

Gene	NCBI Accession*	Change Ratio†	P	Change in NOD
<i>Ifng</i>	NM_008337	14.5	1.2E-06	Increase
<i>Il1b</i>	NM_008361	—	—	—
<i>Il6</i>	NM_031168	—	—	—
<i>Il6ra</i>	NM_010559	2.6	0.1740	No change
<i>Il10</i>	NM_010548	2.6	0.0636	No change
<i>Il10ra</i>	NM_008348	6.1	0.0014	Increase
<i>Il12a</i>	NM_008351	0.8	0.0774	No change
<i>Il15</i>	NM_008357	1.8	0.3883	No change
<i>Tnf</i>	NM_013693	4.6	0.0003	Increase

Il1b and *Il6* mRNA species were not detected above the hybridization noise, and their expression is therefore not shown. *Ifng*, interferon gamma; *Il6*, interleukin 6; *Il6ra*, interleukin 6 receptor alpha; *Il10*, interleukin 10; *Il10ra*, interleukin 10 receptor alpha; *Il15*, interleukin 15; *Tnf*, tumor necrosis factor alpha.

* National Center for Biotechnology Information, Bethesda, MD.

† The change ratio obtained by comparing the hybridization signal of NOD mice to the signal of BALB/c mice. Changes >2-fold with $P < 0.05$ are shown in bold.

TABLE 3. Validation of Microarray Data of Cathepsins by Real-Time RT-PCR

Group	Ctsh	Ctss	Ctsz
12 wk			
BALB/c M	1.0 $\pm$ 0	1.0 $\pm$ 0	1.0 $\pm$ 0
NOD M	8.2 $\pm$ 1.0*	9.3 $\pm$ 0.8*	2.3 $\pm$ 0.2†
NOD SCID M	2.8 $\pm$ 0.3*	2.5 $\pm$ 0.4†	1.6 $\pm$ 0.1
BALB/c F	1.0 $\pm$ 0.1	1.6 $\pm$ 0.2	1.5 $\pm$ 0.1
NOD F	1.4 $\pm$ 0.1	1.2 $\pm$ 0.2	1.5 $\pm$ 0.1
NOD SCID F	1.5 $\pm$ 0.3	1.0 $\pm$ 0.1	1.4 $\pm$ 0.0
4 wk			
BALB/c M	1.0 $\pm$ 0	1.0 $\pm$ 0	1.0 $\pm$ 0
NOD M	1.6 $\pm$ 0.2	1.5 $\pm$ 0.1	1.4 $\pm$ 0.1
BALB/c F	0.8 $\pm$ 0.1	1.2 $\pm$ 0.1	1.3 $\pm$ 0.1
NOD F	1.2 $\pm$ 0.0	0.7 $\pm$ 0.1	1.4 $\pm$ 0.1

Each data point within the 12-week-old groups depicts the average of four to nine PCR assays; for the 4-week old groups, the average was obtained from three PCR assays. Each PCR assay included RNA samples prepared from pooled LGs of three to four mice from separate groups. Data are expressed as the mean $\pm$ SEM. Only changes >2-fold with significant P -value are marked.

* $P < 0.001$.

† $P < 0.01$.

interleukin-10 receptor α (*Il10ra*), and *Tnf* were clearly significantly elevated in the LG of male NOD mice relative to that of matched BALB/c mice.

Data Validation and Quantitative Investigation of Gene Expression

The results of the microarray analysis were validated by real-time RT-PCR for cathepsin family members and cytokines of interest. Beside the total RNAs from the LGs of 12-week-old male NOD and BALB/c mice, total RNAs from age-matched NOD SCID mice of both sexes, female NOD and BALB/c mice, and 4-week-old NOD and BALB/c mice of both sexes were analyzed concomitantly. NOD females share the same genetic background as the male NOD mice, but they do not develop dacryoadenitis as severely or exhibit disease manifestation at 12 weeks. To understand whether any of the changes in gene expression in the male NOD LG relative to male BALB/c LG revealed by microarray might be due to differences in strain background rather than the actual disease process, comparison with the disease-free NOD female mice is therefore informative. BALB/c female mice were likewise used as a control to aid in determining whether any observed changes in female NOD mice were associated with the sex of the mouse. NOD SCID mice were included because they are genetically equivalent to NOD mice, with the exception of the compromised T- and B-cell function and the lack of dacryoadenitis, thus enabling identification of intrinsic changes in the NOD model that may contribute to or potentiate inflammation independent of T- and B-cell action. Finally, 4-week NOD mice exhibit no dacryoadenitis and are thus useful in determining the temporal relationship between changes in gene expression and the establishment of disease, whereas the age-matched male and female BALB/c and female NOD mice provide the necessary developmental and sex-based controls.

The results are summarized in Table 3 (cathepsins) and Table 4 (cytokines). We focused on the elevated expression of *Ctss*, *Ctsh*, and *Ctsz* shown in Table 1, since these gene products are expressed in both mouse and human tissues, are physically enriched within lysosomes and endosomes of antigen-presenting cells (CATS and CATH), and are distributed within all cell types ubiquitously (CATH and CATZ).²⁷ In particular, CATS has an established role in antigen presenta-

TABLE 4. Validation of Microarray Data of Cytokines by Real-Time RT-PCR

Group	Il1b	Il6	Il10	Il12a	Il15	Ifng	Tnf
12 wk							
BALB/c M	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0
NOD M	6.4 ± 0.3*	10.9 ± 1.3*	43.2 ± 2.4*	505.1 ± 33.3*	2.6 ± 0.1*	31.5 ± 5.0*	12.0 ± 1.8†
NOD SCID M	1.6 ± 0.4	2.2 ± 0.3†	4.3 ± 0.8*	2.6 ± 0.9	0.7 ± 0.1	1.7 ± 0.5	2.1 ± 0.4†
BALB/c F	1.9 ± 0.1	1.6 ± 0.3	2.3 ± 0.9	2.8 ± 1.0	1.5 ± 0.1	1.9 ± 1.0	3.8 ± 0.2*
NOD F	1.0 ± 0.2	1.4 ± 0.2	2.4 ± 0.1‡	2.1 ± 0.7	1.2 ± 0.2	1.0 ± 0.4	1.5 ± 0.4
NOD SCID F	0.8 ± 0.2	0.8 ± 0.1	2.2 ± 0.8	1.0 ± 0.4	0.9 ± 0.1	0.5 ± 0.2	0.7 ± 0.1
4 wk							
BALB/c M	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0
NOD M	1.0 ± 0.1	1.8 ± 0.5	5.2 ± 0.2*	0.6 ± 0.2	1.1 ± 0.3	1.2 ± 0.3	2.6 ± 0.8
BALB/c F	2.1 ± 0.9	0.9 ± 0.2	1.4 ± 0.3	1.0 ± 0.5	1.0 ± 0.1	1.6 ± 0.2	3.6 ± 0.6†
NOD F	0.6 ± 0.2	0.5 ± 0.1	1.3 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	1.0 ± 0.4	1.0 ± 0.1

Data for the 12-week-old groups are the average of results in three to seven PCR assays; for data points in the 4-week-old groups, the averages include three PCR assays. Each PCR assay included RNA samples prepared from pooled LG from separate groups of three to four mice. Data are expressed as the mean ± SEM. Only changes >2-fold with significant *P*-value are marked.

* *P* < 0.001.

† *P* < 0.01.

‡ *P* < 0.05.

tion^{23,28} and is associated with obesity²⁹ and lipid-related inflammatory processes.¹⁰ We did not further explore *Ctsr*, since it appears to be expressed only in mouse and not in humans, or *Ctsw*, since it is mainly restricted to the CD8(+) T cells and NK cells,³⁰ which were not the focus of the present study.

In addition to the significantly upregulated genes indicated in Table 2, transcripts for other cytokines which were not detected by microarray, possibly due to low abundance, were re-evaluated (*Il1a*, *Il6*), as were *Il12a* and *Il15* which did not show a significant change by microarray (Table 2) but are linked to macrophage activation.

Consistent with the microarray analysis, the mRNA levels of *Ctsb*, *Ctss*, and *Ctsz* in the 12-week-old male NOD mouse LG were significantly higher than in the matched BALB/c mouse LG. All cytokines tested also showed significantly increased expression in the LG of 12-week-old NOD mice relative to the BALB/c control. The mRNA levels of the *Ctss*, *Ctsb*, *Il6*, *Il10*, and *Tnf* were also all significantly higher in NOD SCID mice than in BALB/c mice, although these levels were still lower than those detected in NOD mice. Male NOD SCID mice, like male NOD mice, exhibit notable lipid deposition in the LGs.⁴ Comparison of gene expression levels in mice aged 4 weeks (i.e., before the onset of lipid deposition and accumulation and before the onset of detectable lymphocytic infiltration), showed small but significant elevations only in *Il10* in male NOD mouse LG relative to the matched BALB/c control.

Lineage Characterization of Infiltrating Immune Cells in LGs

In an earlier study, we revealed the presence of various types of infiltrating immune cells, including macrophages and neutrophilic and eosinophilic granulocytes, B cells, and T cells within the LG of NOD mice at 18 weeks of age.²⁵ Although NOD mice exhibit a diverse immune cell infiltration, NOD SCID mice would not be expected to show marked T- and B-cell infiltration of the LG. Some of the same cathepsins and cytokines were upregulated, however, in NOD and NOD SCID mouse LGs at 12 weeks, when inflammation in NOD mouse LGs is established, but not at 4 weeks. This result led us to investigate the nature of immune cell infiltration in both NOD and NOD SCID mouse LGs at 12 weeks, relative to that in healthy age-matched BALB/c mice. Flow cytometry was performed on pooled cells isolated from LGs of 12-week-old male NOD, NOD SCID, and BALB/c mice. We identified the presence of populations of B- and T-cells, macrophages, neutro-

philic and eosinophilic granulocytes, and NK cells in all LGs (Table 5). B and T cells in NOD SCID mice, although reduced to levels commensurate with those found in healthy BALB/c mouse LG, were still detectable. This result is unsurprising since, although these mice are severely immunodeficient, it has been reported that they do not completely lack T- and B-cell lymphocyte function.³¹ Double-labeling of cells using antibodies against CD11b and GR1 showed that approximately 7% of the BALB/c, 25% of the NOD and 11% of the NOD SCID LG cells were macrophages (CD11b+ GR1-). These data suggest that a source of some upregulated cytokines, and also possibly cathepsins, in NOD and NOD SCID mouse LGs may be these infiltrating macrophages.

CATS Protein Expressed by Macrophages and Acinar Cells in LGs

To elucidate the possible role of CATS in the development of disease in NOD mouse LG, its distribution and abundance in LGs was investigated by immunofluorescence microscopy, as shown in Figure 1. CATS immunofluorescence was highly colocalized with CD68, a lineage marker of macrophages, among the infiltrating cells within the LGs of NOD mice (Fig. 1A). In addition, CATS was detected in acinar cells in BALB/c, NOD, and NOD SCID mice (Fig. 1B). The distributions of CATS

TABLE 5. Results of Flow Cytometry of Inflammatory Cells Isolated from LG of BALB/c, NOD, and NOD SCID Male Mice

Lineage Marker	BALB/c	NOD	NOD SCID
B220+ CD19-	6.8	3.9	5.1
B220+ CD19+	4.1	34.0	0.8
CD4+ CD8-	3.1	13.0	3.1
CD4- CD8+	2.1	7.9	2.0
CD11b+ GR1-	7.0	25.0	11.0
CD11b+ GR1+	2.0	3.5	1.7
CD11b- GR1+	4.2	6.9	3.0
Pan-NK	3.6	5.9	2.2

Data are the percentage of cells positive for each marker of LG from five male mice of each strain, each aged 12 weeks. B220+ CD19-, early B lymphoid progenitor cells; B220+ CD19+, B lymphoid progenitor cells; CD4+ CD8-, mature T helper cells; CD4- CD8+, mature cytotoxic T cells; CD11b+ GR1-, macrophages; CD11b+ GR1+, myeloid immunoregulatory cells; CD11b- GR1+, granulocytes; and Pan-NK, NK cells.

FIGURE 1. Detection of CATS in different locations in LGs from different mouse strains. Triton X-100 and SDS-permeabilized cryosections of LGs from 12-week-old male NOD, NOD SCID, and BALB/c mice were incubated with goat anti-CATS polyclonal antibody and rat anti-CD68 monoclonal antibody followed by the appropriate fluorophore-conjugated secondary antibodies. The sections were imaged by confocal fluorescence microscopy. Bars, 10 μm. (A) Infiltrating immune cells in NOD mouse LG. CATS were marked with rhodamine red-X (red), CD68 with FITC (green), and nuclei with DAPI (blue). Arrows: colocalized CATS and CD68 in cells. (B) Acinar cells in LGs of NOD, NOD SCID, and BALB/c mice. CATS were marked with Alexa Fluor 488 (green), actin filaments visualized with rhodamine-phalloidin (red), and nuclei with DAPI (blue). Arrows: CATS-positive areas at subapical-apical areas; arrowheads: the potential late endosomes/lysosomes.

appeared to be different among the mouse strains. The basolateral punctate labeling that was abundant in the healthy BALB/c mouse LGs was characteristic of a lysosomal labeling pattern. The subapical/apical labeling was prevalent and abundant in both NOD and NOD SCID LGs. Only a trace amount of the positive staining displayed around or within the lumina in the acinar cells was detected in BALB/c mouse LGs. In general, the intensity of CATS immunofluorescence appeared highest in NOD mouse LGs and lowest in BALB/c, consistent with its increased expression in NOD mouse LGs shown by microarray (Table 1) and real-time PCR (Table 3).

To further define the subcellular distribution of CATS protein in the acinar cells, we investigated the colocalization of CATS with the late endosome/lysosome marker Lamp2 by indirect immunofluorescence and confocal fluorescence microscopy (Fig. 2). Consistently, the number and size of Lamp2-positive late endosomes/lysosome and the abundance of the CATS protein appeared to be increased in acinar cells from NOD and NOD SCID mice relative to that in acinar cells of age-matched BALB/c mice. Mild detergent treatment with saponin and a short time (5–10 minutes) of permeabilization were used to elicit maximum Lamp2 immunofluorescence in this experiment. Under these conditions, we noted that the subapical localization of CATS was less evident than under the processing conditions shown previously (Fig. 1B).

Increased Abundance and Activity of CATS in NOD Mouse LG Lysate and Tears

Elevated Ctss mRNA level, increased CATS immunoreactivity in NOD mouse LGs, and increased CATS immunofluorescence detected within subapical compartments in acinar cells all suggest that increased protein abundance and catalytic activity of CATS in LG correlates with development of LG disease. CATS abundance was thus compared in LG of NOD mice with that in LG of BALB/c mice by Western blot analysis (Fig. 3A). Consistently, a 24 kDa protein band corresponding to the molecular mass of the active form of CATS (depicted in lane 1, from Raw264.7 cell lysate) in the gland lysate from NOD mice was detected, compared with a much weaker band to no band at the same position in LG lysate from BALB/c mice. CATS

activities in LGs between the two strains were also compared by enzymatic assays (Fig. 3B). The average CATS activity from NOD mouse LG lysates was significantly greater than that from BALB/c mouse LG lysates.

The altered recovery of CATS immunofluorescence in apparent subapical secretory vesicle-like organelles in the acinar

FIGURE 2. CATS protein distribution in acinar cells from NOD and NOD SCID mice. Saponin-permeabilized cryosections of LGs from 12-week-old male NOD, NOD SCID, and BALB/c mice were incubated with goat anti-CATS polyclonal antibody and rat anti-Lamp2 monoclonal antibody, followed with the appropriate fluorophore-conjugated secondary antibodies. The sections were imaged by confocal fluorescence microscopy. Labeling with DAPI (blue) for nuclei and Alexa Fluor 647 (pink) for actin filaments was conducted in parallel. Lamp2-positive staining marked late endosomes and lysosomes. Arrowheads: the CATS (rhodamine red-X, red)-positive areas on the luminal side; arrows: the CATS and Lamp2 (FITC, green) double-positive areas. Bars, 5 μm.

FIGURE 3. Comparison of CATS abundance and activity in LG from NOD and BALB/c mice. **(A)** Western blot analysis to compare the protein abundance of CATS. LG lysates were prepared from 12-week-old male NOD mice (lanes 3, 5, and 7) or matched BALB/c mice (lanes 2, 4, and 6). Each LG lysate (100 μ g) was loaded on SDS-polyacrylamide gel. Raw264.7 cell lysate (50 μ g; lane 1) was run in parallel as a positive control. *Top*: the lanes were blotted with goat anti-CATS polyclonal antibody, with (lanes 1-5, 1° + 2°) or without (lanes 6 and 7, 2°) primary antibody, then with infrared dye-conjugated secondary antibody (lanes 1-7). *Arrow*: the position of CATS at 24 kDa, comigrating with the positive control in lane 1. *Bottom*: Western blot probed in parallel with rabbit antibody against Rab3D (1° + 2°, lanes 2-7), an LG-specific protein, and secondary antibody as a loading control. **(B)** CATS activity assay. LG lysates were prepared with LG from male NOD and BALB/c mice at the same age as in **(A)**. Each of the paired lysates (10 μ g; $n = 5$) from two strains were incubated for 2 hours in the absence (– inhibitor) or presence (+ inhibitor) of specific inhibitor. No lysate, the reaction background. The resulting fluorescence of the products was measured with appropriate excitation/emission filters. The enzymatic activity is expressed in fluorescent units (FU); error bars, $\pm$ SEM; ** $P < 0.01$.

cells, as well as within the lumina of the acini suggested that CATS is actively secreted at the apical membrane into the tear fluid in NOD mice. The catalytic activities of CATS in tears were measured in parallel with the lysates of LGs from which the tears were collected. The results demonstrated significantly higher CATS activity in the tear fluid of NOD mice than that of BALB/c mice when stimulated with a CCH agonist (Fig. 4A).

Cathepsin D (CATD) is another established endosomal/lysosomal resident peptidase C1 family member similar to CATS and CATH. It has been detected in human saliva,³² human tears,³³ and mouse tears (Wu K, unpublished data, 2006). Unlike CATS and CATH, its expression is not increased in the NOD mouse LG (Table 1). Its catalytic activity was thus measured in LG lysates and tears as a control, showing no significant difference in LG lysates between the strains and a small but significant decrease in activity in NOD mouse tears relative to BALB/c mouse tears (Fig. 4B). This finding suggests that CATS is a selective tear biomarker for dacryoadenitis. This finding also further confirms that NOD mouse LG does not exhibit global disturbances in exocytosis of cathepsins from lysosomes and that the observed increase in CATS activity in tears of male NOD mice is likely from the apparent and abnor-

mal secretory vesicle stores observed beneath the apical membrane.

CATH Protein Expressed by Macrophages but Not Acinar Cells

CATH is defined as an aminopeptidase and an endopeptidase^{34,35} although its cellular function is still obscure to date. Its mRNA was also markedly elevated in the NOD mouse LGs. Immunofluorescence microscopy was conducted to detect and characterize the cells producing CATH (Fig. 5). The CATH protein was observed in some cells in interstitial and elastic tissue within the sac surrounding the LG in the NOD, NOD SCID, and BALB/c mice (Figs. 5a, 5d, 5g). The CATH-positive cells were also enriched at the intercellular spaces between the acini from the NOD (Fig. 5g) and the NOD SCID mice (Fig. 5d), but only a few appeared in the BALB/c mice (Fig. 5a). In addition, CATH-positive cells were observed among the infiltrating foci in the LG of NOD mice (Figs. 5j, 5l). These CATH-positive cells (arrows) were also largely positive for CD68 and were seen either in the extracellular spaces (Figs. 5c, 5f, 5i) or within the foci (Fig. 5l). Unlike CATS, no CATH protein was detected in the acinar cells.

FIGURE 4. Comparison of CATS and CATD activities in stimulated LG and tear fluid from NOD and BALB/c mice. Twelve-week-old male mice were anesthetized and tear fluid collected after stimulation with CCH. LG lysates were prepared from the stimulated glands after tear collection. The whole volume of tear sample and 10 μ g of lysate from each mouse were incubated with substrate in the absence of inhibitor. The resulting fluorescence of the products was measured with appropriate excitation/emission filters. FU, fluorescent unit; error bars, $\pm$ SEM; FC, fold (ratio) change. The enzyme activities presented are the results after subtraction of the reaction background. **(A)** CATS activities in stimulated LG and tears ($n = 7$) assayed for 2 hours. Activities are expressed as FU/10 μ g of lysate or FU/10 μ L of tears. *** $P < 0.001$. **(B)** CATD activities in stimulated LG and tears ($n = 3$) assayed for 1 hour. The activities are expressed as FU/10 μ g of lysate or FU/1 μ L of tears. * $P < 0.05$.

FIGURE 5. Detection of CATH-positive cells in different locations in LG from different mouse strains. Cryo-sections of LG from 12-week-old male NOD, NOD SCID, and BALB/c mice were incubated with goat anti-CATH polyclonal antibody and rat anti-CD68 monoclonal antibody followed with fluorophore-conjugated secondary antibodies. The sections were imaged by confocal fluorescence microscopy. CATH (red) and CD68-enriched macrophages (green) are shown separately in the indicated columns as well as in the merged image. All sections were also labeled to detect nuclei (DAPI, blue) and sections in (a) through (i) were also labeled for actin filaments (pink). (a–i) Parenchymal tissues and surrounding regions of LGs; (j–l) magnification of infiltrating foci. Arrowheads: CATH and CD68 double-positive cells in the surrounding region of the LG (c, f, and i); arrows: double-positive cells in the interior region of the LG (c, f, i) or in the infiltrating foci (l). Magnification: (a–i) 20 μm; (j–l) 10 μm.

Western blot analysis was conducted to investigate whether the abundance of CATH protein also increases in parallel with the elevated mRNA levels in NOD mouse LGs (Fig. 6A). CATH is produced as a proenzyme in cells (335 amino acids [aa] in humans and 333 aa in mice). Proteolytic processing of the single transcript results in the generation of active forms of human CATH containing either a minichain (98–105 aa) and a single chain (116–335 aa) or an active form containing a mini chain, a heavy chain (116–292 aa), and a light chain (293–335 aa), as demonstrated at the protein level.³⁶ The heavy chain and light chains are derived from further proteolytic processing of the single chain without a loss of amino acid residues. The active forms of the mouse CATH protein exhibit an identical organization, existing in two forms which were deduced by UniProtKB (www.UniProt.org/ provided in the public domain by the UniProt Consortium, Swiss Institute of Bioinformatics, Geneva, Switzerland) according to its homology to the human ortholog at the transcriptional level. The minichain,

heavy chain, and light chain of mouse CATH are annotated as 96–103, 114–290, and 291–333 aa, respectively. The major active form of CATH in Raw264.7 cells, used in our study as a positive control for CATH, appears to be the three-chain form, since the major band detected by Western blot analysis is ~23 to 24 kDa, corresponding to the heavy chain of 177 aa (114–290 aa; Fig. 6A, top, lane 3). Both the two-chain and the three-chain forms of CATH are present in mouse LG, as evidenced by detection of both the 23- to 24-kDa band (Fig. 6A, top, lanes 1 and 2) and the 27- to 28-kDa band (Fig. 6A, lane 2, top), corresponding to the heavy chain, and the single chain (220 aa), respectively. In addition, a third band at around 20 kDa was clearly detected only in NOD mouse LG lysate (Fig. 6A, lane 1, top), which may represent a further proteolytic or truncated form of CATH. Densitometry of this Western blot quantified the intensity ratios of 27- and 28-, 23- and 24-, and 20-kDa bands (NOD to BALB/c) as 14.1, 4.7, and 11.3 respectively. These results are typical of those run in multiple experiments comparing NOD

FIGURE 6. Comparison of CATH abundance and activity in LG lysates from 12-week-old male NOD and BALB/c mice. **(A)** Western blot analysis to compare the protein abundance. Lysate (100 μ g) prepared from NOD mouse LGs (lanes 1 and 4), 100 μ g of lysate prepared from BALB/c mouse LGs (lanes 2 and 5), and 30 μ g of Raw264.7 cell lysate (lanes 3 and 6) were loaded onto an SDS-polyacrylamide gel. *Top*: the proteins transferred to nitrocellulose membranes were blotted with rat anti-CATH monoclonal antibody with (1° + 2°, lanes 1–3) or without (2°, lanes 4–6) primary antibody and then with infrared dye-conjugated secondary antibody (lanes 1–6). *Bottom*: The same membrane after being stripped was reblotted with rabbit anti-Rab3D polyclonal antibody and secondary antibody (1° + 2°, lanes 1–6) as a loading control. *Arrows*: major CATH bands corresponding to the single chain of one of the active forms at 27 to 28 kDa and the heavy chain of the other active form at 23 to 24 kDa (comigrating with the positive control), as well as a possibly proteolyzed or truncated species at 20 kDa. These bands were most abundant in NOD mouse LG lysate. A band above the specific 23- to 24-kDa band (lanes 1–3) is visible due to nonspecific reactivity of all samples with the goat anti-rat secondary antibody (lanes 4–6). The molecular masses marked indicate the migration of the molecular mass standards. **(B)** CATH activity assay. Each paired LG lysate (10 μ g; $n = 3$) was incubated with substrate in the absence (– inhibitor) or presence (+ inhibitor) of inhibitor. No lysate, reaction background. Accumulated fluorescence of products from LG lysates was measured at 2 hours. Activity is indicated in fluorescence units (FU)/10 μ g lysate. Errors are $\pm$ SEM; and * $P < 0.05$.

and BALB/c mouse LG lysate and demonstrate the marked upregulation of CATH in the NOD mouse LG versus the BALB/c control as well as illustrate the complexity of CATH processing in different cell types.

The enzymatic activities of CATH were also determined as shown in Figure 6B. Significantly elevated catalytic activity was detected in LG lysates from 12-week-old male NOD mice versus that from the matched BALB/c mice by using a commercial CATH assay. However, the catalytic activity was only partially inhibited by the CATH inhibitor (Fig. 6B). It is unclear whether this indicates hydrolysis by non-CATH enzyme(s) or only partial effectiveness of the inhibitor in this experiment.

DISCUSSION

We have reported in an earlier study that lipid deposition correlates highly with lymphocytic infiltration of the LGs in the male NOD mouse, a model of SjS, suggesting a possible relationship between this autoimmune disorder and other inflammatory diseases associated with abnormalities in lipid deposition, including obesity and atherosclerosis.⁴ Studies in adipose tissue of human or animal subjects with obesity have shown that CATS, TNF α , IL-6, and IL-1 β are upregulated relative to levels in healthy control subjects.^{10,37–40} Moreover, adipocytes themselves were shown to be a major source of CATS,¹⁰ whereas macrophages resident within adipose tissue served as the primary source of the cytokines.^{13,37,41,42} On the other hand, CATS has also been well documented as a protease that functions in MHC II-mediated antigen processing in APCs.²³ For the first time, we report the significant upregulation of gene expression, increased protein abundance, and elevated enzymatic activity of CATS in LGs during the development of autoimmune inflammatory disease.

Consistent with gene expression analysis, increased CATS immunoreactivity is detected in sections from the NOD mouse LG relative to BALB/c mouse LG. CATS protein was detected in many of the infiltrating cells in the NOD mouse LG. Although many of the CATS-enriched cells within inflammatory cell foci in the NOD mouse LG were CATS+/CD68+, confirming their identity as macrophages, some CATS-positive cells were CD68-negative. In addition, a notable proportion of CATS-positive cells were extremely late endosome/lysosome-rich, as evidenced by their enrichment in Lamp2 (data not shown). These observations are consistent with previously established mechanisms for the role of CATS in antigen presentation in macrophages and other APCs (B cells and dendritic cells).⁴³

Beside the CATS-positive immune cells, CATS immunofluorescence and, by extension, protein abundance was also significantly increased in acinar cells from the NOD and NOD SCID mouse LGs relative to BALB/c mouse LGs. A subset of CATS-enriched organelles, mostly localized to basolateral regions within the acinar cells, was significantly colocalized with Lamp2, suggesting stimulated biogenesis of lysosomes in these cells from NOD mouse LGs. Some CATS+/Lamp2+ organelles were extraordinarily large and were located in the middle of the cells (Fig. 3). CATS-positive staining was also observed within secretory vesicle-like organelles at the subapical region, as well as within the lumina of the acini of the NOD and NOD SCID mice, but was rarely seen in the acini of the BALB/c mice, suggesting that altered protein sorting and apical secretion of CATS occur only on the NOD background. This finding was verified in the NOD mouse by the detection of increased CATS activity in tears.

The results of gene expression and Western blot analyses indicated that more CATS protein was expressed in LG lysates from NOD mouse LG relative to BALB/c mouse LG. The measured CATS activities in these lysates showed about a threefold increase in activity in the NOD mouse LG relative to BALB/c mouse LG, which was less than what might be expected, given the apparent differences in protein expression. A challenge for many cells is the regulation of endogenous protease activity, a function accomplished largely by use of proenzymes requiring activation as well as the membrane compartmentalization of proteases. Cells also express endogenous serine and cysteine protease inhibitors that selectively regulate protease activity, and these may also be altered in disease. Significant upregulation of numerous protease inhibitors were detected by microarray analysis in 12-week-old NOD mouse LGs relative to 12-week-old BALB/c mouse LGs. These inhibitors included the serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 11 (Serpinh11, 32-fold increase); secretory leukocyte

peptidase inhibitor (Slpi, 30-fold fold); serine (or cysteine) peptidase inhibitor, clade A, member 3G (Serpina3g, 11-fold); cystatin F (leukocystatin) (Cst7, 6-fold); stefin A3 (Stfa3, 6-fold); serine (or cysteine) peptidase inhibitor, clade B, member 6b (Serpina6b, 5-fold); serine (or cysteine) peptidase inhibitor, clade B, member 9 (Serpina9, 4-fold); and the extracellular proteinase inhibitor (Expi, 4-fold), among others. Some of these inhibitors may inhibit CATS or CATH. In a normal cell, cathepsins are resident lysosomal proteins and, even in NOD mouse LG acinar cells where the distribution of CATS is affected, it remains membrane encapsulated. Cellular regulation of the expression and distribution of inhibitors adds a second level of regulatory complexity that determines how active a cathepsin may be in a given environment. All the activity assays from cell lysates were performed in a cell-free system that lyses membrane compartments and allows mixing of proteins that may normally be sequestered. Thus, the enzymatic activities observed in a cell-free system may not reflect the real activities in vivo.

Routinely, newly synthesized lysosomal proteins such as CATS are processed in the endoplasmic reticulum (ER) and Golgi apparatus, trafficked to the trans-Golgi network (TGN), and then actively sorted to lysosomes via late endosomes as the terminal destination.⁴⁴ This sorting pathway appears to be partially altered in the NOD mouse with a diversion of some upregulated CATS into the regulated apical secretory pathway. This missorting may arise through several pathways that are not mutually exclusive: (1) CATS overproduction may saturate the normal sorting pathways in the TGN that sequester lysosomal proteins into cargo vesicles destined for lysosomes, and thus the extra CATS may traffic through a default pathway into a group of mature secretory vesicles; (2) CATS may be actively missorted into the regulated secretory vesicles due to a fundamental abnormality in the cellular trafficking system of NOD (and NOD SCID) mice. (3) Altered signaling to the acinar cells in the diseased LG may decrease the fidelity of trafficking such that CATS is missorted to alternative membrane compartments. Regardless of cause, the observations of increased endosomal and lysosomal abundance in parallel with missorting of at least one endosomal or lysosomal protein into the regulated secretory pathway demonstrates global changes in protein sorting and processing within the acinar cells that may contribute significantly to disease.

A spectrum of significantly upregulated inflammatory cytokines that have been shown to accompany CATS upregulation in obesity were also detected in the NOD mouse LGs. We hypothesize that these cytokines are at least partially produced by infiltrating macrophages. The LGs of NOD SCID mice retained macrophages at an increased number relative to BALB/c mice, although not as many as in the NOD mice, although it lacked T and B lymphocytes. In parallel, increased mRNAs of Tnf, Il6, and Il10 were detected in the NOD SCID mouse LGs relative to the BALB/c mouse LGs, although to a lesser extent than in NOD mouse LGs.

The abundance of CATH protein, also significantly increased in NOD mouse LGs, was exclusively detected in non-acinar cells by indirect immunofluorescence. We also noted a corresponding increase in CATH enzymatic activity within NOD mouse LG lysates. Colocalization of CATH with CD68 indicated that at least a portion of CATH-positive cells were macrophages. The physical location of CATH suggests its involvement in macrophage and other inflammatory cell functions in the NOD mouse LGs.

What are the consequences of increased CATS and CATH in the LG? An increase in CATS-enriched lysosomes suggests that lysosomal degradative capacity is enhanced, thus raising the possibility that protease degradation of proteins in later endosomes/lysosomes becomes abnormal. Previous work has sug-

gested that alterations in cathepsin activity may expose cryptic epitopes on otherwise tolerated self-proteins, which may be effluxed or recycled into the interstitium from the late endosomal compartments where they may encounter increased proteases.⁴⁵ Cryptic epitopes in the interstitium may encounter primed macrophages and other APCs, potentiating autoimmunity.⁴⁶ On the other hand, the increased CATS and endosomes may stimulate MHC II-mediated autoantigen presentation to T lymphocytes by the NOD mouse LG acinar cells themselves as demonstrated by Saegusa et al.²⁴ in SG acinar cells in a different SJS mouse model.

The detection of elevated proteolytic activity of CATS in the tear fluid of the NOD mouse indicates its increased secretion from acinar cells in this disease model. It is possible that increased CATS in tears digests protein components of the cornea, therefore damaging the integrity of the ocular surface as well as enhancing sensory input from the cornea to the LG and/or inflammation of the ocular surface. Since cathepsins bear collagenase/elastase activity,¹⁵ both CATS and CATH in macrophages and other interstitial cells may also be released to degrade tissue extracellular matrix, thus expediting the infiltration of immune cells. Targeting CATS and CATH may therefore constitute alternative therapeutic strategies in the treatment of chronic autoimmune dacryoadenitis associated with SJS. Although reports of CATS in human tears are lacking, our preliminary clinical data show the presence of CATS activity that varies greatly in levels across patients seen in a corneal clinic (data not shown). Studies exploring its utility as a SJS tear biomarker are currently under way.

In summary, in the NOD mouse model, cathepsin family members and cytokines are upregulated during development and progression of SJS-like disease, with a profile comparable to changes that occur in obesity. These findings are consistent with a causal role for lipid deposition in the autoimmune inflammatory response. The profile of increased cathepsin protease expression and distribution within macrophages, other APCs, and even acinar cells suggest a complex role for these proteases in initiation and progression of autoimmunity. CATS secreted into the tear fluid of NOD mice correlates with the initiation of disease in the mouse model and thus may serve as a biomarker for diagnosis of autoimmune dacryoadenitis in human.

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