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Inflammatory caspases are critical for enhanced cell death in the target tissue of Sjögren's syndrome prior to disease onset

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Abstract

To date, little is known why exocrine glands are subject to immune cell infiltrations in Sjögren's syndrome (SjS). Studies with SjS-prone-C57BL/6.NOD-*Aec1Aec2* mice showed altered glandular homeostasis in the submandibular glands (SMX) at 8 weeks prior to disease onset and suggested potential involvement of inflammatory caspases (caspases-11 and -1). To determine if inflammatory caspases are critical for the increased epithelial cell death prior to SjS-like disease, we investigated molecular events involving caspase-11/caspase-1 axis. Our results revealed concurrent up-regulation of caspase-11 in macrophages, STAT-1 activity, caspase-1 activity, and apoptotic epithelial cells in the SMX of C57BL/6.NOD-*Aec1Aec2* at 8 weeks. Caspase-1, a critical factor for IL-1β and IL-18 secretion, resulted in elevated level of IL-18 in saliva. Interestingly, TUNEL-positive cells in the SMX of C57BL/6.NOD-*Aec1Aec2* were not co-localized with caspase-11, indicating that caspase-11 functions in a non-cell autonomous manner. Increased apoptosis of a human salivary gland (HSG) cell line occurred only in the presence of LPS-and IFN-γ-stimulated human monocytic THP-1 cells, which was reversed when caspase-1 in THP-1 cells was targeted by siRNA. Taken together, our study discovered that inflammatory caspases are essential in promoting pro-inflammatory microenvironment and influencing increased epithelial cell death in the target tissues of SjS before disease onset.

Keywords

Sjögren's syndrome; inflammatory caspases; apoptosis; the C57BL/6.NOD-*Aec1Aec2* mouse model; cytokines

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Conflict of interest

Authors have no financial conflict of interest.

1. Introduction

Sjögren's syndrome (SjS) affects mainly the salivary and lacrimal glands, resulting in dry mouth and/or dry eye conditions in patients as a consequence of autoimmune responses to self-antigens. Despite extensive investigations into the etiology of SjS focusing on genetic, environmental and/or immune factors, neither the triggering nor the disease initiating events in the target exocrine glands are known. Our previous studies examining the salivary glands of the NOD mouse model of SjS indicate the presence of multiple alterations in glandular homeostasis even in the absence of infiltrating immune cells and prior to onset of clinical disease (1–3). These changes include altered cell proliferation at the time of birth, up-regulated apoptosis of acinar tissues, proteolysis of secreted proteins such as sPLUNC, and increased expression of IFN-γ.

In an initial attempt to understand the underlying molecular mechanisms for altered tissue homeostasis prior to disease onset in the NOD mouse model, we previously compared the differential gene expression profiles in the SMX of 8- and 12 week-old C57BL/6.NOD-*Aec1Aec2* mice, a recently generated congenic strain with two genetic intervals derived from the NOD mouse, with those of C57BL/6 mice (4). Utilizing C57BL/6.NOD-*Aec1Aec2* mice allowed us to collect disease-associated genes only rather than strain-associated genes since the congenic strain has the same genetic background as C57BL/6 and to investigate early pathogenesis of SjS without potential complications of Type 1 diabetes occurred in the NOD mouse strain. These microarray analyses revealed that among the apoptosis-related genes present in the microarrays, *caspase-11* was significantly up-regulated at 8 weeks of age in C57BL/6.NOD-*Aec1Aec2* mice, a time when increased apoptosis is seen in the salivary glands, while no significant differences were observed in either *Bcl2* family genes or *caspase-9* (4). Interestingly, caspase-11 is known to be expressed only under pathologic conditions, such as endotoxic shock, multiple sclerosis and brain ischemia.

In addition, caspase-11 is believed to be a dual activator and/or caspase-3(5). Caspase-3 is an executive caspase for apoptotic cell death (apoptotic caspase) while caspase-1 and caspase-11 are involved in inflammatory process (inflammatory caspases). Activation of caspase-1 by caspase-11 can result in the synthesis of the mature form of pro-inflammatory cytokine IL-1β and IL-18 in response to LPS (6); thus, it is not surprising that mice deficient in caspase-11 exhibit a phenotype very similar to *caspase-1* gene-knockout (KO) mice. In addition, embryonic fibroblasts derived from *caspase-11* gene KO mice are resistant to apoptosis induced by ectopic expression of caspase-1, suggesting that caspase-11 is an upstream activator of caspase-1 (6). Beyaert *et al*. (7) reported that binding of NF-kB and STAT-1 to the cloned fragments of 5′-flanking promoter regions of *caspase-11* was necessary, respectively, for LPS- and IFN-γ-inducible expression of caspase-11 in macrophages.

While the human orthologue of caspase-11 has not yet been identified due to insufficient information about the expression, induction and *in vivo* substrates of human proteases associated with caspase-1, it is assumed that caspase-4 or -5 plays a similar role based on sequence homologies (7, 8). To better understand the potential role of up-regulated caspase-11 in the salivary glands of C57BL/6.NOD-*Aec1Aec2* mice prior to onset of SjS-

like disease and to investigate if the molecular events involving caspase-11 are critical for the altered glandular homeostasis, we have examined the expression and activity of both upstream transcription factors and downstream target molecules of the caspase-11 mediated pathway both *in vitro* and *in vivo*, determining the potential consequence of the activated pathway critical to produce pathologic microenvironment that mediates autoimmunity.

2. Results

Caspase-11 gene expression is up-regulated in the exocrine glands of SjS-susceptible mice

To confirm our previous results (4) obtained with microarray analyses showing an increased gene expression of *caspase-11* in the SMX of 8 week-old C57BL/6.NOD-*Aec1Aec2* mice carrying two NOD-derived genetic intervals (Figure 1A), the levels of *caspase-11* mRNA in both the submandibular glands of 8 and 12 week-old NOD/LtJ, C57BL/6.NOD-*Aec1Aec2* and C57BL/6J mice were determined by semi-quantitative RT-PCR. As presented in Figure 1B, *caspase-11* expression was increased 4.6-fold ($p<0.01$) in the SMX of C57BL/6.NOD-*Aec1Aec2* mice compared to C57BL/6 mice at 8 weeks of age, decreasing slightly by 12 weeks of age (bar graphs). Interestingly, *caspase-11* gene expression was also increased 1.7-fold ($p<0.05$) and 1.8-fold ($p<0.05$) in the lacrimal glands of both C57BL/6.NOD-*Aec1Aec2* and NOD/LtJ mice at 8 weeks of age, and remained elevated at 12 weeks of age (data not presented).

To determine if either of known transcription factor STAT-1 and NF-κB for caspase-11 is concomitantly up-regulated with caspase-11, their gene expressions were measured (Figure 1B). *Stat1* gene expression proved to be up-regulated in the SMX of both NOD/LtJ and C57BL/6.NOD-*Aec1Aec2* mice (2.3-fold, $p < 0.01$) at 8 weeks of age. Similarly, expression of *Nfkb1* (p50) were up-regulated in the SMX of C57BL/6.NOD-*Aec1Aec2* mice. In contrast, *Nfkb2* (p52) was slightly down-regulated in the SMX of both C57BL/6.NOD-*Aec1Aec2* and NOD/LtJ mice at 8 weeks of age. By 12 weeks of age, *Nfkb1* was down-regulated in the SMX, while *Nfkb2* remained at low levels.

Caspase-11 expression is detected in macrophage and dendritic cells

Caspase-11 protein expression was confirmed by immunohistochemistry on the SMX of 8 week-old C57BL/6.NOD-*Aec1Aec2* mice (Figure 1C). A slide of human breast cancer tissue was used as a positive control for anti-caspase-11 antibody activity (inset). To localize caspase-11 protein expression in the salivary glands, FITC- or PE- (or Texas Red-) labeled antibodies were used for caspase-11 and other cell type markers. Double-staining caspase-11 expressing cells with a dendritic cell (CD11c) or a macrophage (F4/80) cell marker revealed caspase-11 was expressed by both cells, showing positively stained cells in yellow when the images were merged (Figure 1D). Caspase-11 positive cells surrounded acinar and/or ductal cell units as indicated by the white arrows in Figure 1D.

STAT-1 not NF-kappa B activity is concomitantly up-regulated with caspase-11 in the salivary glands of SjS-susceptible mice

Elevated *Stat1* and *Nfkb1* gene expression in the SMX of 8 week-old C57BL/6.NOD-*Aec1Aec2* mice raised the question, which transcription factor induces *caspase-11* gene expression, leading us to perform electrophoretic gel mobility shift assays (EMSAs). Nuclear extracts were prepared from pooled SMX freshly explanted from 8 week-old female C57BL/6.NOD-*Aec1Aec2* mice, incubated with biotin-labeled oligonucleotides specific for the DNA binding site of either STAT-1 or NF-κB, and separated by PAGE. As shown in Figures 2A and 2B, NF-kB showed no statistically significant increase in activity whereas STAT-1 activity (up-regulated >20%) was found in the SMX of 8 week-old NOD/LtJ and C57BL/6.NOD-*Aec1Aec2* mice, when compared to age-and sex-matched C57BL/6 mice. Furthermore, a doubling of the amount of nuclear extract for the NF-kB assay only slightly increased visualization. In the presence of unlabeled (or cold) oligonucleotide probe, binding was decreased, indicating binding specificity. Unexpectedly, the binding activities of STAT-1 and NF-kB in nuclear extracts prepared from C57BL/6.NOD-*Aec1Aec2* lacrimal glands were consistently below detection levels by EMSA, supporting the dichotomy observed in the underlying pathology and timing of SjS-like disease progression within the submandibular versus lacrimal glands of NOD/LtJ and C57BL/6.NOD-*Aec1Aec2* mice.

Caspase-11 activates caspase-1 but not caspase-3

To better define the consequences of elevated caspase-11 in the SMX of a SjS mouse model during the pre-clinical phase of disease when apoptosis of acinar tissue is prevalent, we analyzed the activity of caspase-1 and caspase-3. As shown in Figure 3A, caspase-1, but not caspase-3, activity was up-regulated in the SMX of 8 week-old C57BL/6.NOD-*Aec1Aec2* mice compared to C57BL/6 mice, although the baseline activities for caspase-3 were higher overall. To confirm activation of the caspase-1 pathway, we examined whether IL-1β and/or IL-18, two factors whose secretion is strongly regulated by the activation of caspase-1 (6), were also up-regulated. RT-PCR analyses indicated highly elevated IL-1β and IL-18 in the SMX of 8 week-old C57BL/6.NOD-*Aec1Aec2* mice (Figure 3B). Interestingly, activation of caspase-1 in the local target tissue (i.e, the salivary glands) of SjS corresponded with up-regulated IL-18 cytokine expression in the saliva but not in sera prior to disease onset (Figure 3C). Gradual increase in IL-18 production in saliva as well as sera was apparent at 12 weeks, which is the time when lymphocytes start infiltrating into the SMX.

Apoptosis is more prevalent in the SMX of SjS-prone mice prior to disease onset in comparison with disease-free mice

To identify if activation of the caspase-11 with subsequent activation of the caspase-1 pathway is associated with apoptotic events in the exocrine glands of SjS-like disease-susceptible mice, apoptotic cell death in the SMX of 8 week-old NOD/LtJ and C57BL/6.NOD-*Aec1Aec2* mice was examined. Histological sections of salivary glands were prepared and TUNEL stained (Figure 4A and B). Visualization using a fluorescent microscope fitted with a red filter for Cy3 revealed that both NOD/LtJ and C57BL/6.NOD-*Aec1Aec2* mice showed more abundant apoptotic cell death than C57BL/6 control mice (Figure 4B). Nuclease-treated slides (PC in Figure 4A) were served as a positive control.

Caspase-11 is not detected in TUNEL-positive acinar or ductal cells

Experiments were carried out to investigate if caspase-11 plays a direct role in increased apoptotic acinar cell death in the salivary glands prior to disease onset. Freshly prepared sections of SMX from 8 week-old C57BL/6.NOD-*Aec1Aec2* mice, first treated to identify TUNEL-positive cells, were counterstained with FITC-conjugated anti-mouse caspase-11 antibody. As presented in Figures 4C, caspase-11 (white arrow) was present in the cells located outside or between the acinar or ductal areas, while TUNEL positive cells (red arrows) were acinar or ductal cells. Although, these dying cells were co-localized with caspase-3 (Figure 4C-a, yellow arrow) showing positivity inside of cytoplasm, our finding indicates that not all TUNEL positive cells were positive for caspase-3.

Caspase-1 in conjunction with IFN-γ is essential to increased apoptotic cell death of human salivary gland epithelial cells

Lack of co-localization between caspase-11 and TUNEL positive cells led us to hypothesize that caspase-11 functions in a non-cell autonomous manner, rather than directly killing the cells, by activating caspase-1 and a subsequent pro-inflammatory cytokine release into the microenvironment, resulting in increase in apoptotic cell death of salivary epithelial cells. This hypothesis was tested using a human salivary gland (HSG) cell line (9) co-cultured with a THP-1 human monocyte cell line, which was stimulated with LPS for the induction of IL-1β and IL-18 in the presence and absence of IFN-γ, in an attempt to duplicate observations in the *in vivo* environment. Our previous data from a SjS mouse model lacking IFN-γ (2) showed the absence of disease and pre-disease phenotype, clearly indicating that IFN-γ is critical not only for the onset of the disease but also for pre-disease stage. In addition, in the NOD mouse, IFN-γ was up-regulated over 2-folds in the SMX prior to disease onset (2). Our current data indicate that increased apoptosis of HSG cells occurred only in the presence of LPS-stimulated THP-1 cells when IFN-γ was present (Figure 5A and B, $p < 0.01$). The increased rate of apoptotic cell death was reversed to a normal level when caspase-1 transcription in THP-1 cells was down-regulated by siRNA (Figure 5). The normalized caspase-1 knockdown efficiency was greater than 70%, as shown in Figure 5C ($p < 0.01$).

3. Discussion

Recent identification of *caspase-11* as one of the differentially expressed genes at 8 weeks of age in SjS -prone C57BL/6.NOD-*Aec1Aec2* mice, together with an abnormal glandular homeostasis in pre-diseased NOD mice, led us to hypothesize the importance of intrinsic properties of target tissues (such as availability of antigen(s) and changed activity of antigen presenting cells by pro-inflammatory cytokines) for the breakdown of peripheral tolerance and the activation of autoreactive immune cells (1, 3, 4, 10, 11). Our efforts to understand initial molecular events triggering onset of SjS identified 3 important findings pertaining to a caspase-11 mediated pathway in the salivary glands. First, *caspase-11* expressed primarily in macrophages/dendritic cells is up-regulated in the SMX prior to disease onset and is apparently associated with enhanced transcriptional activity of STAT-1. Second, these events appear to lead to secretion of pro-inflammatory cytokine production from the local tissue via caspase-1 activation, as indicated by elevated IL-18 levels in saliva, capable of

inducing increased epithelial cell death rather than cell-autonomous killing of epithelial cells. Third, caspase-1 in macrophages/dendritic cells and IFN-γ in the salivary gland microenvironment play a critical role in the death of residential epithelial cells, shown both *in vivo* and *in vitro* analyses. This scheme is illustrated in Figure 6.

We recently postulated that a potential signal for induction of STAT-1 transcriptional activity may come from latent/recurrent viral infection in the salivary glands, especially reactivation of endogenous virus in case of mice housed under SPF conditions, thus promoting interferon induction as an anti-viral defense mechanism by epithelial cells or natural killer (NK) cells in the glands. Increased IFN-γ, known to be present in exocrine glands of NOD-derived mice (2), can enhance the activity of macrophages and/or dendritic cells in the tissues, resulting in production of caspase-11. This is supported by the fact that caspase-11 is known to be induced only when signals from IFN-γ or LPS via the activation of STAT-1 or NF-kappaB transcription factors are synthesized.

A study on experimental autoimmune encephalomyelitis (EAE) indicates that caspase-11 is highly expressed in both oligodendrocytes and infiltrating cells and co-localized with activated caspase-3, suggesting that a pathway involving caspase-11 and caspase-3 is important in the execution of oligodendrocyte death in EAE lesions (5). However, the role of caspase-11 found in the SMX of SjS-prone C57BL/6.NOD-*Aec1Aec2* mice differed from that in EAE models in that caspase-11 in our model was neither produced by dying cells nor co-localized with caspase-3. In our SjS model, caspase-11 apparently is correlated with caspase-1 activity. Furthermore, abundant TUNEL-positive cells in the SMX of C57BL/6.NOD-*Aec1Aec2* mice, together with decreased caspase-3 activity and the fact that not all TUNEL-positive cells were positive for caspase-3, suggest that acinar cell apoptosis induced by pro-inflammatory cytokines in the microenvironment may involve a caspase-3-independent pathway.

During early disease pathogenesis of SjS, the roles of IFN-γ appear to be indispensable based on our previous study (2) as well as our current study. IFN-γ is required but not sufficient for the induction of increased cell death in the target tissue of SjS. In addition, our previous studies (2) indicate that in the absence of IFN-γ, mice exhibited neither secretory dysfunction nor alterations in pre-disease markers, strongly conclusive to our current findings. The requirement for caspase-1 and IFN-γ in enhanced cell death, proven by in vitro co-culture study with siRNA targeting caspase-1 in THP-1 cells, may originate from the fact that the IFN-γ is essential for caspase-1 activation, which cleaves pro-IL-1β and IL-18 to produce mature forms of IL-1β and IL-18 for its release (12). Therefore, synergistic effects between cytokines induced and cleaved by the activation of caspase-11 and caspase-1, and IFN-γ/STAT-1 are essential to drive chronic inflammatory conditions in the targeted glands even before the disease onset.

Increased expression of IL-1β can cause the activation of the signal cascade leading to the activation of several transcription factors involved in inflammatory responses (13). IL-18, originally described as IFN-γ inducing factor, is a potent inflammatory stimulant produced by macrophages and dendritic cells and known to enhances antigen-specific clonal expansion of IFN-γ producing T cells (14), suggesting that IL-18 may impact T cell

immunity in both non-lymphoid and lymphoid tissues by bridging the innate and adaptive arms of the immune system through IFN-γ during the early stage of SjS. A study also indicates that IL-18 produced by Kupffer cells stimulates T_H1 and NK cell cytotoxic activity by increasing their production of FasL (CD 95), which ultimately induces apoptosis in Fas bearing hepatocytes, causing liver injury (15). In a similar manner, IL-18 produced by phagocytic cells may up-regulate FasL on NK cells and Fas on epithelial cells in the SMX via caspase-1 activation, resulting in subsequent apoptotic processes in the epithelial cells. Interestingly, IL-18 and IL-1β have been reported to be up-regulated in both sera and the SMX of SjS patients (16, 17). Considering the results of our current study, one might speculate that IL-1β and IL-18 are up-regulated in the target tissue of SjS patients as well starting at the early disease stage.

Recent studies indicate that a set of caspases (i.e., human caspase-1, caspase-4 and caspase-5, along with murine caspase-11 and caspase-12), considered “inflammatory caspases”, are involved in the proteolytic maturation of inflammatory cytokines (18, 19). Recent studies have identified a complex of proteins, referred to as the “inflammasome”, functions in innate immunity by regulating inflammatory caspase-1 activation (20–22). Proteins that make-up the inflammasomes are members of the NALP (NACHT, LRR and PYD-containing proteins) family of proteins, although information on its exact expression or binding partners is relatively scarce (23). The inflammasomes are hypothesized to act as an early sensor detecting danger signals because the stimulation of the inflammasome triggers a series of internal reactions that ultimately activates caspase-1, which subsequently produces mature IL-1β and IL-18 for regulating immune cells. Important questions that we are currently investigating include if caspase-11 activates caspase-1 as a part of the inflammasomes, if increased epithelial cell death in the target tissues confers target tissue specificity in autoimmune SjS, and if abnormal regulation of the inflammasome translates to SjS in humans. Our current results appear to point to the potential existence of abnormal regulation of the inflammasome in the SMX of NOD-derived SjS-like disease-susceptible mice (Figure 6).

In summary, the present study shows that caspase-11 plays a critical role in the susceptibility of mice to SjS-like disease by up-regulating caspase-1-mediated pathway, which is vital for apoptotic cell death of the neighboring epithelial cells. In addition, presence of IFN-γ in the environment is essential for caspase-1-induced cell death of salivary epithelial cells. The repeated occurrence of pro-inflammatory cytokine secretion and apoptotic cell death following reactivation of latent or persistent viral infection may lead to chronic inflammatory conditions in the salivary glands in SjS. STAT-1 activation rather than NF-κB activation in the SMX of disease-prone mice appears to be responsible for caspase-11 induction. Overall, our observations underscore the potentially critical roles of myeloid cell populations and of intracellular pattern recognition through the inflammasomes activating caspase-1 in the early pathogenesis of SjS. Crossing knockouts onto the SjS-prone mouse strain will confirm critical roles of inflammatory caspases and their therapeutic values in delaying disease onset and progression of SjS.

4. Materials and Methods

Animals

C57BL/6J, C57BL/6.NOD-*Aec1Aec2* and NOD/LtJ were bred and maintained under SPF conditions within the Animal Care Services at the University of Florida, Gainesville. The animals were maintained on a 12 hr light-dark schedule and provided water and food *ad libitum*. For this study, female mice were euthanized at either 8 or 12 weeks of age. Both breeding and use of these animals were approved by the University of Florida IACUC. Euthanasia was carried out using AVMA-approved procedures.

Expression profiles of *caspase-11* and related molecules by RT-PCR

Total RNA was prepared from freshly isolated submandibular glands using the RNeasy Mini Kit (Qiagen, Valencia, CA). Semi-quantitative PCRs were carried out using 1 μg of cDNA as template. Following an initial denaturation at 94°C for 4 min, each PCR was carried out for 34 cycles consisting of 94°C for 30 sec, optimal annealing temperature for 30 sec and 72°C for 1 min. PCR products were analyzed by electrophoresis using 2% agarose gels. PCR band intensities were compared to β-actin using the Flourchem Imaging densitometer system (Alpha Innotech Corporation; San Leandro, CA). The primer sequences are:

β-Actin-forward: 5′-CCTGACCCTAAGGCCAACCG -3′ (398 bps; 57°C)

β-Actin-reverse: 5′-GCTCATAGCTCTTCTCCAGGG -3′

Stat1-forward: 5′-TCCCGTACAGATGTCCATGA-3′ (84 bps; 57°C)

Stat1-reverse: 5′-GCCTGATTAAATCTTTGGGCA-3′

Caspase-11-forward: 5′-ATGGCCGTACACGAAAGGCTCTTA-3′ (376 bps; 57°C)

Caspase-11-reverse: 5′-GCCTGCACAATGATGACTTTGGGT-3′

Nfkb1-forward: 5′-TGAAGCAGCTGACAGAAGACACGA-3′ (350bps; 56°C)

Nfkb1-reverse: 5′-TTCATCTATGTGCTGCCTCGTGGA-3′

Nfkb2-forward: 5′-AGTTGACTGTGGAGCTGAAGTGGA-3′ (336bps; 61°C)

Nfkb2-reverse: 5′-TGGCCTCGGAAGTTTCTTTGGGTA-3′

IL-1β-forward: 5′-CTCCATGAGCTTTGTACAAGG-3′ (245bps; 55°C)

IL-1β-reverse: 5′-TGCTGATGTACCAGTTGGGG-3′

IL-18-forward: 5′-ACTGTACAACCGCAGTAATACGG-3′ (434bps; 55°C)

IL-18-reverse: 5′-AGTGAACATTACAGATTTATCCC-3′

Analysis of STAT-1 and NF-kB transcriptional activity by electrophoretic mobility shift assay (EMSA)

The SMX and lacrimal glands from SjS-prone C57BL/6.NOD-*Aec1Aec2*, NOD/LtJ and disease resistant C57BL/6 mice at 8 weeks were analyzed by EMSA. Pooled glands (0.5 g) from each strain were used to obtain 5–10 μg/μl concentration of nuclear extract following the instructions in the Nuclear Extraction Kit (Panomics, Inc. Redwood City, CA). Nuclear

extracts were incubated with 2.0 μl of 5X Binding Buffer, 1.0 μl of Poly d (I-C) (1 μg/μl), 1.0 μl of biotin-labeled STAT-1 or NF-kB probe (10 ng/μl), and 5.0 μl of distilled water at 15–20°C for 30 min. For negative controls, unlabeled cold STAT-1 or NF-kB probe was added. Samples (5–10 μg/lane) were run on a 6.0% polyacrylamide gel at 4°C using 120V. After electrophoresis, the samples were transferred onto Pall Biodyne B® membranes (Pall Corporation, Ann Arbor, MI) by electroblotting (300 mA). The membrane was baked for 1 hr at 85°C in a dry oven for immobilization. Blocking, incubating with Streptavidin-HRP conjugate, washing, developing with hydrogen-peroxide and luminol, and membrane exposures on Hyperfilm™ ECL for 30 sec were performed following the manufacturer's instruction for the EMSA kit (Panomics, Inc., Redwood, CA).

Caspase activity assay

The SMX from disease prone C57BL/6.NOD-*Aec1Aec2*, NOD/LtJ and disease resistant C57BL/6 mice were analyzed at 8 weeks. From each strain, 1–1½ glands were used to acquire ~3–7 μg/μl concentration of gland lysate following manufacturer's instructions from BioVision (Mountain View, CA). Gland lysates (50 μg/well) were placed into a 96-well flat bottom plate and incubated in the dark with 50 μl of 2X Reaction Buffer (containing 10 mM DTT) and 5.0 μl of either 1 mM YVAD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin) substrate (50 μM final concentration) for caspase-1 or DEVD-AFC for caspase-3 followed by incubation at 37°C for 90 min. Negative controls consisted of the same reactions in the absence of gland lysate. After incubation, the samples were read in a microplate fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter. Experiments were performed in triplicate.

Measurement of apoptotic cells by TUNEL staining and co-localization with caspases

The SMX and lacrimal glands were freshly explanted and fixed in 10% neutral buffered formalin for additional processing. After deparaffinization, slides were placed for antigen-retrieval in 0.1M Citrate Buffer, pH 6.0 (Biogenex, San Ramon, CA), and microwaved (350W) for 6 min. Slides were washed twice in PBS and the tissues were stained following the instructions provided in the *In Situ* Cell Detection Kit, TMR Red (Roche, Indianapolis, IN). Slides were analyzed under a fluorescent microscope using an excitation wavelength in the range of 520–560 nm (maximum 580 nm, red) (Carl Zeiss Inc., Thornwood, NY).

For co-localization with caspase-11, separate sets of TUNEL stained slides were treated first with blocking buffer (Dako, Fort Collins, CO), stained for 1 hr with rabbit anti-mouse caspase-11 antibody (Calbiochem, San Diego, CA) diluted 1:50, and then developed for 45 min using a FITC-conjugated donkey anti-rabbit IgG antibody (Molecular Probes, OR) diluted 1:1000. Since this antibody for murine caspase-11 is cross-reactive with human caspase-4, slides with human breast cancer were used as positive controls following the manufacturer's guideline. For co-localization with caspase-3, TUNEL stained slides were stained with rabbit anti-mouse caspase-3 antibody (Abcam, Cambridge, MA) at 1:50 dilution for 1 hour, and then, incubated with FITC-conjugated donkey anti-rabbit IgG antibody (Molecular Probes, Oregon) at 1:1000 dilution for 45 min. Stained sections were mounted with 4′,6-diamidino-2-phenylindole (DAPI) mounting medium (Vector, Burlingame CA) and observed at 20x and 40x magnifications. Slides were analyzed under a

fluorescence microscope (Carl Zeiss Inc., Thornwood, NY) using an excitation wavelength in the range of 520–560 nm (maximum 580 nm, red) for TUNEL positive cells and 488nm range for caspase-11 or caspase-3 positive cells.

Identification of caspase-11 expressing cells

Slides of freshly explanted SMX from female C57BL/6.NOD-*Aec1Aec2* mice at 8 weeks of age were prepared for immunostaining as described above. Antigen retrieval was performed by incubating the slides in Trilogy (Cell Marque, Austin TX) for 30 min at 95°C. One set of slides were incubated first with rabbit anti-mouse caspase-11 antibody (Calbiochem, San Diego, CA), followed by FITC-conjugated donkey anti-rabbit IgG antibody, as described above. This set of slides were then counterstained with PE-conjugated hamster anti-mouse CD11c antibody (BD Biosciences, San Jose CA) at 1:100 dilution for 45 min. A second set of slides were first incubated with a solution containing both rabbit anti-mouse caspase-11 antibody and rat anti-mouse F4/80 antibody (Serotec, Raleigh, NC) at a 1:50 dilution for 1 hr, then a solution containing both FITC-conjugated donkey anti-rabbit IgG antibody (Molecular Probes, Oregon) at 1:1000 dilution and AffiniPure Texas Red conjugated rabbit anti-rat IgG antibody (Jackson ImmunoResearch, West Grove, PA) at 1:100 dilution and incubated for 45 min. Stained sections were mounted with DAPI mounting medium (Vector, Burlingame CA) and observed at 20x and 40x magnifications.

Measurement of mouse IL-18 by ELISA

Mouse sera and saliva from C57BL/6 and C57BL/6.NOD-*Aec1Aec2* strains (n=5) at 8 and 12 weeks of age were tested for mouse IL-18 with a sandwich ELISA kit (MBL International, Woburn, MA), following the manufacturer's instrunctions.

Detection of apoptotic HSG cells co-cultured with THP-1 cells

THP-1 human monocytes obtained from American Type Culture Collection (Manassas, VA) were cultured in RPMI 1640 medium with supplements. HSG cells were seeded at 5×10^5 cells per well in 6-well plates containing glass coverslips and cultured in complete media. The next day, the HSG culture media was removed from the cells and 5×10^5 THP-1 cells in 1 ml THP-1 growth media containing 2 μg/ml LPS (Sigma, St. Louis, MO) and 10 ng/ml IFN-γ (BD Biosciences, San Jose, CA) were added. The THP-cells were incubated for 48 hours at 37° C before removal. The HSG cells on coverslips were fixed in 4% paraformaldehyde for 1 hour and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate buffer for 2 minutes on ice. To detect apoptotic cells, the *In Situ* Cell Death Detection Kit, TMR red (Roche Applied Science, Indianapolis, IN) was used according to the manufacturer's protocol. Fluorescence images were taken with Zeiss Axiovert 200M microscope and a Zeiss AxioCam MRm camera using the 10x 0.75 NA objectives. Color images were assessed using Adobe Photoshop version 7. Cells were counted using Cell-Profiler image analysis software (24) to detect DAPI staining, and TUNEL positive cells were counted using a cell counter.

Caspase-1 siRNA transfection

siRNA targeting caspase-1 was transfected into THP-1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The siRNA used in this study was purchased from Ambion (Austin, TX) and dissolved in nuclease-free water, and the resulting 20μM stock was stored at -80°C prior to use. The sense and antisense strands are as follows: 5′-GGUUCGAUUUUCAUUUGAGtt-3′ and 5′-CUCAAAUGAAAAUCGAACCtt-3′. THP-1 cells transfected with siRNA targeting caspase-1 were incubated for 48 hours, washed once in growth media, and then co-cultured with HSG cells as described above.

Verification of caspase-1 knockdown by western blotting

THP-1 cells transfected with siRNA targeting caspase-1 were lysed 48 hours after transfection, and cell extracts were loaded onto a 10% SDS-PAGE gel and transferred to nitrocellusose. The following antibodies and dilutions were used: rabbit anti-caspase-1 antibodies at 1:50 (Abcam, Cambridge, MA) and rabbit anti-golgin-97 antibodies at 1:200 (25). Secondary goat anti-rabbit antibodies conjugated to horse radish peroxidase were used at 1:10,000 dilutions (Southern Biotech, Birmingham, AL). Densitometric analysis of the developed film was performed using Image J software. Caspase-1 protein levels were normalized to golgin-97 to determine knockdown efficiency.

Statistical analyses

Statistical significances were determined using the *Student's t* test. Values $p<0.05$ were considered to be significant.

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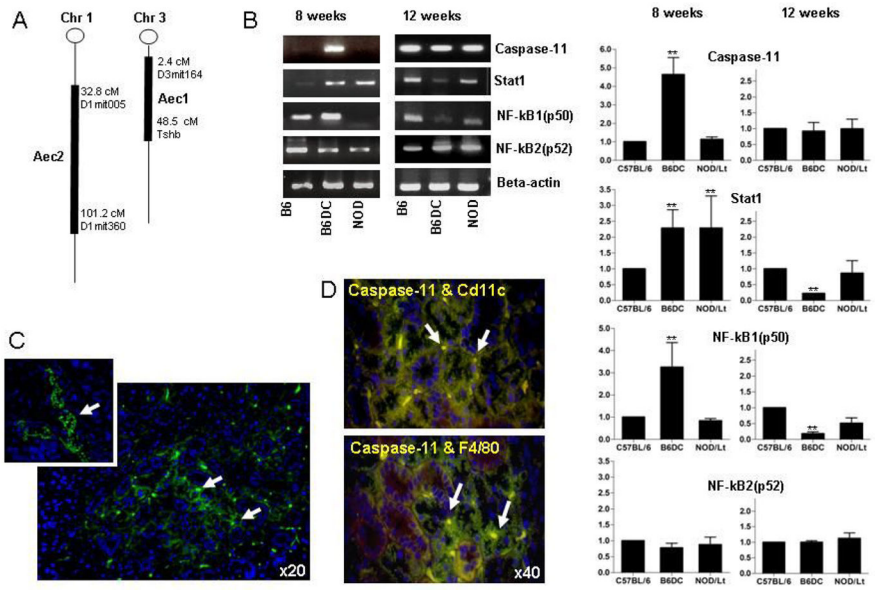

Figure 1. Increased caspase-11 expression in the SMX of the SjS-prone C57BL/6.NOD-*Aec1Aec*2 mouse prior to lymphocytic infiltration

A) Two NOD-derived genetic intervals, namely autoimmune exocrine loci (Aec) 1 and 2 on chromosomes 3 and 1, respectively, in the disease-prone-C57BL/6.NOD-*Aec1Aec*2 mouse are depicted.

B) Elevated caspase-11, its major transcription factors Stat-1, and Nfkb1 in the salivary glands of C57BL/6.NOD-*Aec1Aec*2 were confirmed by semi-quantitative RT-PCR.

C) Caspase-11 in the SMX was stained with FITC-labeled anti-mouse caspase-11 antibody in the C57BL/6.NOD-*Aec1Aec2* at 8 weeks. Arrows indicate positive staining for caspase-11. Magnification: 20x.

D) Double staining of caspase-11 with anti-Cd11c antibody (dendritic cells) and anti-F4/80 antibody (macrophages) revealed that cells positive for caspase-11 were also positive for both cell types. Arrows indicate double-stained cells, which are shown in yellow. Magnifications: 10x and 40x.

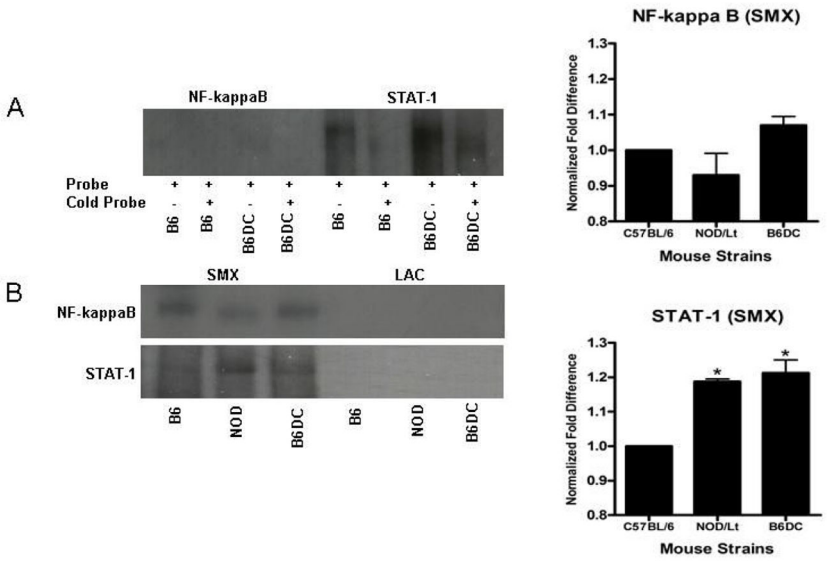

Figure 2. Concomitant increase in STAT-1 activity in the SMX of C57BL/6.NOD-*Aec1Aec2* at 8 weeks

A) Increased STAT-1 activity was detected by EMSA in the glands isolated at 8 weeks. Inhibition of binding is shown with a cold probe (unlabelled probe) incubation. The right panel indicates bar graphs generated by densitometer analyses on EMSA results for NF-kappaB.

B) Absence of STAT-1 and NF-kappaB activity in the LAC at 8 weeks was detected while elevated STAT-1 was shown in the SMX of C57BL/6.NOD-*Aec1Aec2* and NOD/LtJ mouse. Increased amount of nuclear extract (ten micrograms) isolated from 0.5 gram of pooled glands (n=5–7 mice) was used per lane to enhance binding activity for NF-kappa B. The right panel indicates bar graphs generated by densitometer analyses on EMSA results for STAT-1. The experiments were carried out 3 times per molecule of interest. *$p<0.05$ in comparison with C57BL/6.

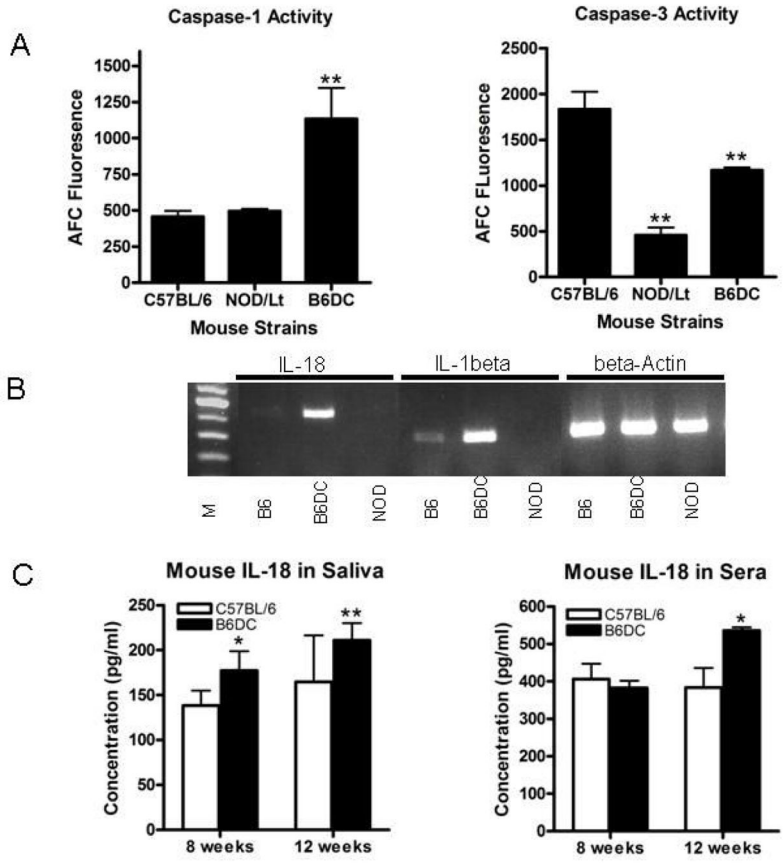

Figure 3. Activation of caspase-1-mediated pathway in C57BL/6.NOD-*Aec1Aec2* prior to disease onset

A) Caspase-1 activity was up-regulated significantly in the SMX of C57BL/6.NOD-*Aec1Aec2* at 8 weeks. Experiments were performed in triplicate. **p<0.01 in comparison with C57BL/6.

B) Genes downstream of caspase-1 such as IL-1β and IL-18 in the C57BL/6.NOD-*Aec1Aec2* mouse at 8 weeks were analyzed by RT-PCR. The C57BL/6.NOD-*Aec1Aec2* mice were positive for these genes while the SMX from other mouse strains showed either negative or weak expression (n=5–7 female mice). Beta-actin was used as a control for normalization.

C) IL-18 protein expression was elevated in the saliva from C57BL/6.NOD-*Aec1Aec2* at 8 weeks by ELISA. Pooled saliva and sera were used for ELISA (n=5 female mice). *p<0.05 and **p<0.01 in comparison with the age-matched C57BL/6 mouse.

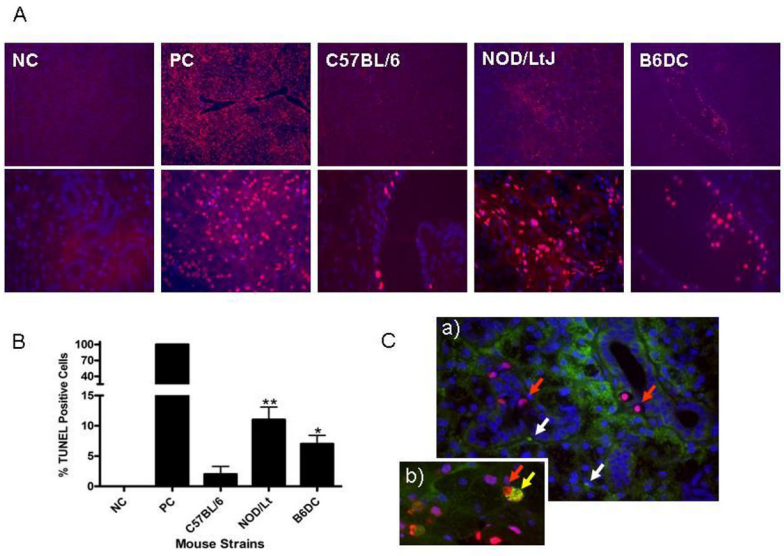

Figure 4. Increased epithelial cell death in the glands of disease-prone mice at 8 weeks and lack of direct co-localization of caspase-11 with TUNEL positive cells

A) TUNEL staining was performed on the pre-diseased salivary glands. NC, negative control; PC, positive control treated with nuclease; upper panel at 10x and lower panel at 40x magnifications.

B) Percentages of TUNEL positive cells are shown as a bar graph. For each mouse, 3 slides were evaluated for TUNEL positive cells, which were counted using a cell counter.

C) Caspase-3 positive cells (yellow arrows in b) were co-localized with TUNEL positive cells (red arrows). White arrows indicate caspase-11 positive cell. Magnification: 40x.

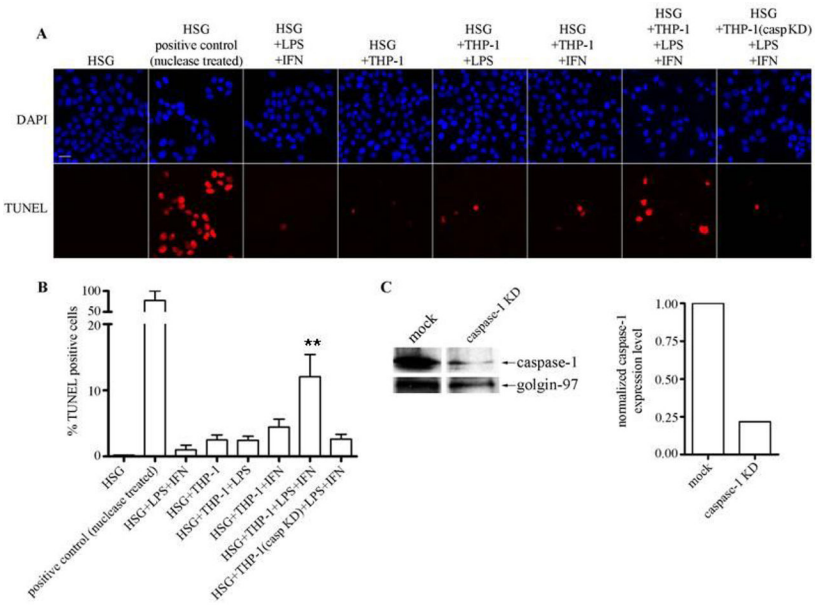

Figure 5. Inhibition of apoptotic cell death of HSG cells by caspase-1 knockdown in THP-1 cells

A) HSG cells were cultured in the absence or presence of LPS-and/or IFN-γ-stimulated THP-1 cells. After removing culture media or stimulated THP-1 cells from the culture, apoptotic HSG cells were analyzed by TUNEL assays. For the caspase-1 inhibition study, siRNA to caspase-1 was transfected into THP-1 cells prior to stimulation with LPS and IFN-γ and co-cultured with HSG cells.

B) Percent TUNEL positive cells were presented as a bar graph. The experiment was repeated 3 times for reproducibility (**$p<0.01$).

C) Knockdown efficiency of caspase-1 was compared with a housekeeping protein golgin-97 by western blotting and depicted as a bar graph. The experiment was repeated twice and caspase-1 protein level was normalized to golgin-97.

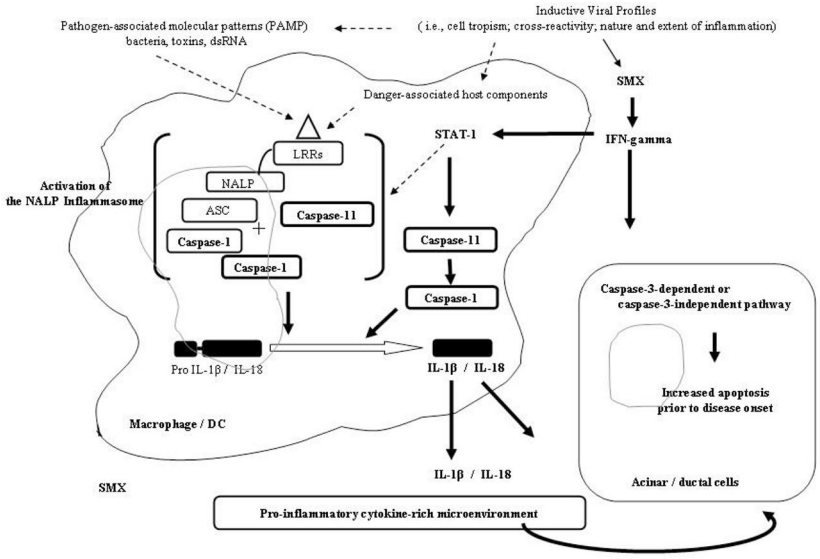

Figure 6. Schematic representation of the current working hypothesis

Inductive viral profile results in alterations in the target tissue via the activation of IFN-STAT and a subsequent induction of caspase-11. Up-regulated caspase-1 activity produces mature IL-1β and IL-18 from macrophages and dendritic cells, which may play a role in the activation of caspase-3 or induction of caspase-3-independent apoptotic factors in neighboring acinar and/or ductal cells. Elevated pro-inflammatory cytokines in the SMX enhances IFN- γ production by epithelial cells, resulting in further activation of macrophages. Areas where further investigation is needed for confirmation are depicted with dotted lines (i.e., questions as to if caspase-11 is a constituent of the NALP inflammasome or why IFN- γ and consequently STAT-1 are up-regulated in the SMX prior to disease onset). The assembled proteins (the inflammasome) in response to signal recognition by LRR of the NALP leads to the activation of caspase-1, depicted in the bracket. Our current findings are depicted in bold arrows. NALP, *NACHT, LRR and PYD containing protein*; LRR, leucine-rich repeats; ASC, apoptosis–associated speck-like protein containing a caspase-activating recruitment domain; STAT-1, Signal Transducers and Activators of Transcription; DC, Dendritic Cells; SMX, submandibular glands.