IDENTIFICATION OF A MOLECULAR PATHWAY INVOLVING CASPASE-11 AND CASPASE-1 IN SJÖGREN'S SYNDROME

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTERS OF SCIENCE

UNIVERSITY OF FLORIDA

2009

To my grandparents, Antonio Madamba Tangonan and Martina Robles Tangonan, for their ever-enduring strength, perseverance and passion for having better lives for their children and future generations

ACKNOWLEDGMENTS

First and foremost I would like to acknowledge God, the ever forgiving and merciful Father. He has created and written this Book of Life for me that went unnoticed in the past, but I have now realized that this master's degree is merely one of the chapters in my book where I would gain so much knowledge.

I would also like to thank my parents, Hilario and Caridad Bulosan, for guiding me to focus my studies on science. Along with their constant support, they have taught me that you reap what you sow, and that doing your best will always benefit in the end. I would also like to show appreciation to my sister, Ate Marilyn, and the Horvit family for always having a home to go to for weekends and holidays.

My friends have played an integral part in boosting my morale in times of struggle, so I would like to thank my V's, Teen, Brudder, Kevtin, Rachel, Ading Veejay, and wonderful brothers and sisters from Centerpoint Christian Fellowship and First Assembly of God for their constant outpour of love not only for each and every day of this process but for the entire time I have known each individual.

I would also like to thank all of the members of the Peck and Cha labs for helping me develop my skills and personality in these past four years of service in both labs. I could always count on Dr. Nguyen for tongue-in-cheek banter, Dr. Peck for the elaborate stories about his many travels, and Ms. Janet Cornelius for her meticulous lab managerial skills that I have tried my best to duplicate for the Cha lab. I appreciate my colleague, Kaleb, for being a precious asset our lab, speeding up our productivity, and contributing her knowledge to each and every troubleshooting experience that all of our lab members had experienced. I would also like to thank Ading Huy for enduring statistics class with me and having "sanity check sessions" over ropa vieja sandwiches at our favorite post-class Latin cafe.

Last but not least, I would like to thank my mentor, Seunghee Cha for even making this educational opportunity possible. She not only possesses the title of Principal Investigator and mentor, but also my boss, guide, role model, leader, friend and surrogate mother. I appreciated her stern words when I was slow in completing my tasks as well as her encouragement not only when experiments faltered, but also for life in general. I recognize the value of this opportunity and only hope that I have reached expectations as her "first" graduate student.

I would also like to thank anyone who I failed to mention specifically or non-specifically, as it was a pleasure to receive a "Hello, how are you?" or a kind smile to get me through the window-less workdays in the lab.

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December 2009

Chair: Seunghee Cha
Major: Medical Sciences

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* (B6DC) mice showed altered glandular homeostasis in the submandibular glands (SMX) at 8 weeks prior to disease onset and suggested potential involvement of the inflammatory caspases, caspase-11 and caspase-1. To determine if inflammatory caspases are critical for the increased epithelial cell death prior to SjS-like disease, this study investigated molecular events involving this caspase-11/caspase-1 axis. 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. Further investigation of upstream stimuli suggest that HSV-1 CpG-DNA stimulated ASC, a key adaptor molecule in the inflammasome, through TLR-9. This was further confirmed by TLR9- siRNA

transfection where knockdown of TLR-9 abolished the induction of ASC. Taken together, this study discovered that inflammatory caspases are essential in promoting a pro-inflammatory microenvironment and influencing increased epithelial cell death in the target of tissues in SjS before disease onset. In addition, Herpes CpG-DNA activation of TLR-9 can induce ASC, suggesting that viral DNA may play a role in triggering an inflammatory process through the inflammasome.

CHAPTER 1
INTRODUCTION

Sjögren's Syndrome (SjS) and the SjS Mouse Model

Sjögren's syndrome (SjS) is a chronic autoimmune disease that targets the exocrine glands. It affects mainly the salivary and lacrimal glands, resulting in dry mouth and/or dry eye conditions as well as subsequent complications in patients as a consequence of autoimmune responses to self-antigens. Interestingly, epithelial cells in the target tissue are actively involved in the immune process by up-regulating co-stimulatory molecules and MHC class II molecules(1). Although involvement of a precise MHC locus has been suggested, SjS has shown weak familiarity, opposed to Type I diabetes. This highlights the importance of environmental factors such as viral infection in the initiation of the disease. Despite extensive research 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. Therefore, mechanisms are poorly defined due to lack of information on SjS pathogenesis during the pre-disease phazes of SjS. To overcome this problem, investigations have turned to mouse models.

The Developed C57BL/6.NOD-*Aec1Aec2* (B6DC) Mouse Strain

Our earlier studies on susceptibility of genes for development of SjS-like disease suggested that two genetic intervals control physiological deviations and immunological responses. Idd5 (insulin dependent diabetes) on chromosome 1 controls physiological aberrations while Idd3 locus on chromosome 3 controls the immunological responses (2). In addition, when these intervals were placed into disease-free C57BL/6 mice, this was sufficient to permit complete development of SjS-like disease phenotype without developing Type I diabetes that is seen in the parental NOD mouse (3). The developed strain has been designated C57BL/6.NOD-*Aec1Aec2*

(B6DC) and has been greatly beneficial to generate congenic, transgenic, or knockout strains with other mouse strains in part due to the wild-type C57BL/6 background.

Our research examining the salivary glands of B6DC indicate the presence of multiple alterations in glandular homeostasis even in the absence of infiltrating immune cells and before onset of clinical disease (4-6). These changes include altered cell proliferation at the time of birth, upregulated apoptosis of acinar tissues, proteolysis of secreted proteins such as sPLUNC and increased expression of interferon-gamma (IFNg).

Caspase-11 as a Differentially Expressed Gene in B6DC Mouse Model During Pre-Disease Stage

In an initial attempt to understand the underlying molecular mechanisms for altered tissue homeostasis before disease onset in the NOD mouse model, experiments previously compared the differential gene expression profiles in the submandibular glands (SMX) of 8- and 12-week-old B6DC, with those of C57BL/6 mice (7). Use of B6DC, in place of the NOD mouse, permitted elimination of strain-associated genes, and rather focused on collection of disease-associated genes as the congenic strain has the same genetic background as C57BL/6. Therefore, the B6DC mouse strain enabled us to therefore investigate early pathogenesis of SjS without potential complications of Type 1 diabetes occurring in the NOD mouse strain. Hybridiations utilizing cDNA probes from respective submandibular glands revealed 75 differentially expressed genes involving fundamental cellular activities such as transcription, translation, DNA replication, and protein folding. Microarray analysis revealed that among the apoptosis-related genes present in the microarrays, caspase-11 was significantly upregulated at 8 weeks of age in C57BL/6.NOD-*Aec1Aec2* mice, a time when increased apoptosis is seen in the salivary glands (indicative of chronic pro-inflammatory stimuli in the salivary glands of SjS). However, no significant differences were observed in either Bcl2 family genes or caspase-9 (7). Interestingly,

what is known is that caspase-11 is expressed only under pathologic conditions, such as endotoxic shock, multiple sclerosis and brain ischemia.

Inflammatory Caspases

Recent studies imply that a subset of caspases is involved in the proteolytic maturation of inflammatory cytokines rather than causing apoptosis. Such caspases (human caspase-1, -4, and -5 and murine caspase-11 and -12) were designated “inflammatory caspases” due to their main function of regulating inflammatory processes. Of note, these caspases also contain a CARD (caspase-recruitment domain) at their N-terminus (8).

Caspase-1 was initially identified as a result of attempts to purify the enzyme responsible for cleaving pro-interleukin-1beta (pro-IL-1beta) (9) for its secretion. Additionally, caspase-1 activates interleukin-18 (IL-18), also referred to as interferon-gamma-inducing factor. Although caspase-1-deficient mice have insufficiency in maturation of proinflammatory cytokines, they show no evidence of a defect in apoptosis (10). Nevertheless, caspase-1 appears to engage neuronal cell death (11), potentially by secreting pro-inflammatory cytokines rather than killing cells in a cell-autonomous manner.

Murine caspase-11 is a poorly characterized inflammatory caspase that is believed to be a dual activator of caspase-1 and/or caspase-3 (12). Caspase-11 is not expressed under normal conditions but is produced in many types of cells in response to cytotoxicity. Caspase-3 is an executionary caspase for apoptotic cell death (apoptotic caspase), whereas 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 interleukin (IL)-1beta and IL-18 in response to lipopolysaccharide (LPS) (13); thus, it is not surprising that mice deficient in caspase-11 exhibit a phenotype very similar to caspase-1 gene-knockout mice. In addition, embryonic fibroblasts derived from caspase-11 gene-knockout

mice are resistant to apoptosis induced by ectopic expression of caspase-1, suggesting that caspase-11 is an upstream activator of caspase-1 (13). Beyaert et al. (14) reported that binding of nuclear factor-B (NF-B) and signal transducers and activators of transcription-1 (STAT-1) to the cloned fragments of 5'-flanking promoter regions of caspase-11 was necessary, respectively, for LPS- and IFN-gamma-inducible expression of caspase-11 in macrophages.

Although the human orthologue of caspase-11 has not yet been identified because of insufficient information about its 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 (14, 15). To better understand the potential role of upregulated caspase-11 in the salivary glands of B6DC mice before onset of SjS-like disease and to investigate whether 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 producing a pathologic microenvironment that mediates autoimmunity.

Inflammasome and ASC

The Inflammasome (Activation Scaffolds of Proinflammatory Caspases)

In 2002, Jurg Tschopp and associates in Switzerland identified and designated a complex of proteins, known as the “inflammasome”, that have distinct roles in the innate defense system by regulating inflammatory caspase-1 activation (16). It has been determined that members of the NALP family of proteins are the main components of the inflammasome although details on the precise expression or associative bindings are unidentified. NALPs exhibit a tripartite PYD-NACHT-LRR structure, and bind to ASC (apoptosis associated speck-like protein containing a caspase-activating domain (CARD). Such binding causes activation of caspase-1 upon over-

expression. Stimulation of the inflammasome triggers a sequence of internal reactions that ultimately activates proteolytic processing of pro-IL1beta and IL-18 into mature IL-1beta and IL-18, respectively. The inflammasome is suggested to act as early sensory machinery to detect danger signals within the cell as well as triggering the host defense system. However, events that trigger the assembly of the inflammasome are still mostly unknown (17).

ASC

ASC is a major adapter protein involved in cleavage of pro-inflammatory cytokines. This cytoplasmic adapter protein is composed of an N-terminal pyrin-paad-dapin domain (PYD) and a C-terminal CARD domain. ASC links Ipaf (ICE-protease activating factor, a.k.a CARD12 or CLAN), a member of Apaf-1-like proteins, to signal-transduction pathways leading to cell death and nuclear factor-kappa B (NF-kB) activation (8). Ipaf-1 enables ASC to recruit NALP3 and Caspase-1 through a CARD-CARD association, however to date, the activation mechanism remains unclear. Ipaf inflammasome molecules include NALP1, NALP3 (cryopyrin), pyrin, and ASC. The inflammasome pathway can be triggered by stimuli such as infection by bacteria or activation by Toll-like receptor agonists such as viral DNA (18). Once activated, the inflammasome causes pyroptosis, an inflammatory form of cell death as a result of activation of caspase-1. Such cell death is caused by oligomerization of ASC adapter protein and low intracellular ionic strength and potassium depletion. One can thus refer to ASC as a pyroptosome (18) since a mature ASC pyroptosome contains both oligomerized ASC dimers and caspase-1, found downstream of the inflammasome. Pyrin is a direct activator of the ASC pyroptosome in innate immunity (19). ASC in particular participates in the immune response by regulating inflammation in response to bacterial and viral pathogens. Under normal conditions, ASC is localized in the cytoplasm. Under apoptotic conditions, ASC condenses to form ball-like aggregates or speck-like structures near the nuclear periphery (20).

Activators of Inflammasome or ASC

CpG-rich DNA from bacteria and viruses stimulate dendritic cells and macrophages to release increased amounts of interferon-gamma (IFN-gamma, Type 1) via activation of the Toll-like receptor 9 (TLR9) pathway (21). CpG-rich DNA also causes an anti-viral response which is dependent on a strong pro-inflammatory component controlled by TNF and IL-1beta (22). Immune-stimulating activity requires short oligonucleotide sequences with unmethylated CpG dinucleotides, which are normally seen at lower frequency in mammalian DNA (23, 24). CpG induces activation of antigen presenting cells (APC) and T-cell proliferation as well as polylonal antibody production in B cells.

Lipopolysaccharide (LPS) is a gram negative, enterobacterial product and a potent activator of mouse macrophages (25). It acts through Toll-like receptor 4 (TLR-4) signaling. TLR 4 is a transmembrane protein that shares a high degree of homology with TLR-9 (26).

In a study conducted by Gao, tumor necrosis factor-alpha, caspase-11, IL-18 and IL-1beta were induced in mouse macrophages by CpG-DNA in a manner similar to or greater than LPS. In comparison to LPS as an activator of the immune response, CpG-DNA inducible genes solely contain a subset of LPS-stimulated genes, which suggest that CpG-DNA employ only a subset of LPS-induced signaling pathways. LPS activates additional signaling pathway(s) not engaged by CpG-DNA (TLR-9) signaling (25). Therefore, Gao concluded that in the presence of CpG-DNA or LPS, macrophages respond rapidly by modifying expression of genes that encode factors responsible for controlling activity in the innate immune system. CpG-DNA upregulates expression of MHC Class-II and respective co-stimulatory molecules, induces production by macrophages and dendritic cells, as well as promotes polyclonal activation of B cells (27-29).

CpG motifs were significantly suppressed in gammaherpesviruses, Epstien-Barr virus (EBV) and herpesvirus saimiri (HVS) compared to alphaherpesvirus (i.e. herpes simplex virus),

in which CpG motifs were relatively enriched. Herpes simplex-1 virus (HSV-1) is a member of the Herpesviridae family (30). HSV-1 infection causes potent caspase-1 activation and pro-IL-1beta maturation as DNA viruses in general activate an IL-1beta-based inflammatory response (17). Lundberg conducted experiments that studied HSV and herpes stromal keratitis (HSK). Lundberg concluded that HSV infected neurons latently in the eye, leading to HSK disease. It is also important to note the intrinsic immunostimulatory ability of HSV-DNA and the tendency for HSV to persist and establish latency in a variety of tissues.

HSV CpG-DNA can activate macrophages to produce effector molecules that are normally induced following encounter with bacterial or viral pathogens or components derived from them. CpG dinucleotides are to a great extent undermethylated in HSV-1 DNA purified from virions. Macrophages and dendritic cells phagocytose dead cells that would activate these cells by their cargo of immunostimulatory HSV-1 DNA to produce proinflammatory cytokines (IL-12, IFN-gamma, IL-6, ad TNF) and chemokines (31). Since macrophages and dendritic cells are fully competent to present antigen, they might orchestrate an autoimmune attack on tissue by presenting cryptic antigens exposed as a consequence of cellular damage to immune cells that escaped thymic or peripheral deletion (32). This may give rise to interesting and novel mechanisms by which HSV-1 and other viruses might contribute to or exacerbate autoimmune diseases such as Sjogren's syndrome.

TLR-9 Activation in Response to Viral CpG

Toll-like receptors regulate activation of the immune system after recognizing pathogens either intracellularly or extracellularly. Toll-like receptor 9 (TLR-9) is a DNA receptor protein for bacterial and viral CpG-DNA released from microbes (33) to deliver intracellular signals (34-37). Expression of TLR-9 is therefore found in endosomal vesicles rather than on the cell surface. Instances such as tissue injury, inflammation or insufficient clearance of self-machinery

can cause higher levels of circulating self-antigens that may stimulate the TLR-9/MyD88-mediated signaling pathway (38, 39).

TLR-9 is located in the endoplasmic reticulum (ER) of resting macrophages and dendritic cells. Latz *et al* (33) reported that upon internalization of CpG-DNA into the subcellular compartment, the TLR-9 receptor is translocated to the same compartment. With CpG-DNA internalization, TLR-9 redistribution occurs, as a portion of the receptor protein travels first to early endosomes and progressively into the tubular lysosomal area. This TLR-9 movement to CpG-rich locations enablesCpG-TLR-9 binding and subsequent signal transduction initiation (33). As a consequence, CpG-DNA activates MAP-kinases that trigger kinase activation that in turn, activate transcription factors NF-kappaB and AP-1. The initiation of these transcription factors leads to enhanced expression of the genes that control immune cells (40). Unmethylated CpG motifs are another molecular mechanism to trigger the innate immune response via the TLR-9-mediated pathway (41, 42).

Importance of Inflammatory Components in SjS

Originally described as IFN-gamma inducing factor, IL-18 is an effective inflammatory stimulant produced by macrophages and dendritic cells (10). IL-18 protein is strongly expressed in periductal mononuclear cells that infiltrate the salivary glands of all SjS patients. Additionally, there are higher levels of IL-18 cytokine detected in patients with primary SjS (43). IL-1beta is one of the important mediators of the body's response to microbial invasion, inflammation, immunological reactions, and tissue injury (44). This same cytokine is also known to be upregulated in sera and inflamed salivary glands of SjS patients (45).

Such findings lead to the importance of analyzing inflammatory components at more refined levels, utilizing *in vivo* and *in vitro* methods to support a working hypothesis of an inductive stimulus triggering IFN-gamma to transcriptionally activate the STAT pathway. This

in turn results in the induction of caspase-11, which activates the caspase-1 pathway to cleave the pro-forms of IL-1beta and IL-18 into mature form to be secreted from the macrophages and dendritic cells. Such a mechanism would initiate inflammation and apoptosis of epithelial cells. Whether caspase-11 activated caspase-1 is part of inflammasome is not known to date. Therefore, to investigate our hypothesis, three specific aims are proposed.

The first specific aim is to identify molecules involved in elevated caspase-1 activation in the salivary gland macrophages of the SjS-prone mouse prior to disease onset. I hypothesize that inflammatory caspases are important in promoting a pro-inflammatory rich microenvironment in the target tissues of SjS prior to disease onset. This question will compare expression levels of inflammatory molecules between B6DC and wild-type B6 mice sera, saliva and tissue samples.

For the second specific aim, it is necessary to analyze the environment of salivary glands *in vitro* by investigating an inductive viral signal such as Herpes Simplex Virus Type 1 (which commonly infects salivary glands) to question whether CpG oligonucleotide (CPG-ODN) viral motif can play a role in the activation of acaspase-1-mediated pathway in macrophages. Focus is placed on the effects of CpG-ODN on inducting inflammation and/or inflammasome components *in vitro* hypothesizing that persistent presence of viral DNA leads to induction of proteins involved in caspase-1-mediated pathways of macrophages. The mouse macrophage RAW 264.7 cell line will be used to analyze cytokine levels of stimulated versus non-stimulated cell conditions.

Finally, the third specific aim questions whether the TLR9 pathway is essential for CpG-mediated ASC expression with subsequent inflammasome activation. I hypothesize that inhibition of the TLR-9 pathway will reduce inflammasome activation in salivary glands, thus preventing disease onset.

All together, these specific aims will attempt to further identify the role of environmental factors in conjunction with the onset of disease in patients with Sjogren's syndrome. By shedding light on potential mechanisms that lead to inflammation of epithelial cells in the salivary glands, such knowledge should expedite the ongoing research needed to alleviate or optimistically prevent autoimmune disease.

CHAPTER 2
MATERIALS AND METHODS

Animals

C57BL/6J (B6), C57BL/6.NOD-*Aec1Aec2* (B6DC) and NOD/ShiLtJ (NOD) (n=35 total) 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-hour light-dark schedule and provided water and food ad libitum. For this study, female mice were killed at either 8 or 12 weeks of age. Both breeding and use of these animals were approved by the University of Florida IACUC. The mice were killed using American Veterinary Medical Association approved procedures.

Expression Profiles of *Caspase-11* and Related Molecules by Semi-Quantitative-PCR

Total RNA was prepared from freshly isolated SMX using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Semiquantitative PCRs were carried out using 1 microgram (ug) of cDNA as the template. Following an initial denaturation at 94 ℃ for 4 min, each PCR was carried out for 34 cycles consisting of 94 ℃ for 30 seconds, optimal annealing temperature for 30 seconds and 72 ℃ for 1 minute. PCR products were analyzed by electrophoresis using 2% agarose gels. PCR band intensities were compared with beta-actin using the Flourchem Imaging densitometer system (Alpha Innotech Corporation; San Leandro, CA, USA). The primer sequences can be found in Table 2-1.

Table 2-1. List of Semi-quantitative PCR (RT-PCR) designed for *Mus musculus* genes.

Gene	Fragment size & Annealing Temperature	Primer Sequence
β-actin-forward	398 bp, 57˚C	5′-CCTGACCCTAAGGCCAACCG-3′
β-actin-reverse		5′-GCTCATAGCTCTTCTCCAGGG-3′
STAT1-forward	84 bp, 57˚C	5'-TCCCGTACAGATGTCCATGA-3'
STAT1-reverse		5'-GCCTGATTAAATCTTTGGGCA-3'
Caspase-11-forward	376 bp, 57˚C	5'-ATGGCCGTACACGAAAGGCTCTTA-3'
Caspase-11-reverse		5'-GCCTGCACAATGATGACTTTGGGT-3'
NF-κB1-forward	350 bp, 56˚C	5'-TGAAGCAGCTGACAGAAGACACGA-3'
NF-κB1-reverse		5'-TTCATCTATGTGCTGCCTCGTGGA-3'
NF-κB2-forward	336 bp, 61˚C	5'-AGTTGACTGTGGAGCTGAAGTGGA-3'
NF-κB2-reverse		5'-TGGCCTCGGAAGTTTCTTTGGGTA-3'
IL-1β-forward	245 bp, 55˚C	5'-CTCCATGAGCTTTGTACAAGG-3'
IL-1β-reverse		5'-TGCTGATGTACCAGTTGGGG-3'
IL-18-forward	434 bp, 55˚C	5'-ACTGTACAACCGCAGTAATACGG-3'
IL-18-reverse		5'-AGTGAACATTACAGATTTATCCC-3'

Analysis of STAT-1 and NF-κB Transcriptional Activity by Electrophoretic Mobility Shift Assay (EMSA)

The SMX and lacrimal glands from SjS-prone B6DC, NOD and disease-resistant B6 mice at 8 weeks were analyzed by EMSA. Pooled glands (0.5 ug) from each strain were used to obtain 5–10 micrograms per microliter (ug/ul) concentration of nuclear extract following the instructions in the Nuclear Extraction Kit (Panomics, Inc., Redwood City, CA, USA). Nuclear extracts were incubated with 2.0 ul of 5X binding buffer, 1.0 l of poly d (I-C) (1 ug/ul), 1.0 ul of biotin-labeled STAT-1 or NF-B probe (10 ng/ul) and 5.0 ul of distilled water at 15–20 ℃ for 30 min. For negative controls, an unlabeled cold STAT-1 or NF-B probe was added. Samples (5–10 ug per lane) were run on a 6.0% polyacrylamide gel at 4 ℃ using 120 V. After electrophoresis, the samples were transferred onto Pall Biodyne B membranes (Pall Corporation, Ann Arbor, MI, USA) by electroblotting (300 mA). The membrane was baked for 1 hour at 85 ℃ 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 seconds were performed following the manufacturer's instruction for the EMSA kit (Panomics, Inc., Redwood, CA).

Caspase Activity Assay

The SMX from disease-prone B6DC, NOD and disease-resistant B6 mice were analyzed at 8 weeks. From each strain, 1–1½ glands were used to acquire 3–7 ug/ul concentration of gland lysate following manufacturer's instructions from BioVision (Mountain View, CA, USA). Gland lysates (50 ug per well) were placed into a 96-well flat bottom plate and incubated in the dark with 50 ul of 2X reaction buffer (containing 10 mm dithiothreitol) and 5.0 ul of either 1 mm YVAD-AFC (7-amino-4-trifluoromethyl coumarin) substrate (50 um final concentration) for caspase-1 or DEVD-AFC for caspase-3 followed by incubation at 37 °C for 90 minutes. 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 Colocalization 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 0.1 m citrate buffer, pH 6.0 (Biogenex, San Ramon, CA, USA), and microwaved (350 W) for 6 min. Slides were washed twice in phosphate-buffered saline and the tissues were stained following the instructions provided in the in situ Cell Detection Kit, TMR Red (Roche Applied Science, Indianapolis, IN, USA). 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, USA).

For colocalization with caspase-11, separate sets of TUNEL-stained slides were treated first with blocking buffer (Dako, Fort Collins, CO, USA), stained for 1 hour with rabbit anti-

mouse caspase-11 antibody (Calbiochem, San Diego, CA, USA) diluted 1:50, and then developed for 45 minutes using a FITC-conjugated donkey anti-rabbit IgG antibody (Molecular Probes, OR, USA) diluted 1:1000. As 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 colocalization with caspase-3, TUNEL-stained slides were stained with rabbit anti-mouse caspase-3 antibody (Abcam, Cambridge, MA, USA) at 1:50 dilution for 1 hour, and then, incubated with FITC-conjugated donkey anti-rabbit IgG antibody (Molecular Probes) at 1:1000 dilution for 45 minutes. Stained sections were mounted with 4',6-diamidino-2-phenylindole mounting medium (Vector, Burlingame, CA, USA) and observed at 20x and 40x magnifications. Slides were analyzed under a fluorescence microscope (Carl Zeiss) using an excitation wavelength in the range of 520–560 nm (maximum 580 nm, red) for TUNEL-positive cells and 488 nm range for caspase-11- or caspase-3-positive cells.

Identification of Caspase-11 Expressing Cells

Slides of freshly explanted SMX from female B6DC 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, USA) for 30 minutes at 95 ℃. One set of slides was 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 was then counterstained with PE-conjugated hamster anti-mouse CD11c antibody (BD Biosciences, San Jose, CA, USA) at 1:100 dilution for 45 minutes. A second set of slides was first incubated with a solution containing both rabbit anti-mouse caspase-11 antibody and rat anti-mouse F4/80 antibody (Serotec, Raleigh, NC, USA) at 1:50 dilution for 1 hour, then a solution containing both FITC-conjugated donkey anti-rabbit IgG antibody (Molecular Probes) at 1:1000 dilution and AffiniPure Texas Red conjugated rabbit anti-rat IgG antibody (Jackson

ImmunoResearch, West Grove, PA, USA) at 1:100 dilution and incubated for 45 minutes. Stained sections were mounted with 4',6-diamidino-2-phenylindole mounting medium (Vector, Burlingame, CA) and observed at 20x and 40x magnifications.

Measurement of IL-18 by Enzyme-Linked Immunosorbent Assay (ELISA)

Mouse sera and saliva from B6 and B6DC 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, USA), following the manufacturer's instructions. RAW 264.7 cell supernatants were also tested for mouse IL-18, while THP-1 cell supernatants were tested from human IL-18 using the Human-IL-18 sandwich kit from MBL International.

Cell Culture of HSG, THP-1 and RAW 264.7 Cell Lines

Human salivary gland cells (HSG) and murine RAW264.7 macrophages were maintained in DMEM media with 4.5g/l glucose and L-glutamine without sodium pyruvate (Meditatech, Manassas, VA). Media is supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Mediatech, Manassas, VA), penicillin at 100 U/ml, and streptomycin at 100 ug/ml (Mediatech, Herndon, CA).

Human monocytic cells (THP-1) grown in suspension were maintained in RPMI media (500 ml) supplemented with 10% FBS (50 ml), 1 mM sodium pyruvate (5 ml), Hepes buffer (5 ml), sodium pyruvate (5 ml), 45% glucose (3 ml), beta-mercaptoethanol (2 ul), and penicillin/streptomycin (5 ml) (Mediatech, Manassas, CA).

All cells are maintained in a humidified chamber at 37 °C with 5% carbon dioxide. All cell culture flasks, cell scrapers and serological pipette supplies were purchased from Fisher Scientific (Fisher, Savannah, GA).

Detection of Apoptotic HSG Cells Cocultured with THP-1 Cells

THP-1 human monocytes obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in RPMI 1640 medium with supplements. HSG cells were seeded at 5 x 105 cells per well in 6-well plates containing glass coverslips and cultured in complete media. The next day, HSG culture media were removed from the cells and 5 x 105 THP-1 cells in 1 ml THP-1 growth media containing 2 ul LPS (Sigma, St Louis, MO, USA) and 10 ul IFN-gamma (BD Biosciences) were added. The THP cells were incubated for 48 hours at 37 °C before removal. 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) was used according to the manufacturer's protocol. Fluorescence images were taken with Zeiss Axiovert 200 M microscope and a Zeiss AxioCam MRm camera using the 10 x 0.75 NA objectives. Color images were assessed using Adobe Photoshop version 7. Cells were counted using Cell-Profiler image analysis software (46) to detect 4',6-diamidino-2-phenylindole staining, and TUNEL-positive cells were counted using a cell counter.

siRNA Transfection

siRNAs targeting caspase-1 and TLR-9 were transfected into THP-1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The siRNA used for caspase-1 was purchased from Ambion (Austin, TX, USA), while siRNA used for THP-1 was purchased from Thermo Scientific (Dharmacon, Lafayette, CO) and dissolved in nuclease-free water, and the resulting 20 um stock was stored at -80 °C before use. The sense and antisense strands for caspase-1, respectively, are as follows: 5'-GGUUCGAUUUUCAUUUGAGtt-3' and 5'-CUCAAAUGAAAAUCGAACCtt-3'.

The target sequence for TLR-9 is CAGACUGGGUGUACAACGA.

THP-1 cells transfected with caspase-1 siRNA were incubated for 48 h, washed once in growth media, and then cocultured with HSG cells as described above. In separate experiments, THP-1 cells transfected with TLR-9 siRNA were incubated for 48 hours and protein analysis. Cell supernatants were saved for ELISA analysis. These TLR-9-transfected cells were also stimulated for 24 hours with NS, LPS, nCpG, and pCpG, respectively. Stimulated siRNA-transfected cells were harvested after 24 hours to collect RNA, protein, and cell supernatant for further analysis.

Cell Lysate Preparation

RAW 264.7 macrophages and THP-1 monocytes were incubated for 24 hours with 1ug/ml LPS, 3μM of nCpG or 3μM pCpG. Three million cells from each condition were then washed with cold PBS and protein lysates were prepared by adding Cell Disruption Buffer using the mirVana Protein and RNA Isolation (PARIS) kit #AM1921 (Ambion, Austin, TX) following the manufacturer's instruction. Bradford Method using BSA as a standard measured protein concentrations of samples to be analyzed by western blot.

Verification of siRNA Knockdown by Western Blotting

For caspase-1 knockdown confirmation, THP-1 cells transfected with siRNA targeting caspase-1 were lysed 48 hours after transfection, and cell extracts were loaded onto a 10% sodium dodecyl sulfate-PAGE gel and transferred to nitrocellulose. 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 (47). Secondary goat anti-rabbit antibodies conjugated to horseradish peroxidase were used at 1:10,000 dilutions (Southern Biotech, Birmingham, AL, USA). Caspase-1 protein levels were normalized to golgin-97 to determine knockdown efficiency. Densitometric analysis of the developed films were performed using Image J software.

THP-1 cells transfected with siRNA targeting TLR-9 were collected and processed in the cell lysate preparation mentioned above. Protein lysates were loaded onto a 4-20% Tris-HCl gel and transferred to nitrocellulose. Rabbit-anti-TLR-9 antibody at 1:250 (ProSci, Poway, CA) and alkaline-phosphatase conjugated goat-anti-rabbit antibody (Sigma, St. Louis, MO) was used at 1:10000 dilutions. To analyze protein expression of siRNA-transfected and stimulated THP-1 cells, nitrocellulose membranes were treated with rabbit-anti-ASC antibody at 1:100 dilution (Axxora, San Diego, CA) followed by secondary goat anti-rabbit antibody as mentioned above. Densitometric analyses of the developed films were performed using the Fluorchem Imaging densitometer system.

CpG-ODN Treatment of RAW264.7 and THP-1 Cells

Cells were plated at one million cells in a well of 6-well plates and set aside for 24 hours to ensure adherence to plate surface. At 80% confluency, concentrations of immunostimulatory CPG-ODN (pCpG, 3µM) and negative CpG-ODN (nCpG, 3µM) were added to respective cell culture wells for 24 hours. Sequences for the CPG-ODN motifs are as follows: nCpG – atAATAGAGCTTCAAGCaag; pCpG – atAATCGACGTTCAAGCaag. In separate experiments, cells were incubated in 1ug/ml of E. coli LPS (InvivoGen, San Diego, CA) as well as non-stimulated conditions to serve as positive and negative controls. Stimulated RAW264.7 cells were harvested by scraping the well surface with a spatula and centrifuging to obtain cell pellets at 1500 rpm for 5 minutes. THP-1 cells incubated under the mentioned conditions were aspirated from wells and also centrifuged at 1500 rpm for 5 minutes to obtain cell pellets for protein and RNA isolation. Cell supernatants were saved for future analysis.

Measure of Activation of ASC and Gene Expression of Related Molecules Using Total RNA Isolation and Quantitative PCR and Confirmation of TLR-9 Gene Knockdown Using qPCR

Cells were washed with cold phosphate buffered saline (PBS) and 300µl Lysis Binding Buffer from Ambion's mirVana kit to each well of a 6-well plate to isolate total RNA following manufacturer's protocol (Ambion, Austin, TX). Fifty nanograms of total RNA was reverse transcribed by Taqman reverse transcriptase (Applied Biosystems, Foster City, CA). Real-time PCR analysis was performed using 2 ul cDNA per condition. Samples were run using the Taqman procedure and an ABI StepOne instrument (Applied Biosystems, Foster City, CA). Primers for PYCARD, Caspase-11, Caspase-1, TLR9, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed according to GenBank on the ABI website. Expression of mRNA values was calculated using the threshold cycle (Ct) value, or the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed threshold. For each sample, ΔCt, sample was calculated by subtracting the Ct value of 18S, from that of each gene of interest to normalize the data. The expression levels relative to control were estimated by calculating ΔΔCt (ΔCt, sample − ΔCt, control) and subsequently using the $2^{-\Delta\Delta Ct}$ method (48).

In-Cell Western Analysis of ASC and Caspase-11 Protein Expression

RAW 264.7 ells were fixed at 15,000 cells per well in a 96-well plate and stimulated with LPS, nCpG, and pCpG to perform a cell-based ELISA. Cells were stained overnight with rabbit-derived primary antibodies specific for ASC and Caspase-11 molecules, respectively at 1:50 dilutions. The secondary antibody conjugated with fluorophore IR 800CW dye was then applied to cells for 1 hour. The Draq5 and Sapphire700 dyes were also used to stain cells in a non-specific manner for normalization of values based on cell numbers. Red fluorescent detection revealed nonspecifically staining of all cells present in each well, while the green fluorophore

staining represented detection of the molecule of interest. Cells were imaged using a LI-COR Odyssey scanner (LI-COR Biosciences, Lincoln, NE).

Detection of ASC Activation Via Immunocytochemistry

RAW264.7 cells were seeded at 15,000 cells per chamber into an 8-chamber slide (BD Falcon, Bedford, MA) and were given 24 hours to adhere to the slide surface. Cells were incubated for 24 hours with 1ug/ml LPS, 3μM of nCpG and 3μM pCpG, respectively. Staining reagents and protocol were provided by Cellnomics NF-κB Activation Kit (Cellnomics, Pittsburg, PA) but antibodies were replaced to analyze ASC protein (primary antibody: rabbit anti-ASC from Alexis Biochemicals, San Diego, CA; secondary antibody: goat anti-rabbit IgG conjugated with Alexa fluor 488 from Invitrogen, Carlsbad, CA). Reagents were scaled to correlate the volumes complementary to chamber area. Cells were fixed with warmed formaldehyde fixation solution for 10 minutes followed by washing and brief permeabilization. Cells were then incubated with rabbit-anti-ASC primary antibody for one hour, rinsed with detergent buffer and wash buffer, and then treated with staining solution containing Alexa Fluor 488 secondary antibody for consecutive hour while covered with foil. After washing, excess well reagents were aspirated the plastic chamber was detached from the slide. Drops of DAPI-mounting medium (Vector, Burlingame, CA) and a glass cover slide were placed on the 8-section slide and cells were viewed at 10X and 40X magnification using DAPI and FITC filters (Zeiss, Thornwood, NY). Whole cell fluorescent staining was analyzed with Image J software.

Statistical Analyses

Statistical significances were determined using the Student's t-test, Tukey Test, and one-way ANOVA. P values less than 0.05 were considered statistically significant.

CHAPTER 3
RESULTS

Specific Aim 1

Caspase-11 Gene Expression is Upregulated in Exocrine Glands of SjS-Susceptible Mice

To confirm earlier results (7) obtained with microarray analyses showing an increased gene expression of caspase-11 in the SMX of 8-week-old C57BL/6.NOD-*Aec1Aec2* (B6DC) mice carrying the two NOD-derived susceptibility loci (*SjS*^{s}) (Figure 3-1a), the levels of caspase-11 mRNA in both the SMX of 8- and 12-week-old NOD/ShiLtJ (NOD), B6DC and C57BL/6 (B6) mice were determined by semiquantitative reverse transcription-PCR. As presented in Figure 3-1b, caspase-11 expression was increased 4.6-fold ($P<0.01$) in the SMX of B6DC mice compared with B6 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 B6DC and NOD mice at 8 weeks of age, and remained elevated at 12 weeks of age (data not presented).

To determine whether either of the known transcription factors STAT-1 and/or NF-kB for caspase-11 is concurrently upregulated with caspase-11, their gene expressions were also measured (Figure 3-1b). *Stat1* gene expression proved to be upregulated in the SMX of both NOD and B6DC mice (2.3-fold, $P<0.01$) at 8 weeks of age. Similarly, expression of *Nfkb1* (p50) was upregulated in the SMX of B6DC mice. In contrast, *Nfkb2* (p52) was slightly downregulated in the SMX of both B6DC and NOD mice at 8 weeks of age. By 12 weeks of age, Nfkb1 was downregulated in the SMX, whereas *Nfkb2* remained at low levels.

Caspase-11 Expression is Detected in Macrophage and Dendritic Cells

Caspase-11 protein expression was confirmed by immunohistochemistry on SMX of 8-week-old B6DC mice (Figure 3-1c). 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, fluorescein isothiocyanate (FITC-) or phycoerytherin (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 that caspase-11 was expressed by both cell stainings, showing positively stained cells in yellow when the images were merged (Figure 3-1d). Caspase-11-positive cells surrounded acinar and/or ductal cell units as indicated by white arrows in Figure 3-1d.

STAT-1 but not NF-κB is Concomitantly Upregulated with Caspase-11 in the Salivary Glands of SjS-Susceptible Mice

Elevated *Stat1* and *NF-kB1* gene expression in the SMX of 8-week-old B6DC 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 B6DC mice, incubated with biotin-labeled oligonucleotides specific for the DNA binding site of either *Stat1* or *Nfkb*, and separated by PAGE. As shown in Figure 3-2a and b, *Nfkb* showed no statistically significant increase in activity, whereas an upregulated *Stat1* activity (>20%) was found in the SMX of 8-week-old NOD/LtJ and C57BL/6.NOD-Aec1Aec2 mice, when compared with 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 B6DC 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 SMX versus lacrimal glands of NOD and B6DC mice.

Caspase-11 Activates Caspase-1, but not Caspase-3

To better define the consequences of elevated caspase-11 in the SMX of the SjS mouse model during the preclinical phase of disease when apoptosis of acinar tissue is prevalent, I analyzed the activity of caspase-1 and caspase-3. As shown in Figure 3-3a, caspase-1, but not caspase-3, activity was upregulated in the SMX of 8-week-old B6DC mice compared with C57BL/6 mice, although the baseline activities for caspase-3 were higher overall. To confirm activation of the caspase-1 pathway, I examined whether IL-1 and/or IL-18, two factors whose secretion is strongly regulated by the activation of caspase-1 (13), were also upregulated. Reverse transcription-PCR analyses indicated highly elevated IL-1 and IL-18 mRNA transcripts in the SMX of 8-week-old B6DC mice (Figure 3-3b). Interestingly, activation of caspase-1 in the local target tissue (that is, the salivary glands) of SjS corresponded with upregulated IL-18 cytokine expression in the saliva but not in sera before disease onset (Figure 3-3c). An 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 Before Disease Onset in Comparison with Disease-Free Mice

To identify whether activation of 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 and B6DC mice was examined. Histological sections of salivary glands were prepared and transferase-mediated dUTP-biotin nick end labeling (TUNEL) stained (Figures 3-4a and b). Visualization using a fluorescent microscope fitted with a red filter for Cy3 revealed that both NOD and B6DC mice showed more abundant apoptotic cell death than C57BL/6 control mice (Figure 3-4b). Nuclease-treated slides (PC in Figure 3-4a) served as a positive control.

Caspase-11 is not Detected in TUNEL-Positive Acinar or Ductal Cells

Experiments were carried out to investigate whether caspase-11 plays a direct role in increased apoptotic acinar cell death in the salivary glands before 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 Figure 3-4c, caspase-11 (white arrow) was present in the cells located outside or between the acinar or ductal areas, whereas TUNEL-positive cells (red arrows) were acinar or ductal cells. Although these dying cells were colocalized with caspase-3 (Figure 3-4c-a, yellow arrow) showing positivity inside the cytoplasm, findings indicate 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 colocalization 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 increased apoptotic cell death of salivary epithelial cells. This hypothesis was tested using a human salivary gland (HSG) cell line (49) cocultured with a THP-1 human monocyte cell line, which was stimulated with LPS for the induction of IL-1beta and IL-18 in the presence and absence of IFN-gamma, in an attempt to duplicate observations in the *in vivo* environment. Earlier data from the SjS mouse model lacking IFN-gamma (5) showed the absence of disease and predisease phenotype, clearly indicating that IFN-gamma is critical not only for the onset of the disease, but also for the predisease stage. In addition, in the NOD mouse, IFN-gamma was upregulated by two-fold in the SMX before disease onset (5). Current data indicates that increased apoptosis of HSG cells occurred only in the presence of LPS-

stimulated THP-1 cells when IFN-gamma was present (Figures 3-5a and b, $P<0.01$). The increased rate of apoptotic cell death was reversed to a normal level when caspase-1 expression in THP-1 cells was downregulated by siRNA (Figure 3-5). The normalized caspase-1 knockdown efficiency was greater than 70%, as shown in Figure 3-5c ($P<0.01$).

This work was published in 2009 in the journal of *Immunology and Cell Biology*, volume 87, pages 81-90.

Specific Aim 2

ASC and Caspase-11 are Upregulated in Murine RAW 264.7 Macrophages in the Presence of CpG-ODN

Earlier data from the SjS mouse model revealed increased gene levels of ASC and Caspase-11, indicating that inflammatory molecules may be required for the disease onset of Sjogren's syndrome. In continuing investigation of inflammatory cytokines in macrophages, I moved onto *in vitro* cell lines to analyze inflammasome component ASC. As a reminder, ASC is an adapter protein necessary for the complex of proteins coined as "the inflammasome" to form (18). Activation of ASC would thus lead to the recruitment of NALP3 and caspase-1 to trigger caspase-1 activation.

Concomitant upregulation of ASC in salivary glands of B6DC at both 8 and 20 weeks of age (data not shown here) prompted investigation of whether the presence of chronic stimulation such as herpes simplex virus (HSV)-derived oligodeoxyribonucleotide CpG-DNA (CPG-ODN) can induce inflammasome components such as ASC, leading to subsequent inflammasome activation, resulting in increased IL-1beta and IL-18 cytokine production.

The mouse macrophage cell line RAW 264.7 was stimulated for 24-hours in the presence of CpG-ODN (pCpG, 3 uM) and negative-CpG (nCpG, 3 uM) compared to non-stimulated cells and LPS-stimulated cells (1 ug/ml) as a positive control for inflammasome activation. To

measure protein expression after CpG-ODN stimulation, In-cell western (ICW) assay was applied. ICW is an immunocytochemical assay that uses an infrared imaging system to detect and quantify proteins in fixed cells. Antibodies to mouse ASC and caspases-11 were incubated at dilutions of 1:50 overnight followed by goat anti-rabbit secondary antibody conjugated with IR Dye 900CW (Li-cor Biosciences, Lincoln, Nebraska). Analysis indicated upregulation of ASC and caspase-11 in mouse macrophages in response to pCpG and LPS stimulation when compared to the non-stimulated cells (Figure 3-6a and b).

Further confirmation of the increased protein expression seen by In-cell western analysis was found by immunocytochemistry of RAW264.7 cells. Staining experiments were carried out to determine whether ASC expression of non-stimulated cells was significantly different than CpG-ODN-stimulated cells. Roughly 15,000 cells were placed in an 8-well chamber slide and stimulated over the course of 24 hours with NS, LPS, nCpG, and pCpG conditions. Cells were then fixed and stained with rabbit-anti-ASC antibody followed by Alexa Fluor-488-anti-mouse antibody. As presented in Figure 3-7a, ASC protein was highly expressed in LPS and pCpG treated cells when compared to non-stimulated cells. Image J analysis of whole cell fluorescent ASC staining revealed that pCpG had significantly different ($P<0.01$) integrated densities of fluorescent protein when compared to that under non-stimulated condition (Figure 3-7b).

Specific Aim 3

TLR-9 siRNA Knockdown Reduces ASC Expression in THP-1 Cells

To better define the role of TLR-9 in increased ASC expression due to CpG-DNA stimulation, the following study analyzed the downstream effects of siRNA knockdown of the TLR-9 pathway. siRNA supplied by Applied Biosystems and Dharmacon were used to transfect THP-1 cells to knock down TLR-9 and GPADH gene expression, respectively. Cells were harvested for qPCR and as shown in Figure 3-8a, after 48 hours of transfection, normalized

TLR-9 knockdown efficiency was 70.9% and 66.3% in GAPDH, which confirmed transfection of siRNA using Lipofectamine.

ASC Expression is Down-Regulated in the Absence of TLR-9

Following confirmation of TLR-9 gene knockdown, TLR-9-transfected THP-1 cells were stimulated over the course of 24 hours under non-simulated or, LPS, nCpG, and pCpG stimulated conditions. Cells were then harvested for qPCR analysis of ASC gene expression. Results revealed down-regulated ASC expression in the absence of TLR-9, in comparison to the three-fold increased rate of expression in non-transfected stimulated cells. ASC gene expression seen in LPS, nCpG, and pCpG conditions were therefore returned to normal levels comparable to the non-stimulated cells (Figure 3-8b).

CHAPTER 4
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* (B6DC) mice, together with an abnormal glandular homeostasis in pre-diseased NOD mice, led to the hypothesis that importance of intrinsic properties of target tissues (such as availability of antigen(s) and changed activity of antigen presenting cells by pro-inflammatory cytokines) contribute to the breakdown of peripheral tolerance and the activation of autoreactive immune cells (2, 4, 6, 7, 50). Efforts at understanding initial molecular events triggering onset of SjS identified five 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 before disease onset and is apparently associated with the enhanced transcriptional activity of STAT-1. Second, these events apparently initiate secretion of pro-inflammatory cytokines from the local tissue through caspase-1 activation, as indicated by elevated IL-18 levels in saliva, an event capable of inducing increased epithelial cell death rather than cell-autonomous killing of epithelial cells. Third, caspase-1 in macrophages/dendritic cells and IFN-gamma in the salivary gland microenvironment play a critical role in the death of residential epithelial cells, shown in both *in vivo* and *in vitro* analyses. Fourth, a viral CPG-DNA stimulus can induce gene and protein expression in macrophages or monocytes. Fifth, knockdown of the TLR-9 reduced ASC expression even in the presence of a viral CpG-DNA stimulus, confirming that ASC induction by CpG-DNA is mediated through TLR-9. This scheme is illustrated in Figure 3-9.

Recently, it has been 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 specific pathogen-free

(SPF) conditions, thus promoting interferon induction as an anti-viral defense mechanism by epithelial cells or natural killer cells in the glands. Increased IFN-gamma, known to be present in exocrine glands of NOD-derived mice (5), can enhance the activity of macrophages and/or dendritic cells in the tissues, resulting in the production of caspase-11. This is supported by the fact that caspase-11 is known to be induced only when signals from IFN-gamma or LPS through the activation of STAT-1 or NF-kappa B 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 colocalized 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 (12). However, the role of caspase-11 found in the SMX of SjS-prone B6DC mice differed from that in EAE models in that caspase-11 in the B6DC model was neither produced by dying cells nor co-localized with caspase-3. In the SjS model, caspase-11 is apparently correlated with caspase-1 activity. Furthermore, abundant TUNEL-positive cells in the SMX of B6DC 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 pathways, as well.

During early disease pathogenesis of SjS, the roles of IFN-gamma seem to be indispensable based on an earlier study (5) as well as current studies (51). IFN-gamma is required but not sufficient for the induction of increased cell death in the target tissue of SjS. In addition, the earlier studies (5) indicate that in the absence of IFN-gamma, mice exhibited neither secretory dysfunction nor alterations in pre-disease markers, strongly conclusive of our current findings. The requirement for caspase-1 and IFN-gamma in enhanced cell death, proven by *in*

vitro co-culture studies with siRNA targeting caspase-1 in THP-1 cells, may originate from the fact that the IFN-gamma is essential for caspase-1 activation, which cleaves pro-IL-1beta and IL-18 to produce mature forms of IL-1beta and IL-18 for its release (52). Therefore, synergistic effects between cytokines induced and cleaved by the activation of caspase-11 and caspase-1, and IFN-gamma/STAT-1 are essential for driving chronic inflammatory conditions in the targeted glands even before disease onset.

Increased expression of IL-1beta can cause the activation of the signal cascade leading to the activation of several transcription factors involved in inflammatory responses (53). IL-18, originally described as an IFN-gamma-inducing factor, is a potent inflammatory stimulant produced by macrophages and dendritic cells and is known to enhance antigen-specific clonal expansion of IFN-gamma-producing T cells (54), 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-gamma during the early stage of SjS. A study also indicates that IL-18 produced by Kupffer cells stimulates TH1 and natural killer cell cytotoxic activity by increasing their production of FasL (CD 95), which ultimately induces apoptosis in Fas-bearing hepatocytes, causing liver injury (55). In a similar manner, IL-18 produced by phagocytic cells may up-regulate FasL on natural killer cells and Fas on epithelial cells in the SMX through caspase-1 activation, resulting in subsequent apoptotic processes in the epithelial cells. Interestingly, IL-18 and IL-1beta have been reported to be up-regulated in both sera and the SMX of SjS patients (56, 57). Considering the results of our current study, one might speculate that IL-1beta 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 (that is, human caspase-1, caspase-4 and caspase-5, along with murine caspase-11 and caspase-12), considered to be 'inflammatory caspases', are involved in the proteolytic maturation of inflammatory cytokines (58, 59). Recent studies have identified a complex of proteins, referred to as the 'inflammasome', functions in innate immunity by regulating inflammatory caspase-1 activation (16, 60, 61). Proteins that make up the inflammasomes are members of the NACHT-, LRR- and PYD-containing proteins (NALP) family of proteins, although information on its exact expression or binding partners is relatively scarce (62). 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-1beta and IL-18 for regulating immune cells. Important questions that are currently being investigated include whether caspase-11 activates caspase-1 as a part of the inflammasome, whether increased epithelial cell death in the target tissues confers target tissue specificity in autoimmune SjS and whether abnormal regulation of the inflammasome translates to SjS in humans. Our current results seem to point to the potential existence of abnormal regulation of the inflammasome in the SMX of NOD-derived SjS-like disease-susceptible mice (Figure 3-9).

As it was mentioned in the results above, a caspase-1-mediated pathway in macrophages residing in the salivary glands, plus an increased IL-18 secretion in saliva are seen in SjS-prone B6DC mice prior to disease onset. In addition, the expression of ASC, a key adaptor for inflammasome activation was also up-regulated in the salivary glands of disease prone mice at 8 weeks. Since the salivary glands are one of the common reservoirs of chronic viral infection such as HSV, I investigated whether DNA derived from HSV is capable of inducing ASC expression, which may play a critical role in inflammasome activation in the salivary glands (data not

presented). As might have been expected, viral CpG-DNA derived from HSV-1 was able to up-regulate ASC expression by 2.5-fold (Figure 3-6b) similar to the increase by LPS stimulation (positive control for inflammasome activation). A study conducted by Pelegrin *et al* (63) indicates that RAW 264.7 macrophages lack ASC and are therefore incapable of caspases-1-mediated processing and release of mature IL-1beta, suggesting that LPS-primed cells are due to activation of a differential pathway (such as $P2X_7$ Receptor-mediated activation). However, the current data clearly indicate that ASC is present and up-regulated in RAW 264.7 cells following LPS stimulation, which differs from Pelegrin's observations and ASC expression was up-regulated in response to LPS and pCpG stimulation, which was detected by RT-PCR and ICW analysis. Normalization of fluorescent readings to the non-stimulated cell condition revealed a 2.5-fold increase in both LPS and pCpG conditions. Experiments to further confirm ASC expression in RAW 264.7 utilized immunocytochemistry analysis, which revealed that LPS stimulation caused an approximate two-fold increase of ASC protein expression when compared to the non-stimulated condition. Furthermore, abundant ASC expression was also found in cells stimulated with pCpG, also suggesting that CpG-DNA causes an increase in ASC protein expression, and that inflammasome activity can by observed via this mouse macrophage cell line. My data therefore, indicate that viral CpG-DNAs can be an inductive signal for ASC, which is important for caspase-1-activation pathway (inflammasome) in macrophages. This finding supports the concept that chronic inflammation in SjS salivary glands prior to disease onset may be caused by chronic viral stimulation in the target tissue.

ASC induction by CpG-DNA was further confirmed by TLR-9 siRNA knockdown experiments where THP-1 cells were transfected with siRNA targeting TLR-9 and analyzed for ASC expression in the presence or absence of immunostimulatory viral CpG-DNA. As shown,

TLR-9 expression was significantly reduced by 70.9% and GAPDH expression was also down-regulated by 66.3% with GAPDH siRNA. As expected, ASC expression in the TLR-9 transfected cells stimulated with CpG-DNA were significantly reduced. Interestingly, transfected THP-1 cells stimulated with nCpG also showed up-regulated ASC expression, which is not consistent with our previous data. Although KPS contamination in this well is suspected, it certainly requires further confirmation. Nevertheless, current findings strongly suggest that ASC induction by CpG-DNA is mediated through TLR-9.

TLR-9 stimulation by CpG induces a signaling pathway involved in the activation of transcription factor NF-kB localized in the cellular endosomal compartments (and downstream of adapter MyD88) to induce pro-IL-1beta and IL-18 expression (64, 65). Therefore it is hypothesized that CpG-DNA stimulation may eventually lead to ASC induction via the activation of NF-kB, leading to cleavage of pro-IL-1beta and pro-IL-18 and subsequent release of mature cytokines. It will be interesting to identify exact molecular pathways involved in this process.

In summary, this study shows that caspase-11 plays a critical role in the susceptibility of mice to SjS-like disease by up-regulating a caspase-1-mediated pathway, which is vital for apoptotic cell death of the neighboring epithelial cells. In addition, the presence of IFN-gamma 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 salivary glands in SjS. STAT-1 activation rather than NF-kB activation in the SMX of disease-prone mice seems to be responsible for caspase-11 induction. ASC induction through TLR-9 in the presence of CpG-DNA revealed that CpG-DNA can also induce activation of caspase-1

inflammasome activation via ASC induction. Knockdown of the TLR-9 gene further confirmed that ASC induction by CpG-DNA is through TLR-9 in THP-1 cells. This scheme is illustrated in Figure 3-9. Further confirmation is required for analysis involving TLR-9 gene knockdown, as protein expression and cytokine production of IL-1beta and IL-18 have yet to be measured to confirm whether the final outcome of the caspases-1 activation pathway is inhibited when ASC expression by CpG-DNA stimulation is reduced by siRNA.

Overall, our observations underscore the potentially critical roles of myeloid cell populations and of intracellular pattern recognition through the inflammasome activation of 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. In addition, based on results found by *in vitro* analysis, future studies would involve *in vivo* analysis of inflammasome-related knockdown in SjS-prone B6DC mice, in which siRNA targeting ASC or other molecules of interest could be adenovirus-associated delivered using gene therapy techniques.

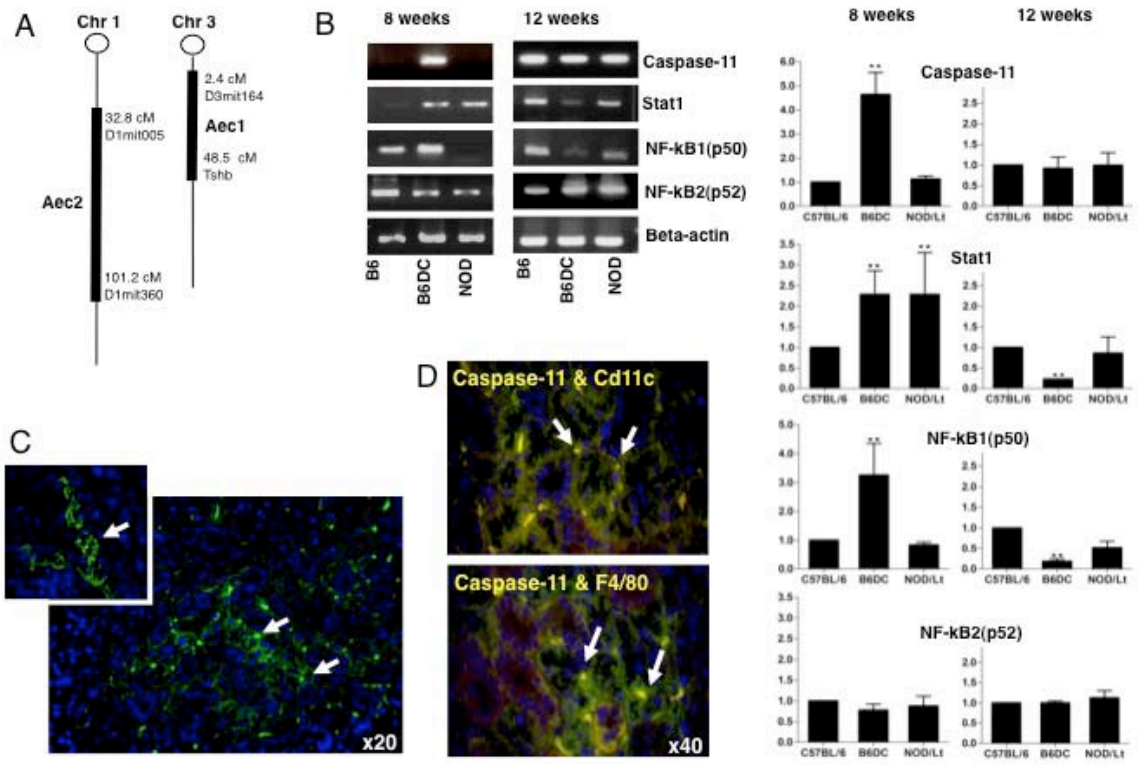

Figure 3-1. Increased caspase-11 expression in the SMX of the SjS-prone C57BL/6.NOD-*Aec1Aec2* mouse before lymphocytic infiltration. A) Two NOD-derived genetic intervals, namely autoimmune exocrine loci 1 and 2 on chromosomes 3 and 1, respectively, in the disease-prone C57BL/6.NOD-*Aec1Aec2* mouse are depicted. B) Elevated caspase-11, its major transcription factors STAT-1 and NF-kB1 in the salivary glands of C57BL/6.NOD-*Aec1Aec2* were confirmed by semiquantitative 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, times 20. 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, times 10 and times 40. Aec, autoimmune exocrine loci; RT-PCR, reverse transcription-polymerase chain reaction; SjS, Sjögren's syndrome; SMX, submandibular glands; STAT, signal transducers and activators of transcription.

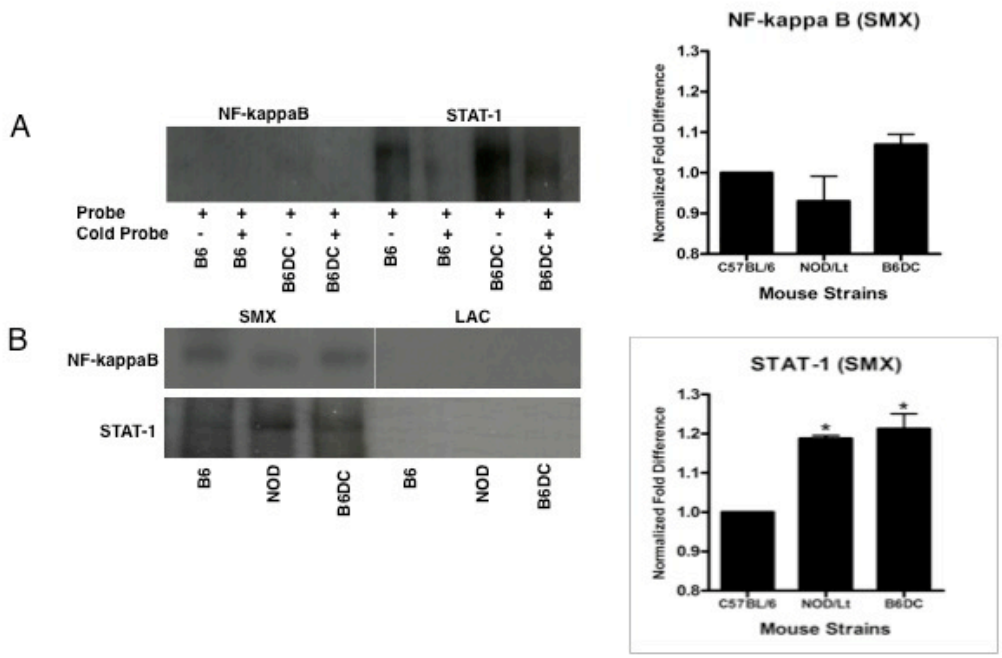

Figure 3-2. Concomitant increase in STAT-1 activity in the SMX of C57BL/6.NOD-Aec1Aec2 at 8 weeks. A) Increased STAT-1 activity was detected by EMSA in the glands isolated at 8 weeks. Inhibition of binding is shown with a cold probe (unlabelled probe) incubation. The right panel indicates bar graphs generated by densitometer analyses on EMSA results for NF-kappaB. B) Absence of STAT-1 and NF-kB activity in the lacrimal gland (LAC) at 8 weeks was detected whereas elevated STAT-1 was shown in the SMX of C57BL/6.NOD-Aec1Aec2 and NOD/ShiLtJ mouse. Increased amount of nuclear extract (ten micrograms) isolated from 0.5 g of pooled glands (n=5–7 mice) was used per lane to enhance binding activity for NF-kB. The right panel indicates bar graphs generated by densitometer analyses on EMSA results for STAT-1. The experiments were carried out three times per molecule of interest. *P<0.05 in comparison with C57BL/6. EMSA, electrophoretic gel mobility shift assay; NF-kappaB, nuclear factor-kappaB; SMX, submandibular glands; STAT, signal transducers and activators of transcription.

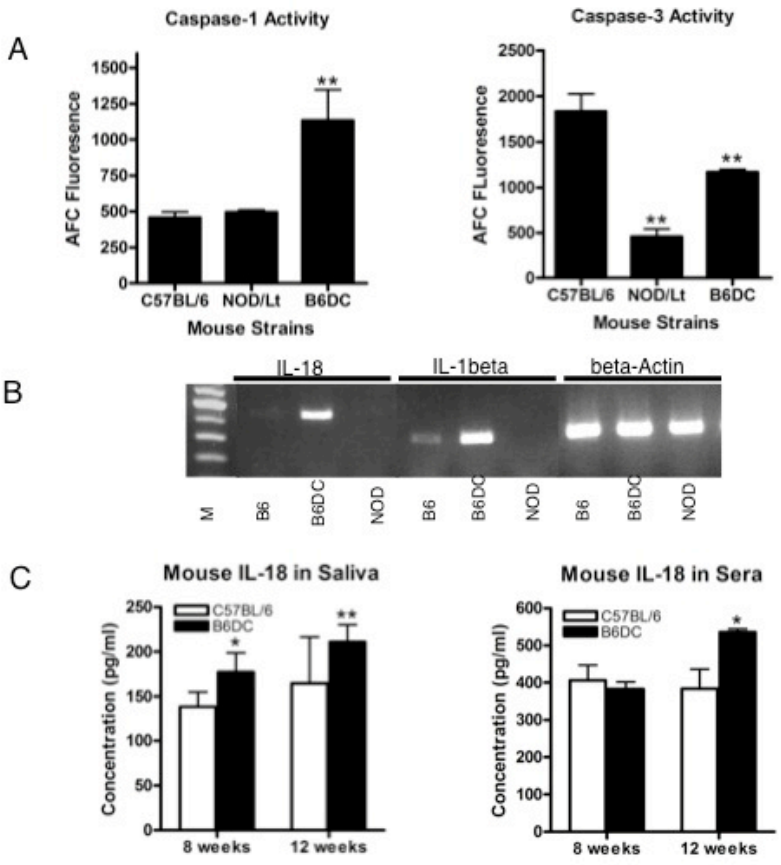

Figure 3-3. Activation of caspase-1-mediated pathway in C57BL/6.NOD-*Aec1Aec2* before disease onset. A) Caspase-1 activity was upregulated significantly in the SMX of C57BL/6.NOD-*Aec1Aec2* at 8 weeks. Experiments were performed in triplicate. **P<0.01 in comparison with C57BL/6. B) Genes downstream of caspase-1 such as IL-1beta 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 whereas 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. IL, interleukin; RT-PCR, reverse transcription-polymerase chain reaction; SMX, submandibular glands; ELISA, enzyme-linked immunosorbent assay.

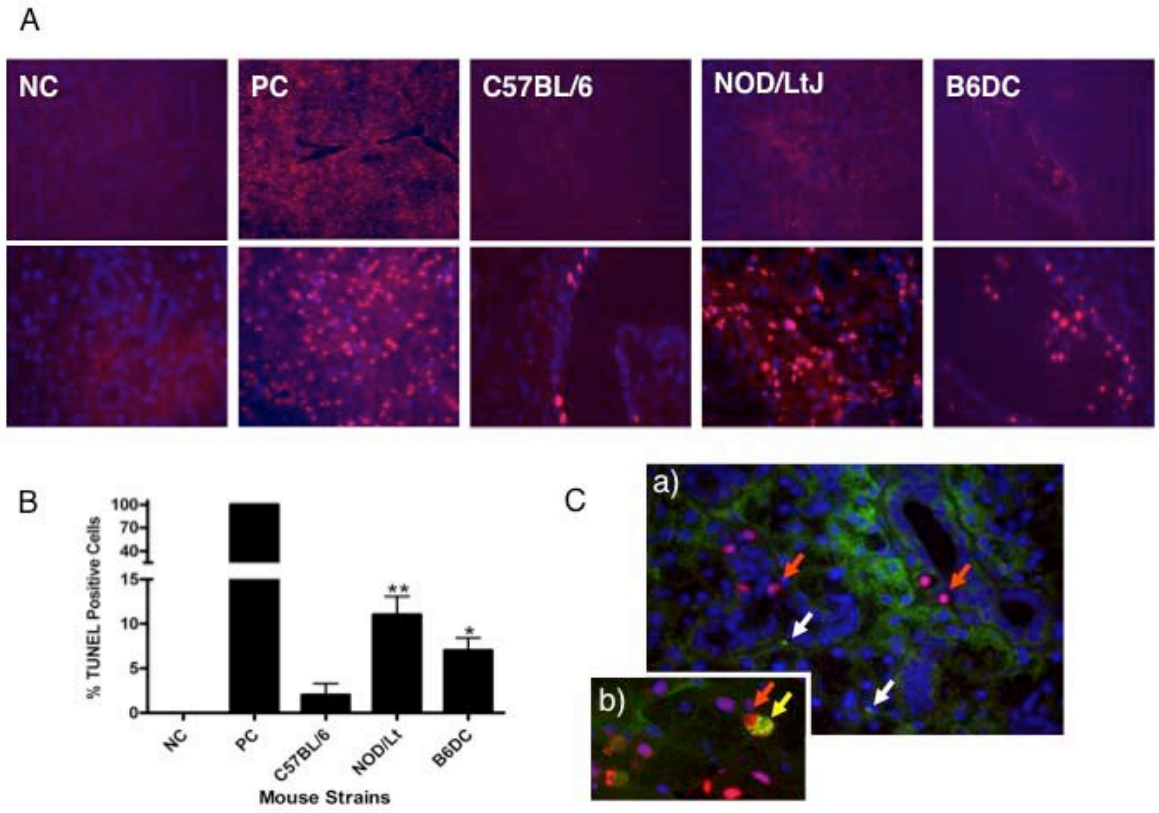

Figure 3-4. Increased epithelial cell death in the glands of disease-prone mice at 8 weeks and lack of direct colocalization of caspase-11 with TUNEL-positive cells. A) TUNEL staining was performed on the prediseased salivary glands; upper panel at times 10 and lower panel at times 40 magnifications. B) Percentages of TUNEL-positive cells are shown as a bar graph. For each mouse, three slides were evaluated for TUNEL-positive cells, which were counted using a cell counter. C) Caspase-3-positive cells (yellow arrows in b) were colocalized with TUNEL-positive cells (red arrows). White arrows indicate caspase-11-positive cell. Magnification, times 40. NC, negative control; PC, positive control treated with nuclease; TUNEL, transferase-mediated dUTP-biotin nick end labeling.

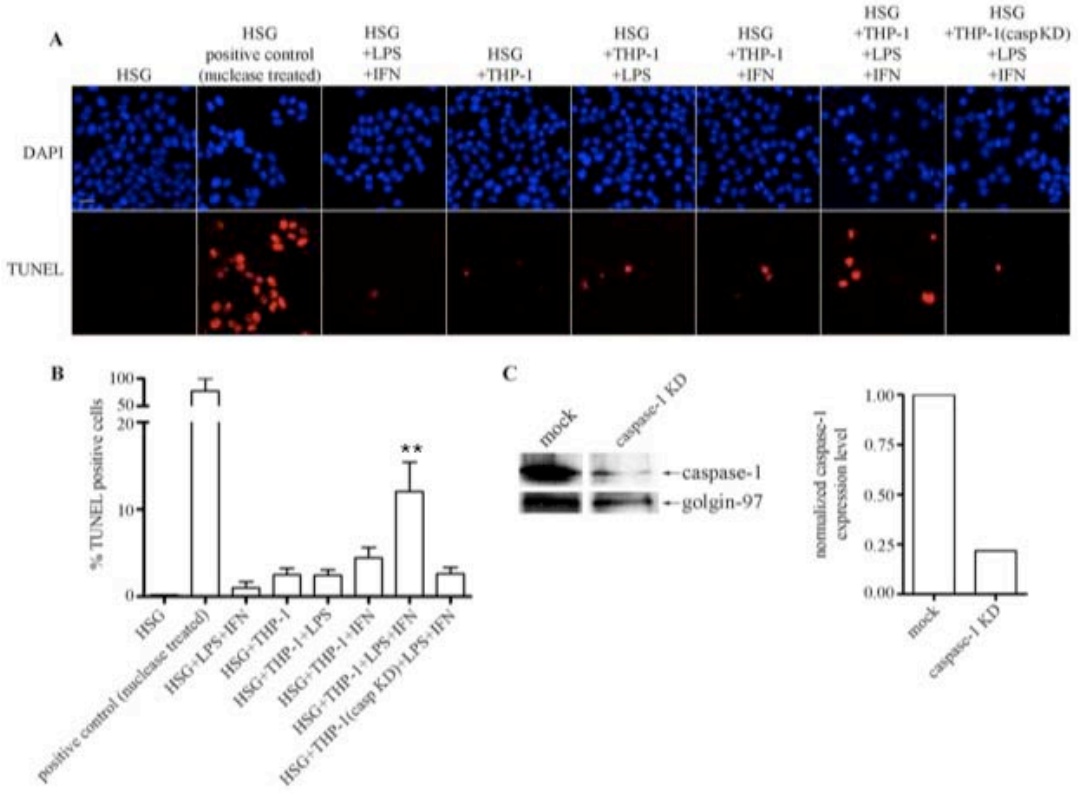

Figure 3-5. Inhibition of apoptotic cell death of HSG cells by caspase-1 knockdown in THP-1 cells. A) HSG cells were cultured in the absence or presence of LPS- and/or IFN-gamma-stimulated THP-1 cells. After removing culture media or stimulated THP-1 cells from the culture, apoptotic HSG cells were analyzed by TUNEL assays. For the caspase-1 inhibition study, siRNA to caspase-1 was transfected into THP-1 cells before stimulation with LPS and IFN-gamma and cocultured with HSG cells. B) Percent TUNEL-positive cells were presented as a bar graph. The experiment was repeated three 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. HSG, human salivary gland; IFN, interferon; LPS, lipopolysaccharide; TUNEL, transferase-mediated dUTP-biotin nick end labeling.

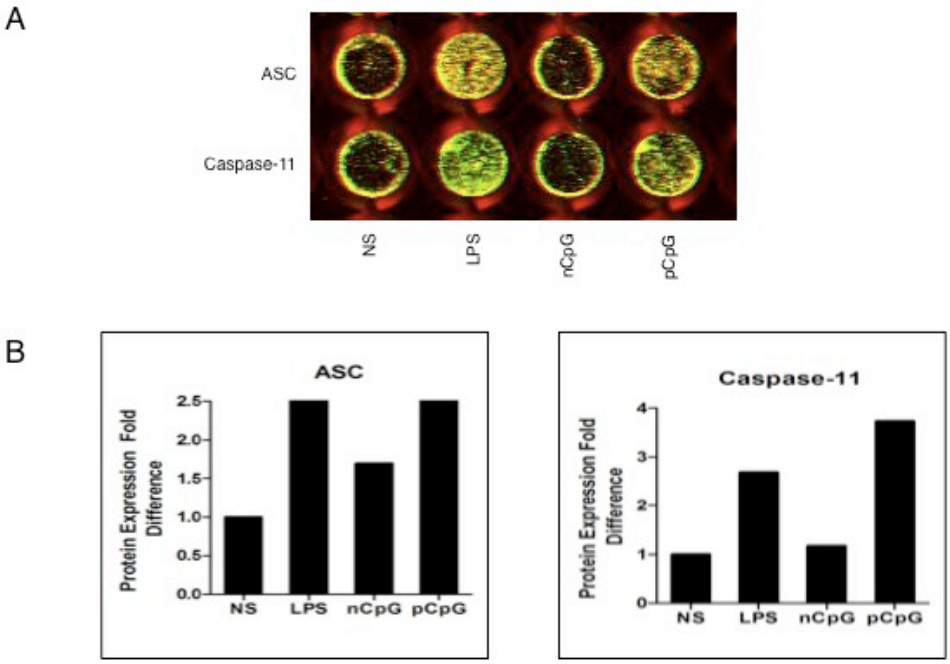

Figure 3-6. Increased ASC and caspase-11 expression in RAW 264.7 macrophages in response to different conditions. A) Image of In-cell Western (ICW) fluorescent imaging of rabbit-anti-ASC cell staining. Red fluorophore dye stained all cells within each well non-specifically. Green fluorophore dye stained molecule(s) of interest. B) Elevated ASC and caspase-11 in mouse macrophage cells by ICW depicted as a bar graph.

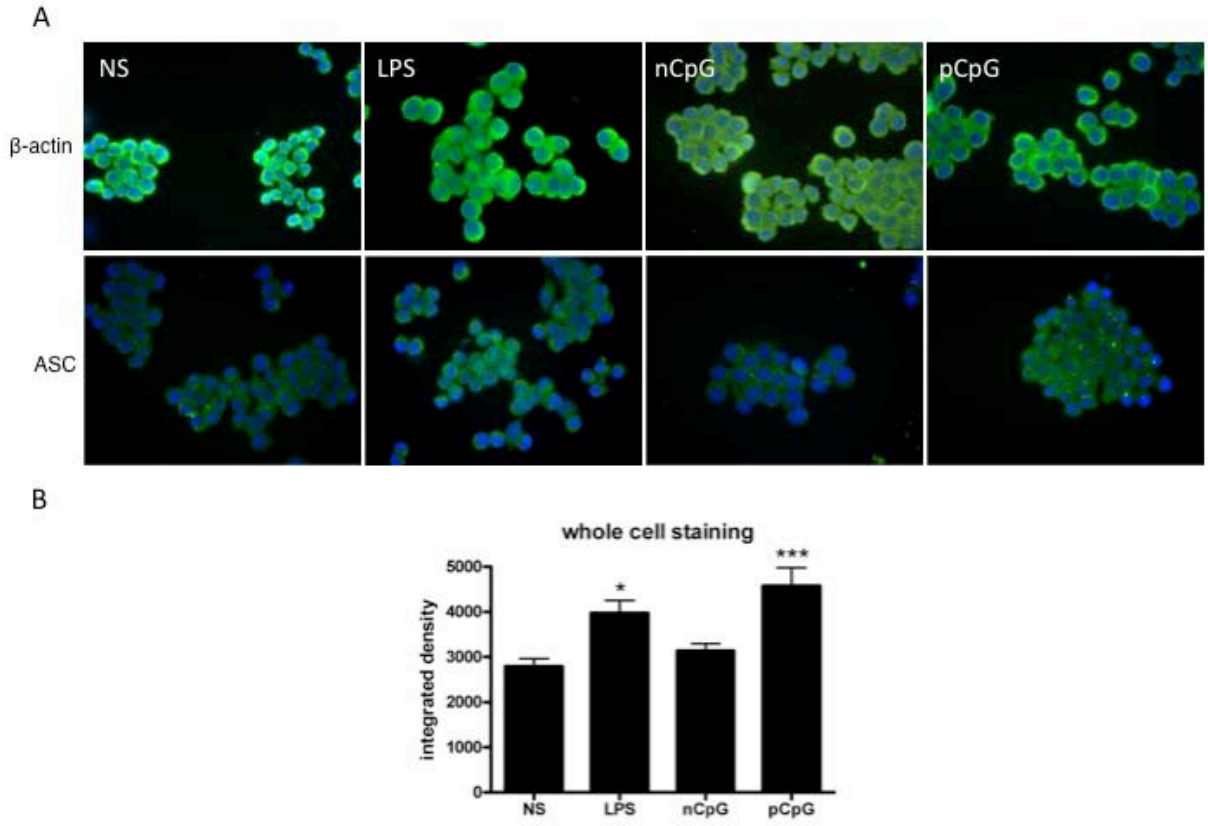

Figure 3-7. Activation of ASC in RAW 264.7 cells by stimulation with pCpG. A) Microscope images of RAW 264.7 cells stained with housekeeping gene, beta-actin (top row) and ASC (bottom row). After stimulating cells for 24 hours under non-stimulated (NS), LPS, nCpG, and pCpG conditions, cells were fixed and stained with rabbit-anti-beta-actin and rabbit-anti-ASC antibodies respectively. Cells were then treated with goat-anti-rabbit IgG conjugated with AlexaFluor488 antibody. Images were taken at 20X magnification. B) Normalized density values of ASC protein expression in cells was measure using Image J analysis software and is depicted as a bar graph. (*$P<0.05$, **$P<0.01$)

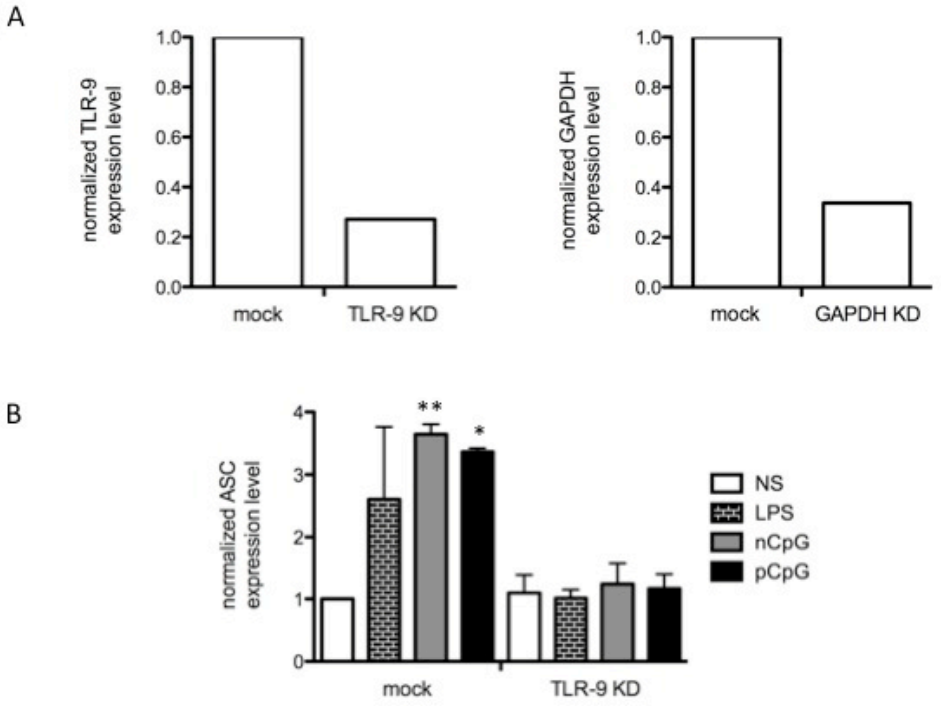

Figure 3-8. Inhibition of TLR-9 (CpG-DNA recognition) pathway via TLR-9-siRNA knockdown in THP-1 cells. A) THP-1 cells were cultured in the absence (mock) and presence of siRNA specific to TLR-9 or GAPDH, respectively. Knockdown efficiency of TLR-9 was compared to a housekeeping gene GAPDH by qPCR and is depicted as bar graphs. B) ASC gene expression of stimulated mock and TLR-9-siRNA-transfected cells represented as a bar graph. Gene expression levels were normalized to the non-stimulated (NS) mock condition (**P<0.01, *P<0.05).

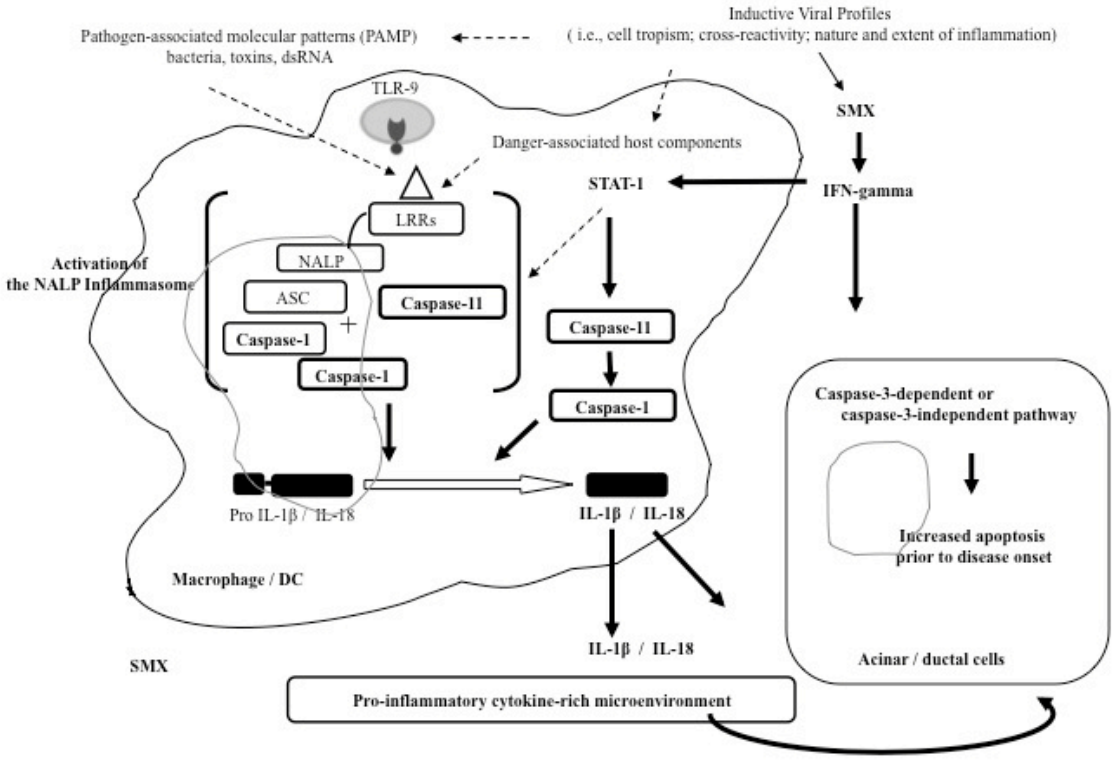

Figure 3-9. Schematic representation of the current working hypothesis. Inductive viral profile results in alterations in the target tissue through the activation of IFN-STAT and a subsequent induction of caspase-11. Upregulated caspase-1 activity produces mature IL-1beta and IL-18 from macrophages and 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-gamma production by epithelial cells, resulting in further activation of macrophages. Areas where further investigation is needed for confirmation are depicted with dotted lines (that is, questions as to whether caspase-11 is a constituent of the NALP inflammasome or why IFN-gamma and consequently STAT-1 are upregulated in the SMX before 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. ASC, apoptosis–associated speck-like protein containing a caspase-activating recruitment domain; DC, dendritic cells; IFN, interferon; IL, interleukin; LRR, leucine-rich repeats; NALP, NACHT-, LRR- and PYD-containing proteins; SMX, submandibular glands; STAT-1, signal transducers and activators of transcription.

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BIOGRAPHICAL SKETCH

Marievic Bulosan was born in Brooklyn, New York in July of 1983 and moved to Florida at the age of seven with aspirations to pursue a medical degree with specialization in dermatology and to start outreach clinics in third world countries. That dream went as far as her pre-med declaration during her freshman year at the University of Florida.

Volunteering in the Department of Pathology in 2004 shifted her concentration to laboratory research, and she graduated in December 2006 with a Bachelor of Science in Microbiology and Cell Science. She became lab manager for Dr. Seunghee Cha in the Department of Oral and Maxillofacial Surgery and Diagnostic Sciences in June 2006 and was urged to pursue a Master's education. Marievic enrolled as a UF student once again in August of 2007 in the Master's of Medical Science program through the College of Medicine. She was accepted into the joint degree program in July 2008 to complete an additional Master's degree in Educational Administration and Policy with hope to finish in May 2010.

While she maintained lab proficiency among volunteers and staff, completed school coursework, and conducted experiments, Marievic co-authored nine abstracts, eight posters, and four manuscripts in which she was primary author for one publication that is currently found in the Journal of Immunology and Cell Biology. Marievic also had the privilege of presenting her work for the International Association of Dental Research in Toronto, Canada in July 2008.

Marievic aspires to become an administrator or director of instruction for community colleges or universities, focusing on improving student outcomes in professional schools. Her goal is to improve teacher accountability and efficacy and encourage professors to teach effectively not only in regards to science but in all course subjects. Later in life, she would like to teach science courses to high school students, to restart the cycle of inspiring students just as she

had been inspired in the past to pursue careers in teaching. She also hopes one day to get married, start a family, and raise her children to have an eager passion for learning and teaching.