Sjögren’s syndrome (SjS) is a chronic, progressive, systemic, human autoimmune disease in which an auto-inflammatory process within the salivary and lacrimal glands results in loss of saliva and tear production, respectively. In-depth analyses of the autoimmune process in humans and animal models of SjS substantiates one of the more important pathoaetiological pathways: an increased level of glandular apoptosis and/or cell lysis. We have hypothesised that failure in clearance of dying cells by macrophages, dendritic cells, and neighbouring tissues results in a sustained innate inflammatory response that transitions to autoimmunity. Since the intrinsic inhibition of inflammation following phagocytosis of dying cells is a function of a family of three receptor tyrosine kinases (RTKs) known as the TAM (Tyro3, Axl, and Mertk), we put forward the following hypothesis: based on published information and analysis of our public microarray data, the failure of TAM RTK signalling, specifically in activating suppressor of cytokine signalling (SOCS) 1 and SOCS3 (which are inhibitors of immune responses), may lead to autoimmunity, and specifically, to SjS-like disease.

Keywords: Sjögren’s syndrome (SjS), autoimmunity, TAM (Tyro3, Axl, and Mertk), receptor tyrosine kinase (RTK), apoptosis, phagocytosis.
**INTRODUCTION**

Sjögren’s syndrome (SjS) is a chronic, systemic, human autoimmune disease in which an immunological attack against the salivary and lacrimal glands results in dry mouth (stomatitis sicca/xerostomia) and dry eye (keratoconjunctivitis sicca/xerophthalmia) disease(s), respectively.\(^1\) Despite efforts to define the genetic, environmental, and immunological basis of SjS, the underlying aetiology remains poorly defined, in part due to diagnoses being made after the onset of the overt disease, which is then further deconvoluted based on disease phenotypes and symptoms. Additionally, disease symptoms can be classified into primary SjS, disease progression symptomatic of xerostomia and/or xerophthalmia, or secondary SjS, SjS disease symptoms compounded from other forms of autoimmunity including lupus, multiple sclerosis, rheumatic arthritis, or other rheumatic autoimmune diseases. In an attempt to better define the nature of autoimmunity in SjS, a variety of mouse models exhibiting various aspects of SjS have been identified and studied extensively, particularly as a means to investigate events associated with early-stage disease.\(^2\) One of the more intensively studied models of SjS is the non-obese diabetic (NOD) mouse,\(^3\) and this model has been shown to closely mimic the generalised SjS phenotype despite the fact that SjS shows great disparities among human patients.

In-depth insight into the autoimmune process in human SjS and animal models with SjS-like diseases substantiates one of the main aetiopathological pathophysiological pathways: an increased, non-compensated, glandular apoptosis and lysis of acinar tissue. Under normal circumstances, the clearance of apoptotic cells by macrophages, dendritic cells (DC), and neighbouring tissue initiates a protective immunosuppressive and anti-inflammatory activity to regulate inflammation, whereas the clearance of lytic cells and the defective clearance of apoptotic cells can activate inflammatory responses that may result in autoimmunity.\(^4\) The intrinsic inhibition of inflammation following phagocytosis of apoptotic cells is mediated by a family of receptor tyrosine kinases (RTKs) and represents a critical mechanism for regulating inflammation and self-recognition, primarily during innate immune responses.\(^5,6\) The focus of this review is to provide an in-depth analysis of RTKs and their functions in apoptosis and autoimmunity, specifically the autoimmune process of SjS.

**SJÖGREN’S SYNDROME IN NON-OBESE DIABETIC-DERIVED ANIMAL MODELS**

Seminal studies and reviews have extensively discussed various animal models of SjS.\(^3,7,8\) Using the NOD and NOD-derived mice, our studies were able to define the genetic predisposition for development and onset of SjS-like disease in both, and offer interesting contrasts and similarities with human SjS. At first glance, SjS-like disease in mice appears to have only a weak association with major histocompatibility complex (MHC) Class I and Class II genes,\(^9,10\) thus apparently mimicking SjS in humans. However, recent human SjS genome-wide association study data have indicated that the highest statistically valued single nucleotide polymorphism association lies within the human leukocyte antigen (HLA) region.\(^11\) Based on extensive studies using our two related SjS-susceptible (SjS\(^3\)) models, NOD/LtJ and C57BL/6-NOD-Aec1Aec2, we have identified multiple physiological, molecular, and immunological features defining the underlying pathophysiology,\(^3\) thus permitting us to divide the disease process into a series of distinct, consecutive, temporal, yet overlapping phases. In the earliest phase (0–8 weeks), multiple aberrant physiological and biochemical activities predominate, associated with changes in cellular junctions, focal adhesions, and increased acinar cell apoptosis and lysis. In the subsequent phase (8–12 weeks), a strong innate autonomous cell response occurs involving the activation of interferon (IFN)-responsive genes in conjunction with the appearance first of macrophages and DC, then of transitional lymphocytes that initiate formation of the signature histological lymphocytic foci consisting mostly of CD4\(^+\) T and B220\(^+\) B cells. In the late phase (12–20 weeks and onward), characterised by an adaptive immune response, an overt clinical disease is seen, defined by a progressive, measurable loss of salivary and lacrimal gland secretory function. This exocrine gland dysfunction in SjS is thought to result from a combination of pro-inflammatory cytokine activity, synthesis of auto-antibodies reactive with the muscarinic acetylcholine and beta adrenergic receptors, plus the direct action of infiltrating T cells, including both CD4\(^+\) T helper 1 and 17 cells.\(^12\) Nevertheless, specific gene knockout and recombinant inbred lines have indicated that the
disease progression can be arrested at various stages of development and onset.

TYRO3, AXL, AND MERTK RECEPTOR Tyrosine Kinases: Structure, Ligands, and Signalling

Tyro3, Axl, and Mertk (TAM) RTKs consist of a cytosolic tyrosine kinase domain, a single hydrophobic transmembrane domain, two extracellular immunoglobulin (Ig)-like domains, and two extracellular fibronectin Type III domains. The Ig-like domains interact with the TAM ligands, either growth arrest-specific protein 6 (Gas6) or protein S (Pros1), through two C-terminal extracellular laminin G domains in a heterotetrameric complex. The TAM ligands bind to the plasma membrane of apoptotic cells at an exposed phosphatidylserine (PtdSer) site through the N-terminus gamma-carboxyglutamic acid domain. Vitamin K reduces the 4-carbon of glutamate in the gamma-carboxyglutamic acid domain, increasing its ability to bind the negatively charged PtdSer. Binding of the TAM RTKs to Gas6 or Pros1 induces autophosphorylation of cytosolic tyrosine residues, which in turn increases phosphorylation of substrates and recruitment of signalling molecules.

TAM RTKs play multiple critical roles in regulating innate immune responses, specifically inhibiting inflammatory responses that might otherwise develop from the phagocytosis of apoptotic cells. Phosphoinositide 3-kinase binds with all TAM RTKs and is responsible for the recruitment of several downstream proteins including the mammalian target of rapamycin. Phospholipase C and growth factor receptor-bound protein 2 bind with Mertk and Axl, a process that regulates calcium channels, while ran-binding protein M associates with Axl and Tyro3, but this binding has an unknown function. Although some molecular pathways can be regulated by more than one of the TAM RTKs, others have been shown to associate with a specific RTK. For example, Shc, Vav1, and activated cell division control protein 42 kinase associate with Mertk, while Nck2, SOCS 1, S-locus receptor kinase (Src)/lymphoid cell kinase, and CI-Ten associate with Axl, and protein phosphatase 1 and Src family kinases with Tyro3. Dysfunction of TAM RTK signalling can result in aberrant phagocytosis of apoptotic particles and membranes, primarily by antigen-presenting cells, leading to subsequent over-expansion of myeloid and lymphoid cell populations' targeting self-antigens that can transition subsequently to autoimmunity.

TYRO3, AXL, AND MERTK SIGNALLING IN CLEARANCE OF APOPTOTIC CELLS

The TAM RTK receptors, together with their major ligands and activations of the SOCS1 and SOCS3 molecules, play critical roles in regulating innate immune responses, particularly inhibiting inflammatory responses associated with phagocytosis of apoptotic cells. A function of GAS6 and Pros1 is to link TAM receptors to PtdSer residues expressed on the membranes of apoptotic cells and debris, facilitating phagocytosis and non-stimulatory degradation. The loss of TAM RTK receptor activations can result in aberrant phagocytosis of apoptotic cells and membranes, primarily in antigen-presenting cells, leading to subsequent over-expansion of myeloid and lymphoid cell populations, which can transition to subsequent autoimmunity or its exacerbation. Loss of normal TAM RTK receptor signalling is also characterised by upregulations of several downstream signalling pathways and pro-inflammatory factors, including toll-like receptors, p38-Mapk, Erk1/2, Traf3, Traf6, and AP-1 transcription factors that regulate multiple pro-inflammatory bioprocesses, including expression of IFN-responsive genes. Our previous studies have shown, by microarray analyses, that the genes of these factors are upregulated in the salivary glands of B6.NOD-Aec1Aec2 mice. Thus, the TAM RTKs appear to connect several early phase pathophysiological processes observed in SjS mice prior to onset of the autoimmune phase of disease.

Apoptosis is one of the major biological events that occur in the exocrine glands of human and animal models of SjS. Two factors shown to be associated with acinar tissue apoptosis are α-fodrin proteolysis and Fas/Fas-ligand interaction. First, salivary glands of NFS/sld mice appear to express the 120 kDa fragment of α-fodrin as an organ-specific autoantigen. Secondly, studies have indicated that the interaction of Fas and Fas-ligand can facilitate a cascade of events that lead to the activation of caspases and proteinases, which serve to fragment DNA.
indicated that the Fas antigen is expressed in ductal epithelial cells of SjS patients with severe sialadenitis, but not in patients with mild sialadenitis, suggesting that Fas-positive ductal cells provide a good target for effector T cells. Okuma et al. intricately demonstrated that dysfunction of epithelial cells, but not haematopoietic cells exhibiting a disruption of Stat3-mediated IκB-ζ induction, resulted in the downstream activation of self-reactive lymphocytes involved in SjS development. An intriguing question plaguing the understanding of autoimmune diseases such as SjS relates to how intracellular self-proteins become autoantigens, presented as dominant neoantigens, and recognised by immune cells following apoptosis. Rosen et al. suggested that cellular apoptosis is one of the early events in SjS development, based on redistribution of molecules within the subcellular compartments. Small membrane blebs were shown to contain the Ro-52 kDa molecule, along with other molecules such as calreticulin, which are normally present within the endoplasmic reticulum lumen. Acinar cellular apoptosis is an ongoing process in SjS that is associated with glandular infiltration by leukocytes, as prevention of high apoptosis in the exocrine glands also prevents leukocyte infiltrations and development of pathology. Thus, it seems imperative to focus on the precise relationship between apoptosis and the normal mechanisms for proper clearance of apoptotic debris, as we and others have shown what are perceived to be deficient regulatory mechanisms associated with the clearance of apoptotic debris; therefore, the focus on TAM RTK receptor-mediated biological mechanisms is discussed below.

TYRO3, AXL, AND MERTK SIGNALLING IN AUTOIMMUNITY

Expression and immune recognition of self versus non-self antigens is a process that requires constant surveillance to prevent auto-inflammation and/or autoimmunity in both predisposed and non-predisposed individuals. The TAM RTKs, via the production primarily of SOCS1 and SOCS3 molecules, represent a powerful system for regulating possible innate and adaptive responses; thus, any failure in the normal function of the TAM RTK-SOCS axis establishes an environment conducive to autoimmune activity, some of which can progress to clinical disease. A close examination of our transcriptomic data publically available at the Gene Expression Omnibus (GSE15640, GSE36378) indicated that the TAM RTK receptors and their ligands are downregulated throughout the entire process of disease progression, as depicted in Figure 1, thereby negating any normal positive feedback during apoptotic cell clearance. Data published by Lu and Lemke indicated that mice deficient in TAM RTK expressions appear to show normal peripheral lymphoid organs at birth, but by 4 weeks of age they display a marked increase in spleen and lymph node size relative to wild-type, and by 1 year of age, the spleen weights were on average 10-times that of the wild-type. The aberrant growth of peripheral lymphoid organs was primarily due to hyperproliferation and constitutively activated B and T cells. Nevertheless, Caraux et al. demonstrated that all three TAM RTK receptors plus their ligands (Gas6 and Pros1) are expressed by natural killer (NK) cells and bone marrow stromal cells, respectively, pointing to the fact that these receptors appear essential in the formation of the NK cell receptor repertoire and in the functional maturation of NK cells in the spleen, whereas Gas6 and Pros1 promoted the growth and maturation of NK cell precursors in vitro. These data suggest a crucial role of TAM receptors and their ligands in proliferation and maturation of lymphoid cells, especially in inhibiting production of autoreactive lymphocytes. As predicated, therefore, such mutants exhibit a broad spectrum of autoimmunity, including symptom-like clinical pathologies associated with rheumatic arthritis, pemphigus vulgaris, and systemic lupus erythematosus with elevated blood titres of antibodies directed against normal cellular antigens, including nucleoproteins and double-stranded DNA. It must also be noted that the Socs3 gene knockout mouse is a lethal mutation, suggesting that these systems have additional roles in overall health and development.

TYRO3, AXL, AND MERTK SIGNALLING IN SJÖGREN’S SYNDROME

In recent studies, Wallet et al. reported that DC from NOD mice deficient in Mertk expression not only played a pivotal role in negative selection of T cells within the thymus, but also failed to exhibit apoptotic cell-induced immunosuppression, resulting in a more severe...
Type 1 diabetes. The significance of these studies by Wallet and colleagues is critical due to the fact that we have previously reported a decreased production of SOCS3, together with a complete lack of any detectable upregulated Socs1/SOCS1 expression in the salivary glands of C57BL/6.NOD-Aec1Aec2 mice. This is highlighted even more by the fact that in the salivary glands of SjS-non-susceptible (SjS<sup NS></sup>) B6 mice, Socs3 is one of the highest upregulated genes observed within our microarray analysis (Figure 2). It is well documented that salivary glands of B6 mice, like most strains of inbred mice, will show varying degrees of histological evidence for lymphocytic infiltration as the mice age, but the levels of such infiltration are usually low and the composition of the infiltrate is quite different from that seen in C57BL/6.NOD-Aec1Aec2 and NOD/LtJ mice. Because the expression of Socs1 and Socs3 are strongly associated with activation of the TAM RTK receptors, these data are consistent with the concept that the TAM RTK signalling pathways in macrophages and DC of the parental B6 mice are capable of inhibiting subsequent inflammatory responses despite infiltration of salivary glands by CD4<sup+</sup> T cells, whereas this is not achieved in our SjS<sup S></sup> mice. Most importantly, TAM RTKs and their signalling pathways lead directly to transcription of Socs1 and Socs3 genes, and SOCS3 is known to play a critical role in inhibiting pro-inflammatory responses induced by macrophages and DC following engulfment of apoptotic (or infected) cells, thereby acting as a ‘go/no go’ system for innate to adaptive immune responses. The focus of the following discussion is to provide logical support to the hypothesis that the TAM receptors and their regulatory ligands represent a critical bioprocess for controlling immune homeostasis during the innate phase of an immune response. However, in the development of SjS-like disease, the normal bioprocess is either dysregulated, subverted, or actively suppressed.

Figure 1: Differential gene expression of the Tyro3, Axl, and Mertk receptor tyrosine kinase signalling in SjS<sup S></sup>-C57BL/6.NOD-Aec1Aec2 mice.

Microarray data at 8 weeks of age were presented for C57BL/6.NOD-Aec1Aec2 and C57BL/6J mice. Hybridisations were carried out with individual RNA samples using Affymetrix GeneChip® Mouse Genome 430 2.0 Array in accordance with the instructions of the manufacturer (Affymetrix, Santa Clara, CA, USA). Microarray data were normalised using the guanine-cytosine robust multi-array average algorithm and analysed using the Linear Models for Microarray Analysis package from the R Development Core Team (The R Project for Statistical Computing) to perform differential expression analyses. B-statistics (the log of the odds of a gene showing either positive or negative trends over time) were calculated for each gene. Genes exhibiting a B-statistic of >1.5 were considered differentially expressed and represents a >82% level of probability that a gene is differentially expressed. Gas6: growth arrest specific protein 6; SjS: Sjögren’s syndrome.
In addition to phagocytic activity, TAM RTK receptors and their ligands are directly involved in the suppression of inflammation by physically forming a complex with the IFN-α/β receptor signalling pathway. Humans and animal models of SjS have been shown to express elevated levels of IFN proteins, both IFN-α/β (Type I) and IFN-γ (Type II), as well as multiple IFN-regulated genes, often referred to collectively as IFN-stimulated genes. Earlier studies have indicated that primary SjS patients exhibit an activated Type I IFN system. A recent study demonstrated that the Type I IFN signature was highly upregulated in peripheral blood, while Type II IFNs predominated in gland tissues of primary SjS patients. Epigenetic mapping has identified hypomethylation of IFN-regulated genes in whole blood and B cells, and B cell methylation alterations in B cells were more rampant in IFN-regulated gene pathways. Interestingly, Hall et al. have demonstrated that 58% of SjS patients had high IFN activity and these patients were associated with a more severe disease phenotype, in particular focus score. The data suggest that the recruitment of innate cells to the salivary glands leads to high levels of IFNs in response to gland aberration. The inability of TAM receptors to regulate this rapid induction of IFNs potentially results in further gland destruction observed in SjS. Using animal models, Cha et al. revealed that high levels of IFN-γ are detected in mice of the NOD/ShiLtJ and B6.NOD-Aec1Aec2 lines as early as time of birth, and may suggest a vertical transmission of IFN during pregnancy. In contrast, if these SjS mice expressed a non-functional IFN-γ or IFN-γ-receptor encoding gene, they failed to develop any aspect of SjS-like disease, revealing an absolute requirement for IFN-γ in the development and onset of SjS. Further studies by Szczerba et al. and Nandula et al. confirmed that Type I IFN signalling is required for the development of SjS, especially in the development of the glandular pathology. As mentioned earlier, apoptotic cellular debris tends to express PtdSer; it can be recognised directly by the PtdSer receptor or several additional receptors present on phagocytic cells via bridging molecules, including the TAM receptors via Gas6 or Pros1, the integrin molecule αvβ5 via thrombospondin-1, the integrin molecule αvβ3 via the Megf8 molecule, and even the IFN receptor IFN-αR via IFN-α. In addition, phagocytic cells can bind apoptotic debris via CD31 crosslinking, CD36-oxidised low density lipoprotein, and ICAM3 crosslinking. Again, an examination of our

Figure 2: Differential gene expression of Socs1 and Socs3 and their natural inhibitors in SjS-C57BL/6. NOD-Aec1Aec2 mice. Detailed methodology was described previously. In brief, total RNAs were isolated from salivary glands of C57BL/6.NOD-Aec1Aec2 and C57BL/6J mice at 4, 8, 12, 16, or 20 weeks of age (n=5 per strain per age group). Here, data at 8 weeks of age are presented. Detailed methodology is presented in Figure 1 and elsewhere. B-statistics were calculated for each gene. Genes exhibiting a B-statistic of >1.5 were considered differentially expressed and represent a >82% level of probability that a gene is differentially expressed.
transcriptome database from B6.NOD-Aec1Aec2 mice indicate that the genes for each of these processes, except the PtdSer receptor, are highly upregulated, as are their downstream signal transduction pathways. The intrinsic inhibition of inflammation following phagocytosis of apoptotic cells is mediated, in large part, by the TAM RTKs present on phagocytic and antigen-presenting cells by interacting with the IFN-αRs. However, the importance of the TAM RTKs in regulating inflammation and self-recognition through the inhibition of innate and adaptive immune responses remains unclear. Nevertheless, there is indirect evidence of a significant role in the TAM RTK-mediated induction of SOCS1 and SOCS3. SOCS1 and SOCS3 normally inhibit the Janus kinase/signal transducers and activators of transcription (STAT) and p130 pathways, respectively, as well as the MyD88 and Trif pathways in toll-like receptor signalling. It is not surprising, then, that the transcriptome data from the salivary glands of C57BL/6.NOD-Aec1Aec2 mice indicate that neither Socs1 nor Socs3 expressions are upregulated, yet their intrinsic regulatory molecules, Trim8 for Socs1 and IRF8 for SOCS3, and SOCS2 for both Socs1 and SOCS3 each exhibit upregulated gene expressions compared to B6 mice (Figure 2).

However, Socs3 is upregulated, and even co-expressed with the pro-inflammatory cytokine interleukin 17 in SjS patients’ peripheral blood mononuclear cells and salivary gland cells, implying that there is a disruption in the normal regulatory activity of the SOCS3 against STAT3. This however reveals a greater complexity in the disease; as patients are usually diagnosed with SjS later in life it is not unlikely that Socs3-based regulation could have degenerated over the span of chronic inflammation.

**CONCLUSION**

Rheumatic diseases, especially SjS in humans, are characterised by a sustained activation of IFN-stimulated genes during the development and onset of clinical autoimmunity. Under normal circumstances, the IFN signalling pathways, and thereby any subsequent adaptive immune responses, are highly regulated. The lack of regulation results in a dysregulated and activated IFN response. We conceptualise that a series of normal activation-inhibitory interactions at the level of the macrophage during the early innate response to tissue are the underlying cause of development of adaptive autoimmunity leading to SjS.

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