Murine Cytomegalovirus-Induced Salivary Gland Disease

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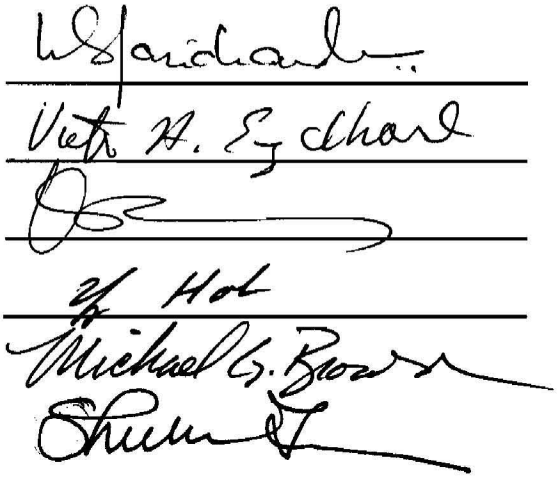

Abstract

Sjögren's Syndrome (SS) is a complex autoimmune disease with unknown etiology. The hallmarks of SS are focal inflammation and dysfunction of the exocrine glands, tissues that secrete saliva and tears. To investigate disease pathogenesis, spontaneous mouse models have been studied which recapitulate certain human disease features. For example, the non-obese diabetic mouse has been helpful in the identification of autoantibodies as one cause of salivary gland hypofunction. However, the genetic and environmental factors contributing to SS are not understood. Viruses which infect the glands have long been suspected to initiate disease. Indeed, human viruses HCV or HIV can lead to a sicca syndrome resembling SS.

In this dissertation, the ability of murine cytomegalovirus (MCMV) to induce SS disease features was characterized in the autoimmune mouse strain, NZM2328. MCMV persisted in the salivary gland for many weeks and was associated with a strong inflammatory response within the gland. After viral latency was established, severe focal inflammation in the salivary and lacrimal glands was observed in females. Focal inflammation induced by MCMV depended on genetic factors, since B6 mice were protected. MCMV infection of NZM2328 mice led to production of autoantibodies reactive with salivary and lacrimal gland proteins. In addition, secretory function was progressively lost in a subset of females. Therefore, MCMV infection of NZM2328 female mice led to many of the disease manifestations of SS.

Xerostomia, or chronic dry mouth, is a complication of SS as well as certain human viral infections. Further studies showed that MCMV impaired saliva secretion within the first few days of infection. Remarkably, secretory dysfunction early after infection did not depend on viral load in the gland or require lymphocytes or inflammatory monocytes which suggested that a soluble factor was responsible. NK cells, beyond their recognized antiviral role in MCMV infection, were critical in protecting the host from damaging inflammatory responses and functional loss of the salivary gland.

This work establishes novel virus-induced models of SS and xerostomia. It distinguishes focal inflammation and organ dysfunction as distinct disease features and defines an important role for NK cells in regulating tissue pathology after viral infection.

Dedication

I dedicate this dissertation to my parents.

As my first teachers, they instilled a love of learning that continues beyond the completion of a doctoral degree. Their support throughout my education was essential to my success.

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List of Abbreviations

7 Amino-actinomycin D	7-AAD
Antinuclear antibody	ANA
C57BL/6	B6
Chromosome	Chr
Chronic glomerulonephritis in NZM 1	Cgnz1
Days post infection	dpi
Dendritic cell	DC
Diaminobenzidine	DAB
Diffuse infiltrative lymphocytosis syndrome	DILS
Fas ligand	FasL
Female	F
Glomerulonephritis	GN
Granulated convoluted tubules	GCT
Hepatitis C virus	HCV
Histocompatibility-2	H-2
Human cytomegalovirus	HCMV
Human herpesvirus-6	HHV-6
Human T lymphotropic virus type 1	HTLV-1
Immediate early gene 1	IE1
Inhibitor of DNA binding 3	Id3
Lymphoproliferation	lpr
Male	M
Matrix metalloproteinase	MMP
Murine cytomegalovirus	MCMV
Murine embryonic fibroblast	MEF
New Zealand black	NZB
New Zealand mixed	NZM
New Zealand white	NZW
Newborn calf serum	NCS
Quantitative real-time PCR	QPCR
Salivary gland	SG
Sjogren's syndrome	SS
Submandibular gland	SMG
Systemic lupus erythematosus	SLE

Chapter I. Introduction

A. Sjögren's Syndrome

i. Disease features

Sjögren's Syndrome (SS) is a systemic autoimmune disease mainly involving the salivary and lacrimal glands. The disease disproportionately affects women and has a population prevalence of about 0.5% (1). SS is characterized by focal lymphocytic infiltration of the salivary glands and lacrimal glands, dry mouth (xerostomia), dry eyes (keratoconjunctivitis sicca), and autoantibodies to the ubiquitous Ro/SSA and La/SSB antigens. Hypergammaglobulinemia is often associated with SS, and in some cases salivary gland and/or lacrimal gland enlargement is also found (2). Primary SS occurs alone while secondary SS accompanies another autoimmune disorder, usually a connective tissue disease such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE). In addition, SS patients have a 40-fold increased risk for B-cell lymphoma (3). This complex disease results in loss of secretory function, and must be distinguished from a variety of conditions that can lead to sicca symptoms such as infections or drug therapy. Lack of saliva in the oral cavity leaves the patient susceptible to infections; similarly, lack of tear production leads to irritation, blurring of vision, and ocular surface disease. Current treatment is only palliative, relieving the discomfort of dryness. Understanding the underlying disease factors is critical in preventing and treating SS.

Rheumatic diseases often lack a single distinguishing feature and thus diagnosis relies on the presence of a specified combination of clinical and laboratory findings. For SS, the revised classification criteria defined by the American-European Consensus Group are outlined below (4).

I. Ocular Symptoms. Positive response to at least one of the following questions:

1. Have you had daily, persistent, troublesome dry eyes for more than 3 months?

2. Do you have a recurrent sensation of sand or gravel in the eyes?

3. Do you use tear substitutes more than 3 times a day?

II. Oral Symptoms. Positive response to at least one of the following questions:

1. Have you had a daily feeling of dry mouth for more than 3 months?

2. Have you had recurrently or persistently swollen salivary glands as an adult?

3. Do you frequently drink liquids to aid in swallowing dry food?

III. Ocular Signs. Positive objective test result for decreased tear flow by either Schirmer's I test performed without anesthesia (≤5 mm in 5 minutes) or Rose Bengal score or other ocular dye score

IV. Histopathology. In minor salivary glands, focal lymphocytic sialadenitis evaluated by an expert histopathologist with a focus score ≥1, defined as number of lymphocytic foci (adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue

V. Salivary Gland Involvement. Positive objective test result for decreased saliva flow by either unstimulated whole salivary flow (≤1.5 ml in 15 minutes), parotid sialography, or salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer

VI. Autoantibodies. Presence in the serum of antibodies to Ro(SSA) and/or La(SSB) antigens

Primary SS may be defined as the presence of any 4 of the 6 items, as long as either item IV (Histopathology) or VI (Serology) is positive, or the presence of any 3 of the 4 objective criteria (items III, IV, V, VI). In patients with a potentially associated disease, secondary SS is defined by the presence of item I or II plus any 2 from items III, IV, and V.

ii. Human viruses and SS-like disease

Exclusion criteria for SS list conditions which can cause similar manifestations. These include past head and neck radiation treatment, pre-existing lymphoma, sarcoidosis, graft versus host disease, and use of anticholinergic drugs. In addition, patients with acquired immunodeficiency disease caused by HIV (AIDS) or, more recently, Hepatitis C virus (HCV) infection are excluded from diagnosis of SS. These chronic viral infections can lead to a clinical picture similar to SS, but there are key differences (Table 1). For example, diffuse infiltrative lymphocytic syndrome (DILS) is a disorder in some HIV patients characterized in part by sicca symptoms. Upon biopsy of minor salivary glands, predominant CD8 T cell infiltration is observed instead of the CD4 T cells typically seen in SS (5). In addition, DILS patients lack the systemic

Table 1. Comparison of Sjögren's Syndrome and viral infections with similar disease features

Variable	Sjögren's Syndrome	DILS	Hepatitis C	HTLV-1
Sicca symptoms	Present	Present	Present	Present
Parotid gland enlargement	Moderate	Moderate to severe	Mild to moderate	May be present
Extraglandular manifestations	Pulmonary Gastrointestinal Neurologic Renal	Pulmonary Gastrointestinal Neurologic Musculoskeletal	Gastrointestinal Musculoskeletal	Pulmonary Neurologic Uveitis
Salivary gland infiltrating lymphocyte	CD4	CD8	CD4	CD8
Autoantibodies	Anti-Ro/La, RF, ANA	Low frequency RF, rare ANA and anti-Ro	Low frequency anti-Ro, high frequency RF, ANA	Unknown

DILS, diffuse infiltrative lymphocytosis syndrome; HTLV-1, human T lymphotropic virus type 1; RF, rheumatoid factor; ANA, antinuclear antibodies

Adapted from (6)

markers indicative of SS including antinuclear antibodies and rheumatoid factor (6). Another retrovirus associated with a sicca syndrome is human T lymphotropic virus type 1 (HTLV-1) particularly in endemic areas such as Japan (7, 8).

HCV can cause a disorder most like SS. Extrapolating from multiple studies, xerostomia was reported in 158 (18%) of 859 HCV patients (9). Conversely, HCV antibodies were detected in 156 (14%) of 1309 SS patients which provided grounds for its exclusion in the revised classification criteria. The prevalence of HCV in SS patients varies with geographic location and the SS criteria used. It has been difficult to differentiate SS secondary to HCV from classic primary SS since a proportion of HCV patients fulfill the outlined criteria, but there are limited studies suggesting key differences. HCV positive SS patients generally lack anti-Ro/SSA and anti-La/SSB autoantibodies, show no female bias, and display milder histopathology (10-12). Though salivary gland histopathology is on average more severe in primary SS, the composition of lymphocytic foci is similar in both groups: mainly T cells with a CD4 predominance over CD8 and a minor population of B cells (13, 14). Taken together, it is clear that HCV can cause a sicca syndrome resembling primary SS. Whether the virus causes disease directly by persistent infection of salivary glands or initiates disease by another mechanism remains a subject of debate. Of note, the presence of negative strand HCV RNA *in situ* in salivary gland epithelium has been demonstrated, indicating sialotropism in addition to its well known hepatotropism (15, 16). Therefore, persistent infection within the gland itself may lead to disease in genetically susceptible individuals.

Viruses have been implicated as a trigger for idiopathic SS. Infection of salivary gland cells themselves could initiate a pathogenic process in different ways: 1) by stimulating pathogenic anti-viral immune responses via persistent and/or reactivating virus, 2) by priming autoimmune responses to glandular antigens via inflammatory signals in the context of tissue destruction, 3) by molecular mimicry whereby T or B cells generated against viral antigens crossreact with host proteins. The salivary gland serves as a site of viral persistence and latency for a number of viruses, including herpesviruses CMV and EBV. Studies looking at the presence of CMV, EBV and HHV-6 seropositivity in SS patients have shown no increase in prevalence or antibody titers compared to healthy subjects (17, 18) in contrast to other reports that found increased prevalence of HHV-6 (36% versus 10%) (19) and elevated titers of antibodies to CMV and EBV (20, 21). The conflicting results from seroepidemiology may stem from small sample sizes or false positive results due to rheumatoid factor, found in SS patients. A majority of studies find increased detection of EBV proteins or DNA within ductal or acinar epithelium of SS patients compared to healthy controls (22-25) but not always (26, 27). Since herpesviruses are highly prevalent in the general population, it is difficult to assess their role in the etiology of SS.

iii. Mouse models of exocrinopathy

Mouse models of SS have advantages and disadvantages for studying certain aspects of the disease. Disease features of four models are shown in Table 2, though there are others with known single gene defects in immune function such as *Id3* or *Fas* (28, 29). The first model was described by Kessler in 1968 who documented spontaneous focal inflammation in salivary and lacrimal glands of NZB and NZB/NZW F1 mice by 4 months of age (30). The majority of these mice do not develop spontaneous loss of secretory function however (31).

The most widely studied model is the nonobese diabetic mouse (32) mouse. This strain develops spontaneous lymphocytic infiltrates first in the pancreas at 4 weeks, and then in other exocrine organs at about 8 weeks including the submandibular gland, lacrimal gland, and thyroid. By 24 weeks, saliva secretion is reduced to about 30% compared to 8 week old NOD or age-matched BALB/c (33, 34). Genetic studies have shown that two diabetes susceptibility loci, *Idd3* and *Idd5*, are sufficient for development of sialadenitis and dacryoadenitis with loss of salivary gland function on a C57BL/6 background (35). NOD-scid mice lacking T and B cells are protected from spontaneous glandular inflammation and loss of function, indicating a pathogenic role for these cells (36). Interestingly, impaired secretory function in NOD mice was not associated with the development of focal sialadenitis over time, but correlated with increased levels of cytokines IL-4, IFNg, and TNFa in saliva (34). The NOD mouse was particularly informative in identifying a role for autoantibodies in secretory dysfunction. This strain does not

Table 2. Mouse Models for Sjögren's Syndrome

	NZB/W F_1	NOD	d3Tx NFS/*sld*	C57BL/6-*lpr* Day 100 post MCMV
Sialadenitis	4 mo	2-4 mo	1 mo	5 mo
Secretory Function	n.d.	4 mo	18 mo	n.d.
Other organ involvement	Lacrimal	Pancreas Lacrimal Thyroid	Lacrimal	Kidney
Autoantibodies against SG		•		
Anti-Ro and anti-La		°		•
Anti-muscarinic receptor 3		•		
Anti-a-fodrin		•	•	

° indicates the lack of antibodies in serum

• indicates the presence of antibodies in serum

No symbol represents not done (n.d.)

Adapted from (37)

develop the characteristic anti-Ro/SSA or anti-La/SSB (38), but develops autoantibodies to other self proteins targeted in human SS including the muscarinic receptor M3, responsible for stimulating secretion in the salivary gland, and to α-fodrin, a ubiquitous cytoskeletal molecule whose cleaved 120 kD form is found in apoptotic glandular epithelium. IgG-purified sera from diseased NOD mice or primary SS patients reversibly decreases induced saliva secretion in $Ig\mu^{null}$ NOD mice, indicating a role for autoantibodies in decreased salivary gland function (39). Thus, the NOD mouse models the autoantibody response. Autoantibodies may be triggered by submandibular gland abnormalities intrinsic to the NOD background, as suggested by altered gland morphology in the neonatal mouse (40). Compared to C57BL/6 mice, neonatal NOD salivary glands display increased expression of Fas and FasL and decreased acinar proliferation. In addition, the authors found increased glandular expression of MMP-2 and MMP-9, enzymes that regulate epithelial branching morphogenesis during glandular development. Thus, both immune and non-immune factors contribute to the development of SS-like disease in NOD mice. A disadvantage of this model is the development of diabetes which is not associated with human SS.

Another model of SS is the thymectomized NFS/*sld* mouse (41, 42). This strain has a mutation affecting sublingual gland differentiation (*sld*). Four weeks following thymectomy at day 3, infiltration of the submandibular, parotid, lacrimal, but not sublingual gland occurs. While this model was useful for identifying the α-fodrin autoantigen, these thymectomized mice do not develop secretory dysfunction until after

18 months of age (43) indicating that the presence of inflammation does not necessarily incite functional loss. The specific consequences of thymectomy are not defined.

In both the NOD and NFS/*sld* models, an underlying genetic alteration in glandular morphology or homeostasis in combination with a propensity for autoimmunity precedes disease. Environmental insults to the salivary gland alter homeostasis as well. One group has described virus-induced focal sialadenitis in mice which have a defect in Fas-mediated apoptosis, an important mechanism of downregulating T cell responses (44). When C57BL/6-*lpr/lpr* mice are infected with murine CMV (MCMV), following acute infection they develop chronic sialadenitis without the presence of infectious virus. 100 days post infection (dpi), the submandibular gland displayed modest mononuclear infiltrates with 1-2 foci per lobe. This strain does not spontaneously develop significant salivary gland disease but exhibits glomerulonephritis after 6-8 months of age due to the Fas mutation. Since C57BL/6 mice do not develop MCMV-induced sialadenitis, it was concluded that Fas-mediated apoptosis is necessary for downregulation of the immune response in the salivary gland. The authors did not determine the impact of focal inflammation on secretory function. Though there are limitations to its usefulness as a model of SS given the modest focal inflammation in context of a known genetic defect, the observations of Fleck prompted the question of whether viral infection of the gland may elicit SS-like disease in an immunocompetent host.

B. Murine Cytomegalovirus

i. Characteristics of infection

CMVs belong to the β-herpesvirus subfamily. They are characterized by strict species specificity, tropism for hematopoietic tissue and secretory glands, and lifelong infection (45). Depending on the immunocompetence of the individual, the virus can exist as a low-level persistent infection or remain latent with periodic reactivation. It is estimated that 50-80% of people in the United States are infected by the age of 40 (46). CMV infection is generally asymptomatic, but can cause severe disease in newborns or the immunocompromised, particularly AIDS patients and organ transplant recipients. In these individuals, clinical manifestations which can cause significant morbidity and mortality include hepatitis and pneumonitis. CMV is most likely transmitted via close contact with bodily fluids from an infected host since the virus can be shed in saliva, tears, urine, breast milk, and genital secretions for months to years.

MCMV infection of mice resembles HCMV infection of humans and thus has been used as a model to study pathogenesis and viral immune responses. MCMV spreads via a myelomonocytic cell type in the blood to many tissues and replicates in various cell types, including epithelium, endothelium, myocytes, bone marrow stromal cells, dendritic cells and macrophages (47). HCMV can abortively infect lymphocytes *in vitro* (48) but there is a general consensus that lymphocytes are rare targets of infection. Disseminated infection in organs such as spleen, liver, kidney, adrenals, and lung is controlled by the immune system within 1-2 weeks, but virion production persists in the salivary gland at high levels for 1-2 months, a result of viral immune evasion. The tropism for salivary

glands is conserved among CMVs of different species (49). Virus obtained from mouse salivary gland homogenates is more virulent than *in vitro* passaged virus, and is routinely used as a source for experimental stocks (50). The mouse salivary gland is composed of three sets of lobes, the submandibular, parotid, and sublingual. In particular, ultrastructural studies show that MCMV predominantly replicates in the seromucous acinar cells of the submandibular gland, the largest lobe (51). The nuclei of infected acinar cells become enlarged with intranuclear inclusions and hundred of virions are visualized within cytoplasmic vesicles. By hijacking the specialized secretory pathway of acinar cells, the vesicles rupture at the apical side and release virions into the lumen for secretion through the ducts. Because cortisone treatment prevented necrosis of acini based on electron micrographs, Henson and Strano concluded that MCMV infection of the salivary gland is not cytolytic in itself, but induces a prolonged destructive immune response resulting in epithelial cell death and regeneration. However, MCMV is also considered a cytopathic virus since tissues such as liver exhibit cytopathogenic effect in immunocompromised recipients (49).

Following resolution of productive infection, MCMV establishes latency. Latency is classically defined as the point when infectious virus cannot be recovered from the various organs in a viral plaque assay (52). Whether MCMV achieves true molecular latency or persists at a low level has been addressed with sensitive plaque assays detecting 1 PFU; BALB/c mice achieve latency without low-level persistence of infectious virions in spleen, kidney, salivary gland and lung (53, 54). This evidence does not preclude low-level persistence in hosts with other genetic backgrounds. It is clear that

a functional viral DNA genome is retained since the virus can reactivate in various organs once immune control is withdrawn, either by immunodepletion regimens, γ-irradiation, or tissue explant (55-58). In one mouse model of latency, depletion of T and NK cells caused reactivation in 100% of mice by 14 days (59). Reactivation occurred most rapidly in salivary glands, followed by lungs and spleen. Thus, lymphocytes actively control MCMV latency, mediated in part by IFNγ. It should be noted that these studies were performed in μMT-/- mice to aid in detection of reactivated virus. Previous work has shown that B cells and antibodies are not required for resolution of infection, but do play a role in limiting recurrent virus after immunodepletion (60). MCMV can also reactivate in the lung after bacterial sepsis or direct intraperitoneal administration of inflammatory cytokines TNFα and IL-1β in immunocompetent mice (61, 62). Given that MCMV-infected hosts are exposed to many other pathogens throughout life, control of MCMV latency is therefore an active process and depends on constant immunosurveillance.

Following infection of adult mice, latency has not been reported to cause disease in immunocompetent hosts though one group has characterized mild myocarditis 100 days post infection which depended on genetic background (63, 64). Though not outwardly pathogenic, broad alterations in T cell subsets have been documented in CMV seropositive individuals. CMV infection of humans and mice elicits a unique pattern of memory inflation, in that during viral latency large populations of CMV-specific T cells accumulate over the lifetime of the host (65, 66). One mouse study found 20% of all CD8 T cells at 1 year post infection specific for a single MCMV epitope, IE1, suggesting repetitive antigen stimulation (67). Furthermore, these MCMV-specific T cells are

functional *ex vivo* which contrasts sharply with clonally exhausted T cells found in other models of persistent infection (68, 69). In summary, other than the expansion of specific T cells, relatively little is known about the consequences of long-term infection.

ii. MCMV immune responses

Both innate and adaptive immunity are critical for MCMV control. Resolution of primary infection is a coordinated effort of cytokines, NK cells, and T cells. After intraperitoneal infection, systemic cytokine responses peak at 36 hours. These include type I interferons, IL-1, IL-6, IL-12, IFNγ and TNFα among others (70, 71). Cytokines have direct antiviral functions but also shape downstream immune responses by enhancing effector functions of NK cells and by regulating adaptive T cell responses. In contrast to these beneficial effects, excessive production of systemic cytokines or "cytokine storm" can lead to cachexia, a wasting disease characterized by fever, severe weight loss, fatigue, and loss of appetite. These manifestations can be observed in mice under high viral burdens and may cause mortality in extreme cases. Therefore, regulation of inflammatory cytokines is paramount to preserving the health of the host and promoting antiviral immunity.

NK cells are critically important in defense against herpesviruses, as evidenced by recurrent infections in NK cell deficient patients (72, 73). The mouse model of infection has repeatedly shown that NK cells rapidly control MCMV titers in various organs during the first few days of infection (74-76). NK cells are activated by cytokines or by specific signals through various cell surface receptors. NK cells, but not NKT, mediate virus control in the spleen and liver by killing infected cells via perforin and granzymes and by producing IFNγ (77). Depletion of NK cells with monoclonal antibodies either before or

during persistent infection have shown a role for NK cells in controlling salivary gland virus as well (74, 78). Studies to date have focused on responses in *Cmv1* mice, where a C57BL/6 derived genetic interval encoding the NK activating receptor Ly49H enables recognition of the virally encoded m157 protein on infected cells. Efficient early control of virus replication by NK cells has striking effects on downstream immune responses. One outcome of efficient viral control is limiting IFNα and IL-12 production by plasmacytoid DC (70). This results in prevention of DC loss and acceleration of CD8 T cell priming compared to susceptible BALB/c mice. On the other hand, inefficient NK cell-mediated virus control was linked with high type I IFN and delayed but exaggerated CD8 T cell responses. Thus, in addition to their well-described role in limiting viral burden, NK cells can regulate both innate and adaptive immune responses.

T cells are required for the termination of productive MCMV infection and establishment of latency. Animals deficient in T cells cannot control viral replication and die in a matter of weeks (79). In particular, cytotoxic CD8 T cells mediate defense in all tissues except the salivary gland. In this organ, CD4 T cell production of IFNγ is required (80, 81). It is not clear why the salivary gland is exempt from $CD8^+$ T cell control, although viral proteins which interfere with MHC Class I antigen presentation contribute (82). The inflammatory infiltrate within the salivary gland during infection has been characterized in BALB/c mice (83). CD8 T cells are recruited to the gland in high numbers 2-4 weeks post infection along with smaller numbers of γδ T cells and CD4 T cells. Interestingly, the number of DCs in the gland is also increased by nearly 40 fold. High levels of IFNγ produced by both CD4 and CD8 T cells and IL-10 produced by CD4

T cells are found (84). IL-10 is a key modulator of CD4 T cell IFNγ responses and actively contributes to virus persistence in the salivary gland. Eventually, CD4 T cells control glandular MCMV to undetectable levels. The immune response to MCMV is multifaceted and reflects the highly evolved interplay of virus and host.

C. New Zealand Inbred Mice

i. Origins of lupus-prone NZM2328 mice

Inbred mouse strains are instrumental for investigating the pathogenesis and genetic basis of disease. F_1 hybrids of New Zealand Black (NZB) and New Zealand White (NZW) mice were first described in 1963 to develop renal failure with histological similarities to human SLE (85). In 1980, an accidental mating of a female F_1 with a littermate (F_2) or its NZW father (N_2) resulted in offspring with mixed coat colors (Figure 1). Some of these mice were bred for their unusual coat colors of grey or tan with pink eyes. Inbreeding began with a single grey pair. Mice were selected first by coat color, then by renal pathology and differences in time to disease. Twelve of 27 original inbred lines homozygous for H-2^z were characterized for lupus nephritis through 10 months of age (86). Six lines survive today and have varied renal disease phenotypes: NZM 2328, 2410, 2758, 64, 88 and 391 (87). NZM2328 mice display disease features most like human SLE, including acute and chronic glomerulonephritis, severe proteinuria, and autoantibodies ANA and anti-dsDNA. This spontaneous disease model mimics the female predominance observed in patients, as the incidence of severe proteinuria is higher in females compared to males, 70% vs. 20% by 12 months of age (88). NZM2328

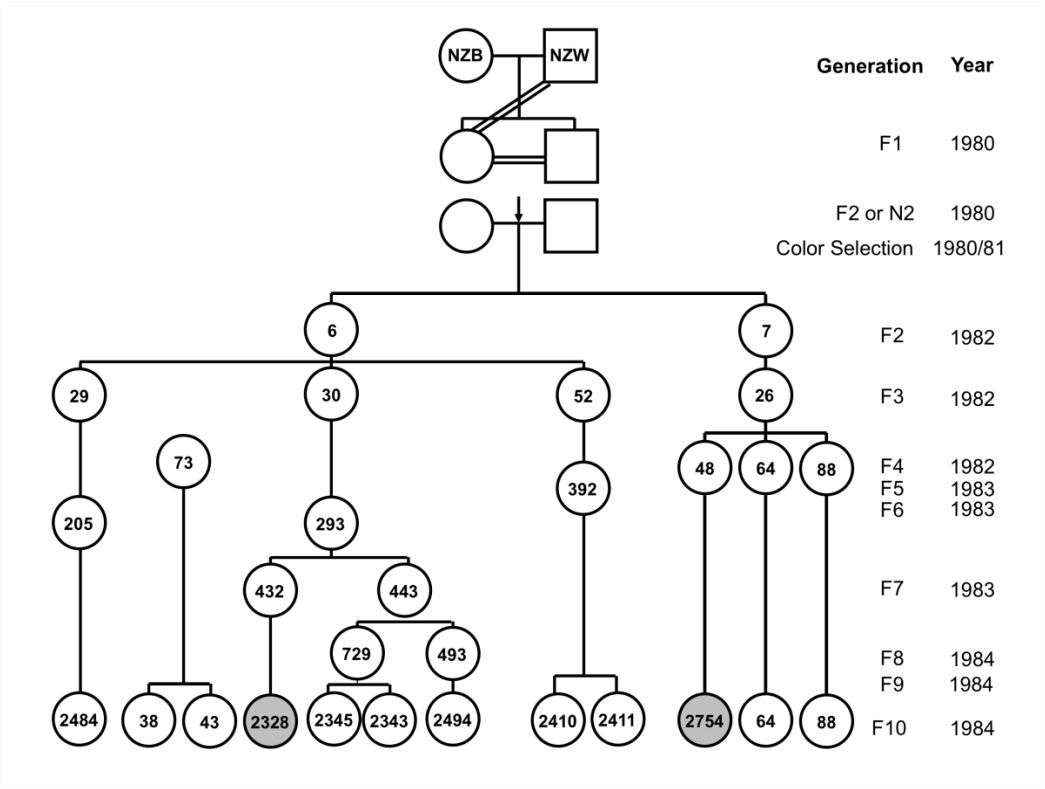

Figure 1. Origins of New Zealand Mixed strains. Mice used in this study are shaded and include NZM2328 and NZM2758 (derived from 2754).

females begin to show severe disease at 5 months. In contrast to NZM2328, NZM2758 is resistant to lupus-like disease despite sharing a majority of alleles (87). Due to their inherent genetic similarities yet distinct disease phenotypes, the panel of NZM strains and parental strains NZB and NZW offer a unique genetic system to understand and examine disease pathogenesis.

ii. NZM mice: a rich genetic resource to examine autoimmunity and disease

Much progress has been made on defining genetic contributions to lupus susceptibility in NZM2328. Notably, backcross analysis of (NZM2328 X C57L/J)F_1 X NZM2328 identified a significant genetic linkage on chromosome 1 with chronic GN and severe proteinuria (*Cgnz1*). In addition, a locus on chromosome 4 was suggestively linked to plasma levels of anti-dsDNA autoantibodies (*Adnz1*) (88). Subsequently, congenic strains were created whereby the genetic intervals linked with disease were replaced with those from C57L/J, a non-lupus prone strain (89). NZM.C57Lc1 are protected from kidney disease, while NZM.C57Lc4 are not. Both congenic strains display reduced incidence of autoantibodies to dsDNA and nuclear antigens. These studies show that anti-dsDNA and ANA production and chronic GN are under separate genetic control and that breaking tolerance to these antigens is not required for disease pathogenesis. The *Cgnz1* locus has been refined by generation of the NZM.Lc1R27 recombinant congenic strain. Though a majority of R27 female mice develop acute GN, they do not progress to chronic GN indicating that acute GN is under independent genetic control and does not necessarily lead to renal failure (90). The genes on chromosome 1 that are responsible for the development of chronic GN have been narrowed to a 1.3 Mb region containing 45 genes.

Overexpression of some of these genes has been documented in kidney and lymphocytes from young NZM2328 vs. R27 mice. Therefore, intrinsic abnormalities in the immune compartment as well as the target organ may contribute to SLE pathogenesis.

iii. Innate resistance of NZ mice to MCMV

Innate immunity to MCMV is under genetic control. NZ mice have been utilized as an alternative genetic system to study *Cmv1*-independent genes required for early viral resistance (91, 92). Relative virus levels in the various strains are shown in Figure 2 ((92), reprinted with permission). NZW mice control virus levels in spleen and liver 3.5 days post infection by an NK-dependent mechanism termed *Cmv2*. In contrast, NZB are relatively susceptible. Genetic mapping for MCMV resistance trait loci in (NZW X NZB)F_2 cohorts identified a chromosome X locus that confers MCMV resistance in F_2 male mice. Further study is required to identify the specific gene or genes which may impact innate viral immunity in NZ, however it is clear that *Cmv2* represents a complex genetic trait that is independent of known mechanisms of MCMV control including Ly49H and H-2.

NZM mice (all H-2^z) vary in innate immunity to MCMV (92). Pertinent to the current study, NZM2328 are moderately susceptible to MCMV particularly in the spleen. NZM2758 display slightly higher splenic virus levels than NZM2328. Despite high virus levels, NZM2328 and NZM2758 do not exhibit clinical signs of infection such as decreased activity, rough haircoat, or squinted eyes (unpublished observations). Moderate weight loss (7-10%) is observed 2-3 days post infection in both resistant NZW and susceptible NZM2328 mice ((92) and data not shown). Therefore, virus levels and

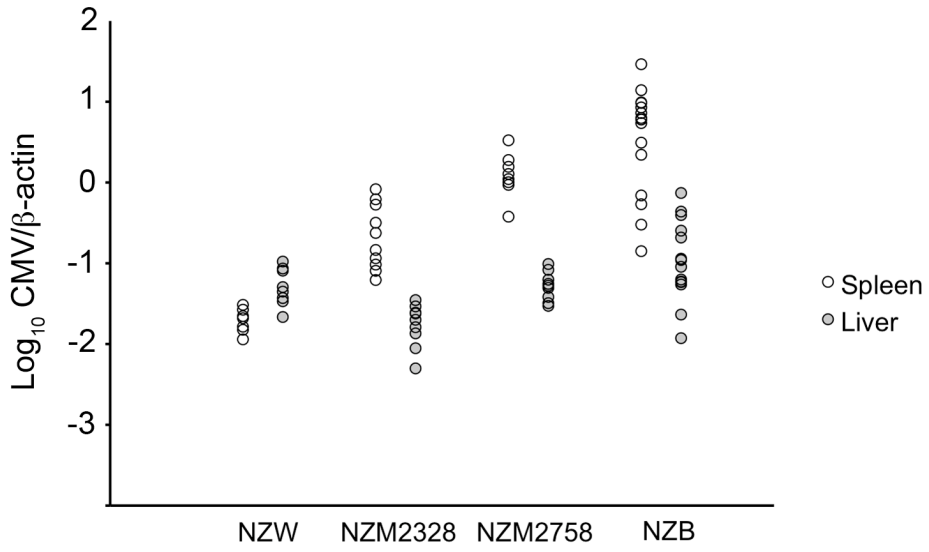

Figure 2. Assessment of early MCMV control in NZ mice. Mice were infected with 10^5 PFU MCMV. Spleen and liver MCMV levels at day 3.5 are shown. In the spleen, NZW virus levels are significantly lower than all other strains ($p < 0.01$ by Mann Whitney test).

clinical manifestations are not correlated at a viral dose of 10^5 PFU. At this high dose, no differences in viral immunity are observed between male and female NZ animals. In summary, NZ inbred mice are a valuable tool to uncover novel genetic modifiers of innate immunity to MCMV as well as the pathogenesis of autoimmunity.

D. Salivary Gland Function

i. Mechanisms of normal secretion of saliva

Saliva, a hypotonic solution of water, ions and proteins, serves many important functions in the oral mucosa. Lubrication is provided by mucins, while the first steps of digestion begin with enzymes such as amylase and lipase. Proline rich proteins maintain the integrity of teeth by controlling mineralization, and antimicrobial components lysozyme, lactoferrin, and IgA protect the mucosal surface from infection (93). The salivary glands are divided into three pairs of lobes: the parotid, sublingual and submandibular. In mice, the submandibular gland is the largest and is composed of seromucous acini, granulated convoluted tubules (GCTs), ductal epithelium, a dense network of blood vessels, and some myoepithelium. Secretion begins within acini, a cluster of specialized epithelial cells. Resting salivary flow is stimulated by acetylcholine released from parasympathetic nerves located throughout the gland. Acetylcholine binds to M3 receptors on acinar cells and results in water transport to the acinar lumen mainly through aquaporin 5 (94, 95). Fluid then travels through the ductal system and is enriched with proteins produced by the GCTs. Secretion of salivary proteins is controlled by the sympathetic nerves acting on α– and β-adrenergic receptors. An enhancement of saliva flow rate can occur through

sympathetic stimulation by mastication or taste. Signaling events downstream of M3 receptor activation have been studied in great detail and reveal many points of regulation (96). For example, the mobilization of Ca^{++} required for opening ion channels and creating an osmotic gradient depends on the levels of many second messengers such as cyclic ADP-ribose and nitric oxide (NO). Thus, secretion is a highly coordinated process involving many molecular players and can instantaneously adapt to the needs of the animal.

ii. Measures of secretory function

Unlike humans, unstimulated salivary flow in mice is not measurable. Therefore, salivary gland function is measured by the volume of saliva produced in response to muscarinic receptor agonists. Current methods allow collection of saliva directly from the oral cavity without resorting to cannulation with anesthesia which can reduce salivary flow itself (97, 98). Pilocarpine is a muscarinic receptor agonist naturally found in the South American shrub *Pilocarpus jaborandi* (99). Interestingly, leaves of this plant were originally used in rituals by Brazilian natives to cause profuse sweating and salivation. In 1873, this was observed by visiting French physicians and then introduced to Western medicine. It is commonly used to treat xerostomia under the trade name Salagen (100). In mice, a weight-adjusted dose of pilocarpine hydrochloride is administered intraperitoneally and the volume saliva secreted over time is collected. For this study, a low dose of 0.5 mg/kg was used to stimulate a gradual response over 25 minutes (Figure 3A). We found that responses to pilocarpine are sex and strain dependent thus appropriate controls are required. Assessment of salivary gland function can be

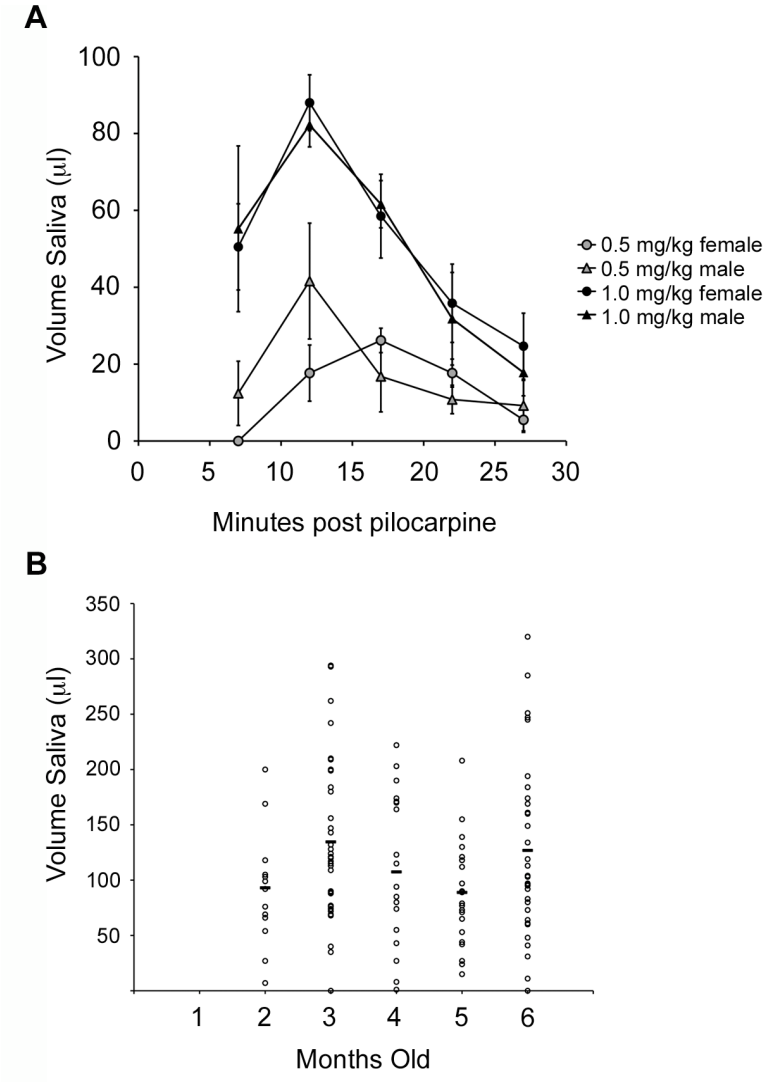

Figure 3. Saliva secretion assay. A, Dose response to pilocarpine in NZM2328 mice at 6 months of age. Beginning 2 minutes post injection, saliva was collected in 5 minute increments and measured by micropipette. 5-6 mice per group, error bars indicate SEM. B, Saliva production in untreated or PBS treated NZM2328 females over time. Data are accumulated from all studies performed. Total volume in 25 minutes is plotted.

repeated on the same animal with reproducible results, and average secretion does not change over the experimental time course of 6 months in untreated animals (Figure 3B). Agonist-induced saliva secretion serves as a direct readout of salivary gland function and allows for the evaluation of xerostomia in mice.

iii. Xerostomia: a variety of causes

Xerostomia, or dry mouth, affects many patient populations besides SS. This list includes those treated with radiation for head and neck cancers, recipients of anticholinergic and other drugs (101), methamphetamine abusers (102), patients with biliary cirrhosis, graft versus host disease, and certain chronic viral infections described above. It is generally thought that xerostomia can originate from direct damage to acinar cells, defects in fluid transport, or cholinergic dysfunction, however experimental evidence is limited. Rat models of irradiation-induced xerostomia show serous acini are particularly radiosensitive and become damaged (97). This may be due to a richness of heavy metals like Zn, Mn and Fe in secretory granules which can oxidize lipids upon radiation exposure, causing leakage of lytic enzymes and autolysis (103). The exact causes of xerostomia in other conditions remain elusive. Liver damage is found in methamphetamine abusers, biliary cirrhosis and chronic HCV infection and is hypothesized to be one common link between these disparate conditions and xerostomia (104, 105).

Acute MCMV infection has recently been used as a correlative model to examine a possible link between viral hepatitis and xerostomia (106). Not surprisingly, elevated liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

were found in sera of BALB/c mice 5 days after infection, the peak of viral hepatitis. At this point, mice secrete about 50% of normal before reaching a transient nadir of 8% secretion at day 7. Mice carrying *Cmv1* resistance were relatively protected from liver damage and hyposalivation after infection, though the mechanism was not addressed. How MCMV infection is causing acute secretory dysfunction is an important question.

Rationale and Research Goals

The genetic and environmental factors that control the development of SS are poorly understood. It is hypothesized that viruses may initiate the disease which includes focal sialadenitis, autoantibodies, and loss of secretory function. While viruses such as HCV can cause similar manifestations during active infections, a role for viruses in SS has not been substantiated. Human studies are limited by the fact that the infectious insult and disease are separated in time. In addition, a virus causes disease in only a subset of people based on genetic factors. An animal model of virus-induced SS would enable the mechanistic study of disease. To physiologically mimic human SS, the model would utilize a natural infection of an immunocompetent host.

Xerostomia is a debilitating condition of SS and of various human viral infections. Current treatment is palliative. Understanding the mechanism of glandular hypofunction will eventually lead to novel therapies and improved quality of life for these individuals. It is hypothesized that viral infection of salivary glands leads to tissue damage by the immune system, resulting in salivary deficiency. Specifically, lymphocytes or other inflammatory cells may destroy glandular acini and result in secretory dysfunction.

Therefore, the following aims are proposed:

Specific Aim 1. Establish a virus-induced mouse model of Sjögren's Syndrome

CMV was chosen to induce SS-like disease due to its tropism for salivary glands in mouse and human. SS disease features after MCMV infection of the autoimmune mouse strain NZM2328 are characterized.

Specific Aim 2. Investigate mechanisms of virus-induced secretory dysfunction

Using a novel mouse model of virus-induced xerostomia, the roles of 1) viral burden, 2) inflammatory monocytes and 3) lymphocytes including NK and T cells in secretory dysfunction are tested.

Chapter II.

Severe Focal Sialadenitis and Dacryoadenitis in NZM2328 Mice Induced by MCMV: A Novel Model for Human Sjögren's Syndrome

Ohyama, Y., Carroll, V.A., Deshmukh, U., Gaskin, F., Brown, M.G., and Fu, S.M. *Severe focal sialadenitis and dacryoadenitis in NZM2328 mice induced by MCMV: a novel model for human Sjögren's syndrome.* J Immunol, 2006. **177**(10): p. 7391-7.

Abstract

The genetic and environmental factors that control the development of Sjögren's Syndrome, an autoimmune disease mainly involving the salivary and lacrimal glands, are poorly understood. Viruses which infect the glands may act as a trigger for disease. The ability of sialotropic MCMV to induce acute and chronic glandular disease was characterized in an autoimmune prone mouse strain, NZM2328. MCMV levels were detectable in the salivary and lacrimal glands 14-28 days after intraperitoneal infection and correlated with acute inflammation in the submandibular gland. After latency, virus was undetectable in the glands by PCR. At this stage, NZM2328 female mice developed severe chronic periductal inflammation in both submandibular and lacrimal glands in contrast to the much milder infiltrates found in female B6-*lpr* and male NZM2328. The focal infiltrates consisted of $CD4^+$ and $B220^+$ cells as opposed to diffuse $CD4^+$, $CD8^+$, and $B220^+$ cells during acute infection. Salivary gland functional studies revealed a gender-specific progressive loss of secretory function between day 90 and 125 post infection. Latent MCMV infection did not significantly affect the low incidence of autoantibodies to Ro/SSA and La/SSB antigens in NZM2328 mice. However, reactivities to other salivary and lacrimal gland proteins were readily detected. MCMV infection did not significantly alter the spontaneous onset of kidney disease in NZM2328. Thus chronic inflammation induced by MCMV with decreased secretory function in NZM2328 mice resembles the disease manifestations of human Sjögren's Syndrome.

Introduction

Sjögren's Syndrome (SS) is a systemic autoimmune disease characterized by focal lymphocytic infiltration of the salivary and lacrimal glands, dry mouth (xerostomia), dry eyes (keratoconjunctivitis sicca), and autoantibodies to the ubiquitous Ro/SSA and La/SSB antigens. The disease disproportionately affects women and has a population prevalence of about 0.5% (1). Primary SS occurs alone while secondary SS accompanies another autoimmune disorder, usually a connective tissue disease such as rheumatoid arthritis or systemic lupus erythematosus. In addition, SS patients have a 40-fold increased risk for B-cell lymphoma. This complex disease results in loss of secretory function and must be distinguished from a variety of conditions that can lead to sicca symptoms such as active infections or drug therapy (4). The underlying causes of SS are unknown. It is thought that an environmental insult to the gland causes cell death in context of danger signals, which initiates an autoimmune response to the gland in genetically predisposed individuals.

A clinically important sialotropic virus is CMV. This prevalent β-herpesvirus persists in its host for life. Depending on the immunocompetence of the individual, the virus can exist as a low-level chronic infection or remain latent with periodic reactivation. Human CMV preferentially replicates in the ductal epithelium of the salivary glands, while murine CMV (MCMV) principally replicates in the acinar cells of the submandibular gland (SMG) long after it is cleared from other organs (83, 107). MCMV infection of the salivary gland is not cytolytic in itself, but induces a prolonged destructive immune response resulting in epithelial cell death and regeneration (51).

Thus, CMV dissemination in mice and humans involves viral replication in salivary gland epithelium and consequent immune cell destruction of the gland. However, it is not known whether immune cell exposure to salivary gland antigens during acute virus infection and glandular destruction may elicit autoreactive lymphocytes that can initiate autoimmune disease even after viral latency is established.

B6-*lpr* mice have been reported to develop mild chronic sialadenitis following acute infection without the presence of infectious virus (44). In this study, we sought to determine the ability of MCMV to induce SS-like disease in an immunocompetent autoimmune prone strain, NZM2328. This strain spontaneously develops SLE-like disease features with a female bias beginning around 5 months of age, including anti-dsDNA antibodies, glomerulonephritis, and severe proteinuria (88). Here we show that although MCMV replicates to very high levels in the SMG and lacrimal glands, it is efficiently cleared from the exocrine glands after 8-10 weeks. In addition to recruitment of mixed inflammatory cells during active infection, MCMV caused severe chronic focal inflammatory lesions in both the submandibular and lacrimal glands when virus was undetectable by PCR. Other features of human SS, including salivary gland dysfunction and autoantibody production, were investigated to evaluate MCMV infection of NZM2328 mice as a novel model for Sjögren's Syndrome.

Results

Kinetics of MCMV clearance in the salivary and lacrimal glands. Delayed MCMV clearance in the salivary gland of B6-*lpr* mice compared to B6 has been reported (44). We sought to determine the kinetics of viral clearance in the autoimmune prone strain NZM2328. SMG MCMV levels were assessed over a 100 day time course. As shown in Figure 4, 14-28 days post infection (dpi) infectious MCMV was readily detected in the SMG of all strains; NZM2328 female virus titers were highest at 14 dpi. At later times, MCMV titers declined until it was no longer detectable by plaque assay in all groups (Figure 4A). A second NZM2328 cohort infected with a lower dose of an alternate MCMV virus stock gave similar results by QPCR (Figure 4B). Importantly, QPCR detection of MCMV genomes from the SMG correlated with detection by plaque assay, although the second cohort cleared MCMV more rapidly as active MCMV replication was not apparent in NZM2328 after day 56, possibly because less MCMV was injected into this cohort.

To determine whether high MCMV levels and consequent tissue pathology are unique in the SMG, we also examined the lacrimal glands of MCMV-infected mice. As with SMG virus levels, lacrimal gland MCMV was readily detected in NZM2328 mice by 14-28 dpi, but overall virus levels were substantially lower (Figure 4C).

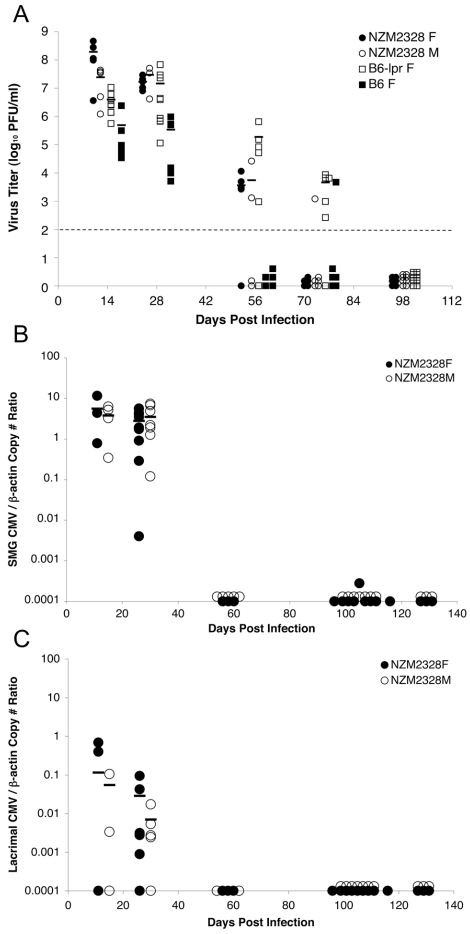

Figure 4. Kinetics of MCMV infection in the salivary and lacrimal glands. A, Female and male NZM2328, female B6-*lpr* and female B6 mice were infected i.p. with MCMV (Stock 1, 10^6 PFU). Infectious MCMV levels in SG homogenates were quantified on MEFs by plaque assay. The limit of detection is indicated (dotted line). B & C, NZM2328 mice were infected i.p. with MCMV (Stock 2, 10^5 PFU). SMG and lacrimal gland DNA samples were quantitated by QPCR. The limit of detection in this study was 0.0001 ie1/β-actin copy number ratio. SMG MCMV was detected by QPCR after 56 days in 1 of 15 female (F) mice.

Acute and chronic inflammatory lesions in NZM2328 after MCMV infection. During acute MCMV infection, when virus is abundant in the SMG, a diffuse mixed leukocytic infiltrate is found throughout the SMG (83). This type of inflammation can be distinguished from chronic inflammatory lesions with large foci (aggregates >50 mononuclear cells) including T and B cells. B6-*lpr*, but not B6 mice, develop mild focal inflammation at 100 dpi (1-2 foci per lobe of SMG), despite the absence of detectable virus (44). To examine whether the autoimmune prone strain NZM2328 also develops chronic inflammation in the salivary gland upon MCMV infection and whether it was specific to this organ, we examined exocrine glands (submandibular, parotid, sublingual, and lacrimal gland) and kidneys to assess the type and severity of inflammation histologically. The latest time point studied corresponds to almost 6 months of age, the time at which females of this strain may begin to spontaneously develop glomerulonephritis, mild chronic sialadenitis and dacryoadenitis.

Table 3 summarizes the histological findings after MCMV infection in the SMG. Two doses of MCMV were used with similar results. For days 14-28, the period when high titers of infectious MCMV were detected in SMG of all mice, diffuse infiltration of mixed inflammatory cell types was observed in all groups. The most severe infiltrations were seen in the SMG of female NZM2328 at day 14 and day 28, such that glandular epithelium was not visible (Figure 5). Diffuse infiltration of inflammatory cells was much milder in the SMG of male NZM2328 and female B6-*lpr*. The acute infiltration decreased at day 56 and was no longer apparent by day 75 in all groups. The presence of diffuse infiltrates correlated with the presence of virus. Acute inflammation was fairly

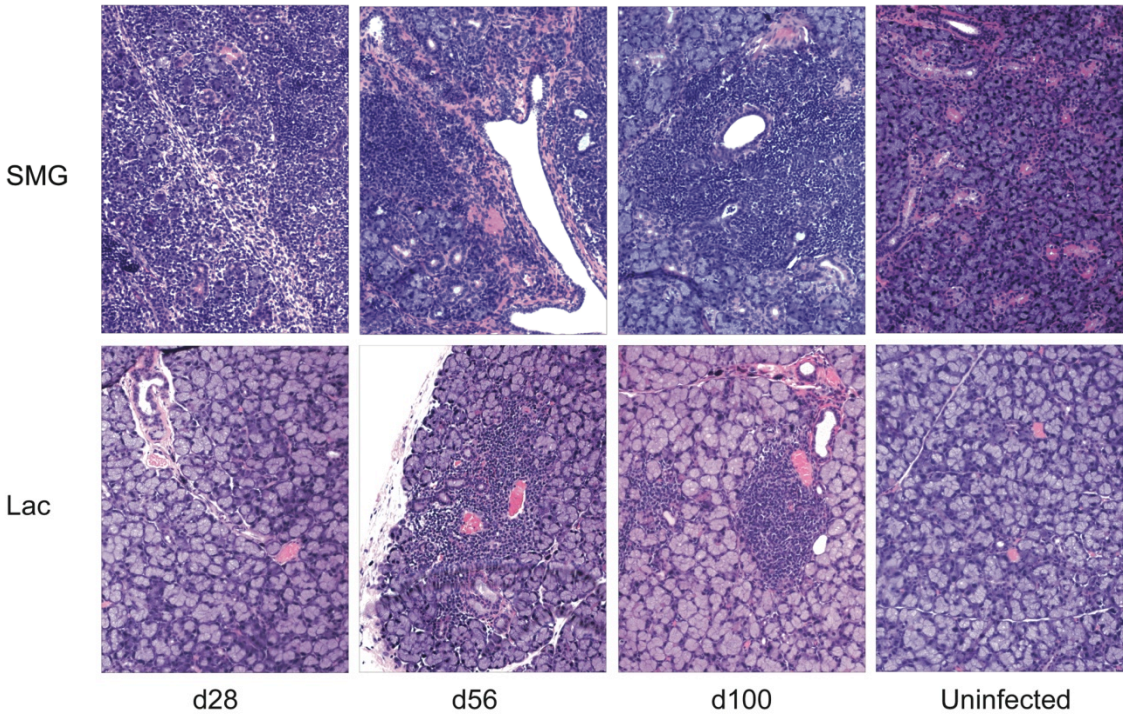

Figure 5. Acute and chronic inflammation after MCMV infection. NZM2328 female mice were infected with MCMV (10^5 PFU). At various times post infection, tissues were formalin-fixed, sectioned, and stained with H&E. Note the diffuse SMG infiltrate at day 28 during acute virus infection. This was followed by development of large periductal foci in both the SMG and lacrimal gland at day 56 and day 100 during viral latency. Uninfected mice were age matched to d100, corresponding to 5.5 months of age. Images shown at 100x.

Table 3. Summary of Histological Analysis of SMG

		Days Post Infection					
		14	28	56	75	100	Control (100)
10^6 pfu MCMV Stock 1[a]							
	n	5	9	6	6	8	4
NZM2328 F	Acute-Diffuse	2.8	2.4	1	0	0	0
	Chronic-Periductal	0	0.2	2.2	3.3	2.9	0.5
	n	5	5	5	6	7	6
NZM2328 M	Acute-Diffuse	1	2	0.2	0	0	0
	Chronic-Periductal	0	0	1.6	2	1.4	0
	n	6	8	6	6	9	4
B6-*lpr* F	Acute-Diffuse	1.5	1	0.6	0	0	0
	Chronic-Periductal	0	0.4	0.3	0.8	0.7	0

[a]Diffuse inflammation of mixed cell types scored 0 = none, 1 = diffuse inflammatory cell infiltration less than 2500/mm^2, 2 = diffuse inflammatory cell infiltration less than 10,000/mm^2, 3 = diffuse inflammatory cell infiltration more than 10,000/mm^2.
Chronic inflammation scored 0 = none, 1 = one periductal mononuclear cell focus/4 mm^2, 2 = two periductal mononuclear cell foci/4 mm^2, 3 = more than three periductal mononuclear cell foci/4 mm^2, 4 = confluent infiltration with glandular destruction.

10^5 pfu MCMV Stock 2[b]							
	n	3	6			12	6
NZM2328 F	Acute-Diffuse	2.2	4.3			0	0
	Chronic-Periductal	0	0			1.9	0.4
	n	4	3			9	7
NZM2328 M	Acute-Diffuse	0.5	1.5			0	0
	Chronic-Periductal	0	0			0.7	0.1

[b]Acute inflammation scored 0 = none, 1 = diffuse infiltrate <25% area, 2 = 25-50% area, 3 = 50-75% area, 4 = 75-100% area, 5 = 100% area. Chronic inflammation scored as above.

Table 4. Summary of Histological Analysis of Lacrimal Gland

		Days Post Infection			
		14	28	100	Control (100)
10^5 pfu MCMV Stock 2[a]					
	n	3	6	12	4
NZM2328 F	Acute-Diffuse	0	0	0	0
	Chronic-Periductal	0	0	2.2	0.8
	n	4	3	9	1
NZM2328 M	Acute-Diffuse	0	0	0	0
	Chronic-Periductal	0.5	0	0.2	0.5

[a]Acute inflammation scored 0 = none, 1 = diffuse infiltrate <25% area, 2 = 25-50% area, 3 = 50-75% area, 4 = 75-100% area, 5 = 100% area. Chronic inflammation scored 0 = none, 1 = one periductal mononuclear cell focus/4 mm^2, 2 = two periductal mononuclear cell foci/4 mm^2, 3 = more than three periductal mononuclear cell foci/4 mm^2, 4 = confluent infiltration with glandular destruction.

restricted to the SMG, since slight infiltration (histological score 1) was found in the sublingual and parotid glands of all groups for days 14-28 (data not shown).

Interestingly, as diffuse inflammation abated over time in the SMG, MCMV-infected NZM2328 females developed focal mononuclear cell infiltration in the periductal area that was evident at 56 dpi and severe by 75-100 dpi. Focal inflammation was restricted to the SMG since infiltrates were rarely observed in the mucinous sublingual and serous parotid glands. This is of interest since MCMV prefers to replicate in the mixed acinar epithelium of the submandibular gland. Severe focal inflammation persisted through 200 dpi without detectable virus while spontaneous sialadenitis of uninfected age matched controls was only mild if even present (Figure 5 and data not shown). In comparison to female NZM2328, the size and number of foci were reduced in male NZM2328 and female B6-*lpr* at all time points studied after day 56. These findings suggest that MCMV-induced chronic inflammation in the SMG is dependent on both genetic and sex-linked factors.

In addition to the SMG, the lacrimal gland was also studied for histological changes (Figure 5, Table 4). Curiously, despite the presence of MCMV at 14-28 dpi, diffuse inflammation could not be found in this exocrine organ. However, at 100 dpi NZM2328 females developed focal periductal dacryoadenitis that was more severe than age matched controls. The lacrimal glands of NZM2328 males were unaffected by MCMV infection, and few infiltrates were seen in B6-*lpr* females (data not shown). The lacrimal gland findings support the notion that NZM2328 females are uniquely susceptible to MCMV-induced focal inflammation in the exocrine glands. MCMV infection did not significantly

increase the incidence of severe proteinuria in NZM2328 females by 28 weeks of age (140 dpi, data not shown).

Changes in the composition of the mononuclear cell infiltrate over the course of MCMV infection. To characterize the cell populations associated with the acute and chronic infiltrate, SMG were obtained from NZM2328 females at different times following MCMV infection and analyzed by immunohistochemistry (Figure 6). At day 28 after MCMV infection, when high titers of infectious MCMV were detected, diffuse infiltration of mixed inflammatory cells including Th1.2+, CD4+, CD8+ and B220+ cells were observed throughout the SMG. In contrast, at day 100 after MCMV infection, when MCMV became undetectable, mononuclear cells infiltrated around ductal structures in defined areas and CD8+ cells were not found. CD4+ and B220+ cells segregated in separate areas and a predominance of $CD4^+$ T cells over $B220^+$ cells was generally observed. These immunohistochemical findings are similar to those of SS (13).

Focal sialadenitis was accompanied by interstitial fibrosis and ductal destruction evident by Trichrome staining (Figure 7). In contrast to an uninfected age-matched control gland, blue staining collagen surrounding the ducts indicative of basement membrane structure was missing in NZM2328 females 100 dpi. In addition, apoptosis of lymphocytes and SMG ductal epithelium was detected 100 dpi by TUNEL staining that was not found in an uninfected age-matched control gland (data not shown). These histological findings suggest that previous MCMV infection of NZM2328 females

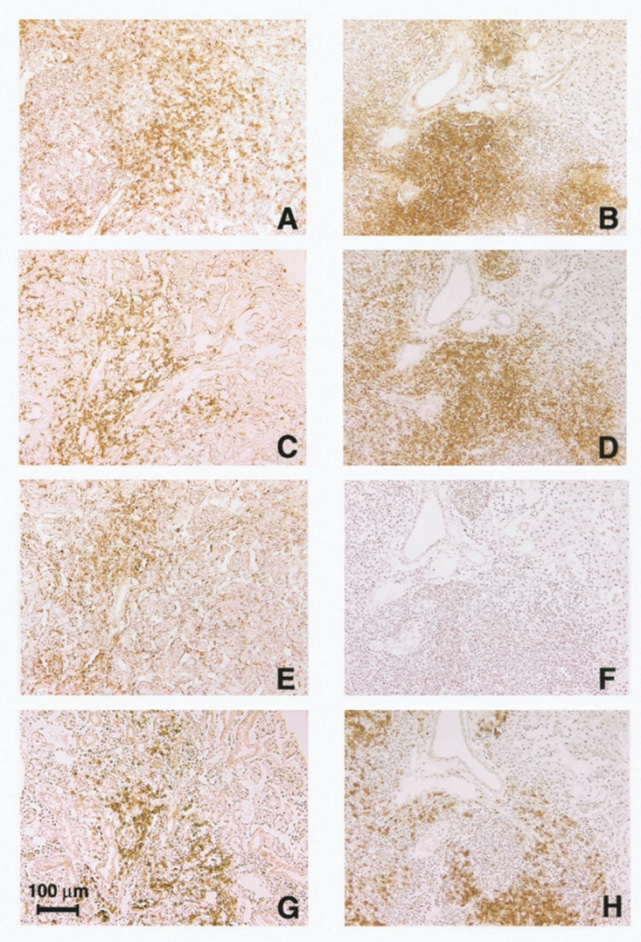

Figure 6. Composition of infiltrating cells in the submandibular gland in female NZM2328. Frozen serial sections were stained with monoclonal antibodies and detected with diaminobenzidine (brown). Left, 28 days post infection; right, 100 days post infection. A & B, Thy1.2; C & D, CD4; E & F, CD8; G & H, B220. Note the absence of CD8 cells and the separate areas of $Thy1.2^{+}$ and $B220^{+}$ cells at the chronic time point (100 dpi).

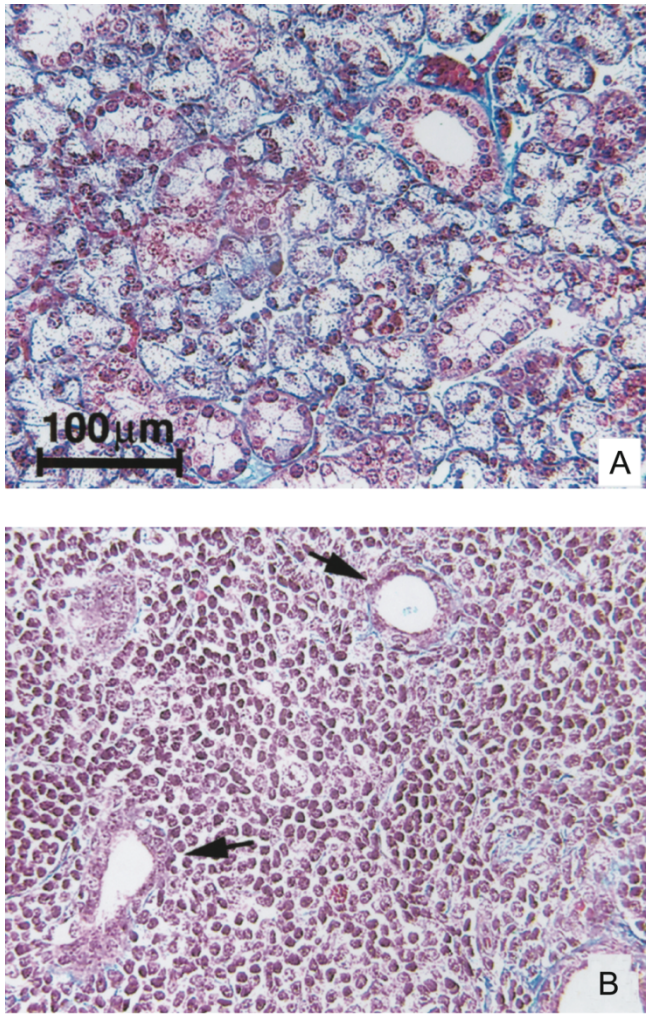

Figure 7. Submandibular gland destruction in female NZM2328. Formalin-fixed tissue sections were stained with Trichrome to visualize collagen basement membrane surrounding ducts (arrows, blue stain). A, Normal submandibular gland showing contiguous staining around the ducts in an age matched control. This is a representative of 2 sections. B, Disruption of basement membrane 100 dpi of 10^6 PFU MCMV. This is a representative of 5 sections.

compromises the structural integrity and homeostasis of the submandibular gland after 100 dpi.

Loss of saliva production in MCMV-infected NZM2328 with chronic inflammatory lesions. To further investigate the consequences of MCMV-induced chronic inflammatory lesions within the salivary gland, we measured salivation following induction with pilocarpine, a muscarinic receptor agonist. In uninfected NZM2328 control animals saliva yields did not change over the course of study (Figure 8A). Unexpectedly we found that saliva yields were higher in infected animals than in age- and gender-matched control animals (Figure 8B and 8D). However, despite having increased pilocarpine-induced salivation during latent virus infection, only MCMV-infected NZM2328 females progressively lost this function between 90-125 days after infection (Figure 8B and 8D). Thus, MCMV infection in NZM2328 leads to gender-specific differences in inflammatory lesions observed in the exocrine glands that apparently coincide with the onset of salivary gland dysfunction.

Production of autoantibodies in MCMV-infected NZM2328. In addition to salivary gland dysfunction, SS patients often display autoantibodies to Ro/SSA and La/SSB antigens. MCMV infection did not lead to elevated levels of anti-Ro60, anti-Ro52 or anti-La/SSB autoantibodies in NZM2328 or B6-*lpr* (Figure 9). Since

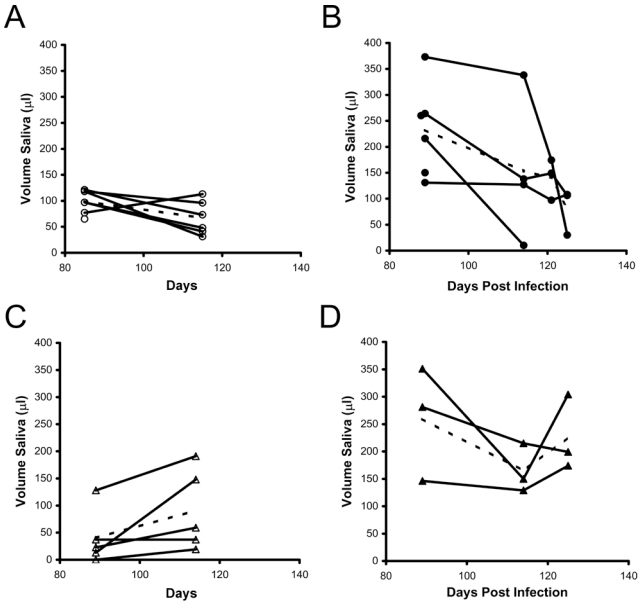

Figure 8. Salivary gland function is altered upon MCMV infection in NZM2328 mice. The secretory response to pilocarpine (0.5 mg/kg body weight) over a continuous 25 minute collection period is reported as total volume saliva. Individual mice were followed over a time course corresponding to 90-125 days post infection with 10^5 PFU MCMV from Stock 2. Dashed line indicates the mean of each group. A. Uninfected female NZM2328 (n=6). B. MCMV-infected female NZM2328 (n=6, 3 animals sacrificed prior to 125 days post infection for histology) C. Uninfected male NZM2328 (n=5). D. MCMV-infected male NZM2328 (n=3).

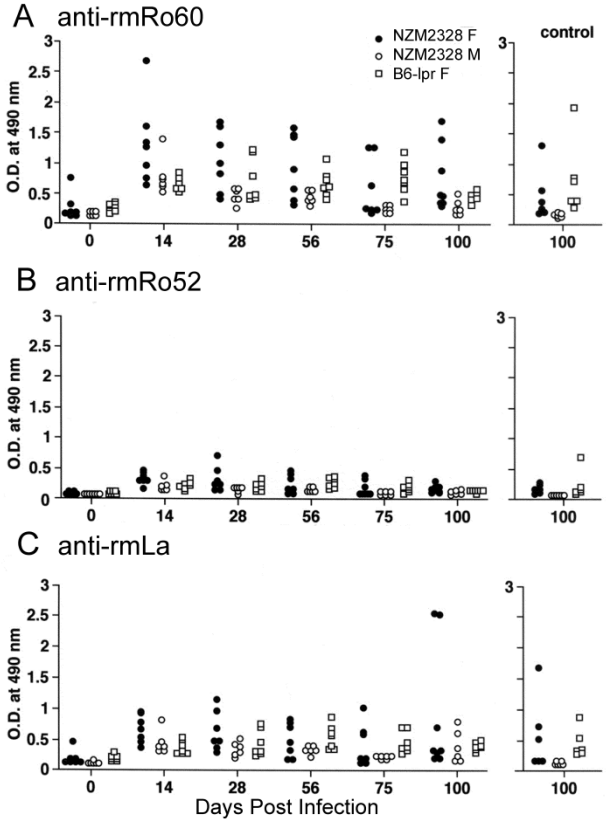

Figure 9. MCMV infection does not enhance the development of autoantibodies against Ro and La. Sera from indicated mice were collected at various times post infection with 10^6 PFU MCMV from Stock 1 or from uninfected mice at the 100 day time point corresponding to 5.5 months of age. Relative titers of autoantibodies against recombinant murine (rm)Ro60, rmRo52, and rmLa were determined by ELISA at 1:250 dilution.

autoantibodies to salivary and lacrimal gland antigens such as anti-muscarinic receptor 3 have also been documented in SS (108), we examined whether organ targeted autoantibodies develop in MCMV-infected NZM2328. As shown in Figure 10, autoantibodies recognizing exocrine gland antigens are apparent in infected NZM2328 females. In addition, we could immunoprecipitate three distinct protein bands from a human salivary gland cell line extract with one of these sera that were not similarly immunoprecipitated using control sera (Y.O. and S.M.F., unpublished data). Taken together, these data substantiate a role for viral induction of autoantibodies to glandular antigens.

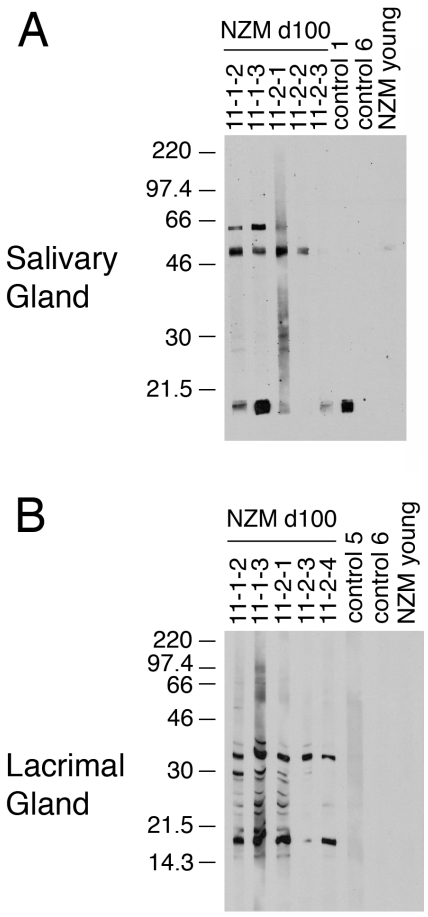

Figure 10. Development of autoantibodies to salivary and lacrimal gland antigens upon MCMV infection. Organ homogenates (50 μg/lane) from an uninfected mouse were probed with sera diluted 1:100 from the following mice: NZM2328 females 100 days post infection (NZM d100), age matched NZM2328 females (46), 8-12 week old NZM2328 female (NZM young). Five representative day 100 sera are shown. Molecular weight in kD is indicated.

Discussion

In this study, we show that NZM2328 lymphocytes respond to acute MCMV infection of the exocrine glands with similar kinetics and vigor as for other strains. However, NZM2328 female mice were distinguished by severe acute and chronic inflammation in the salivary and lacrimal glands. Focal inflammation persisted for at least 200 days without detectable replicating virus. This phenotype is not a general feature of MCMV-induced pathogenesis since B6 mice do not develop such lesions (44). As previously shown, we also noted delayed SMG viral clearance and mild chronic infiltrates in MCMV infected B6-*lpr* since Fas may play a role in apoptosis of infected glandular epithelium and Fas-mediated cell death of lymphocytes is important for the downregulation of immune responses. Although immunocompetent NZM2328 mice are genetically prone to develop spontaneous lupus-like disease including anti-dsDNA autoantibodies and acute and chronic glomerulonephritis, MCMV did not accelerate the incidence of severe proteinuria in this study (Figure A1), suggesting that MCMV-induced pathology in the salivary and lacrimal glands does not accelerate overt autoimmune disease in general. Furthermore, MCMV failed to elicit anti-Ro60 or anti-La autoantibodies. Instead, autoantibodies with specificity for salivary and lacrimal gland antigens were detected in latently infected NZM2328 females when focal inflammation in the exocrine glands was severe. Though the target antigens have yet to be identified, we did not observe significant reactivity with proteins in the size range of α-fodrin (120 kD), a previously discovered target antigen in SS. Correspondingly, a progressive loss in

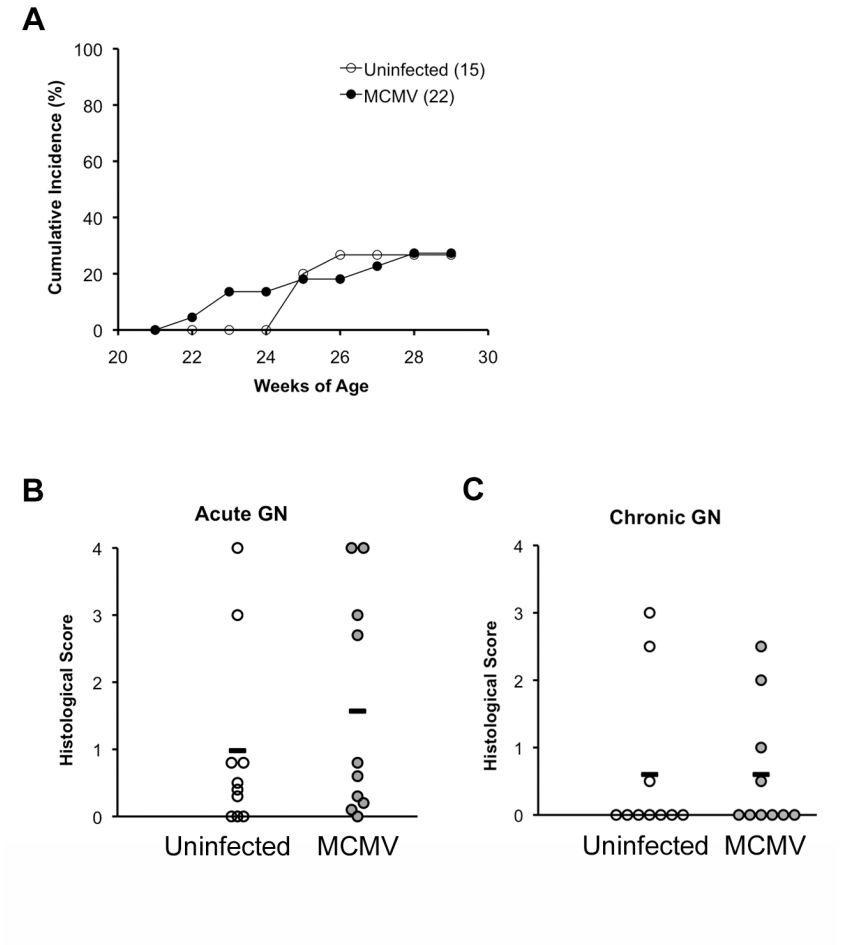

Figure 11. MCMV does not accelerate kidney disease in NZM2328 females. A, Cumulative incidence of proteinuria as measured by urine dipstick. B and C, Acute and chronic GN was evaluated at 6 months of age by a pathologist.

salivary gland function was also observed in NZM2328 females during latent infection. Interestingly, salivary gland function was enhanced by 90 days post infection in NZM2328. This phenomenon remains to be explained but may be due to epithelial regeneration after infection. Taken together, MCMV infection of NZM2328 females displays various features of human SS.

While MCMV is cleared from systemic organs after 1-2 weeks, it persists in the SMG for 6-10 weeks depending on viral dose and mouse strain. Recent work indicates viral interference with antigen presentation to $CD8^+$ T cells contributes to viral persistence of this organ (82). $CD4^+$ lymphocytes have a key role in restraining MCMV (80), and salivary gland derived virus is more virulent than that derived from other organs or grown *in vitro* (109). The SMG therefore provides an important niche for efficient MCMV replication, dissemination, and evasion of host immune responses. MCMV tropism and relative persistence in the SMG may be important for induction of chronic inflammation in this organ in NZM2328 after virus is cleared.

We also noted focal dacryoadenitis in latently infected NZM2328 females. The lacrimal and salivary gland both drain to the cervical lymph nodes, which were enlarged in many of the affected animals (V.C. and M.G.B., unpublished observations). There are several potential explanations for the organ specific infiltrates. Since both of the exocrine glands become infected during early times, $CD4^+$ cells specific for viral peptide may accumulate to control sporadic reactivation from latency in the target organ. Low level transcription and translation of viral gene products may be sufficient for activation and retention of lymphocytes in the tissue. In this case, the dysregulation of the immune

response inherent to NZM2328 would be responsible for the chronic inflammation observed since B6, a non-autoimmune prone strain, is protected. The nature of immune dysregulation in NZM2328 is under investigation by other groups. Of note is the finding that NZM2328 do not possess a global defect in $CD4^{+}CD25^{+}$ T_{reg} cells, however they may require a higher threshold for suppression of particular d3tx-induced autoimmune diseases, namely glomerulonephritis and sialadenitis but not dacryoadenitis, thyroiditis, or prostatitis (110). On the other hand, some NZM2328 mice spontaneously develop mild infiltrates into the submandibular and lacrimal glands after 7 months of age. Therefore NZM2328 may possess an intrinsic potential for autoimmune lymphocytes specific for glandular self-antigen, and viral infection may activate these cells perhaps through massive glandular destruction during acute infection. A third possibility is molecular mimicry (111), where the MCMV response cross-reacts with glandular antigens. Each of these scenarios is consistent with a genetically programmed failure to control immune responses in the salivary and lacrimal glands, whether to foreign or self antigen. The specificity of the infiltrating cells in our model remains an open question, and a more detailed study of the SMG-infiltrating lymphocytes is important to delineate these possibilities.

In summary, we describe the development of focal inflammation in the submandibular and lacrimal glands upon MCMV infection of NZM2328 female mice. Furthermore, severe focal inflammation was accompanied by production of autoantibodies to glandular antigens and a progressive loss of salivary gland function. These manifestations support MCMV infection of NZM2328 as a novel virus-induced

model for human SS. Viral infection and autoimmune disease have long been associated, however the mechanism remains elusive. Our model offers a novel system to study the intricacies of the relationship between virus and host, and highlights the interplay of virus and host factors in development of organ-specific chronic inflammation.

Chapter III.

NK Cell Regulation of Salivary Gland Inflammation and Disease after Viral Infection

Abstract

The salivary gland represents a major site of persistent viral replication for many viruses, including cytomegalovirus. We established a mouse model of salivary gland dysfunction after acute viral infection and investigated the cellular requirements for loss of secretion. MCMV infection severely impaired saliva secretion independently of salivary gland virus levels. Lymphocytes or circulating monocytes/macrophages were not required for secretory dysfunction. Dysfunction occurred before glandular inflammation, suggesting a soluble mediator initiated the disruption of acinar cell function. Despite genetic differences in innate resistance to MCMV, NK cells protected the host against acinar atrophy and loss of secretions under conditions of exceedingly low virus inoculum. Therefore, beyond their recognized role in controlling MCMV replication, NK cells preserve organ integrity and function by regulating the innate inflammatory response.

Introduction

Cytomegalovirus, a beta-herpesvirus, establishes widespread infection which culminates in a persistent infection of the salivary glands (49). The specialized secretory machinery of glandular epithelial cells is utilized by the virus for highly efficient virion production and for excretion in saliva for long periods of time. While CMV infection can cause morbidity and mortality in neonates and immunocompromised individuals, infection of immunocompetent individuals is largely asymptomatic. Within the salivary gland, the ability of CMV to both evade immune responses and hijack the secretory pathway without pathological consequences is an elegant example of co-evolution between virus and host. Indeed, the ability to secrete saliva must be preserved for both virus transmission and the well-being of the host.

Several human viruses have been associated with sicca symptoms. An association of sicca syndrome with herpesviruses CMV or EBV has been hypothesized, but supporting studies are limited due to the high prevalence of these viruses in the population, variable times of infection, and differing methods of viral detection (22, 23, 26, 27). Diffuse infiltrative lymphocytosis syndrome (DILS), a disorder of HIV-positive patients, is characterized in part by sicca symptoms (112). In addition, one study found submandibular gland secretion of early-stage HIV patients is decreased 50-60% compared to controls (113). Chronic HCV infection causes a sicca syndrome in a proportion of patients and was recently excluded from Sjögren's syndrome classification criteria, differentiating it as a separate disease entity (4, 114, 115). The mechanism(s) by which viral infections cause secretory dysfunction are unknown.

Loss of saliva secretion, or xerostomia, is a common disorder shared by a diverse group of patients, including those suffering from SS, primary biliary cirrhosis, side effects of drugs or radiation therapy, and viral infection. Of these, SS has been studied in most detail yet the cause of dysfunction is not fully understood. Accumulating evidence in humans and mice suggests dysfunction may not require glandular destruction (96). For example, SS patients often display only partial destruction of salivary gland tissue, and some respond to pilocarpine indicating the remaining tissue is functional. Anti-muscarinic autoantibodies have been documented to inhibit secretory function in mice (39). Other hypotheses proposed to explain dysfunction of the salivary glands include changes in water channels, neurological abnormalities, and activation of innate immunity (116). The causes of secretory dysfunction are likely heterogeneous.

Mouse models of virus-induced dysfunction are needed. NZM2328 mice spontaneously develop systemic lupus erythematosus-like disease features with a female bias beginning at 5 months of age (88). Infection of NZM2328 mice with MCMV induces SS-like disease, characterized by severe focal inflammation of the exocrine glands (117). Loss of secretory function occurred in only a subset of these infected mice, indicating focal inflammation was distinct from organ dysfunction. To focus on mechanisms of virus-induced dysfunction, we examined secretion over the course of infection in this strain. By examining mice early after MCMV infection, we sought to understand causes of virus-induced dysfunction and how the immune response regulates this consequence of infection.

Natural killer cells are lymphocytes of the innate immune system important in tumor and viral immunity. Upon stimulation, they can rapidly produce pro-inflammatory cytokines such as IFNγ and kill transformed or virally infected cells. NK cells have a critical role in control of viral replication and survival after MCMV infection (74, 118). There is evidence that NK cells may serve an immunoregulatory role by producing IL-10 under extreme inflammatory conditions. First, NK-derived IL-10 has been shown to temper pathogenic CD8+ T cell responses after MCMV infection of perforin 1-deficient mice, though IL-10 was not detectable in NK cells of wild-type mice (119). In addition, NK cells can provide IL-10 to dampen IL-12 release by dendritic cells after systemic infection with disseminating pathogens like *T. gondii* (120). Given that NK cells are essential innate immune responders to MCMV infection, we hypothesized that they may protect the host from damaging inflammatory responses targeting the salivary gland.

We examined the consequences of MCMV infection during the first week of infection, including sialadenitis and secretory dysfunction. We investigated the mechanism of loss of secretions using several strategies to ablate lymphocytes or other inflammatory leukocytes in the model of virus-induced salivary gland dysfunction. These studies revealed a novel regulatory role of NK cells in limiting downstream tissue damage after MCMV infection.

Results

MCMV infection leads to secretory dysfunction

The acinar epithelium of the salivary gland is an important site of replication for cytomegalovirus. The ability of the gland to secrete saliva during active viral infection was investigated. The functional output of the salivary gland can be measured by the volume of saliva collected after administration of pilocarpine, a muscarinic receptor agonist. All NZM2328 mice failed to produce saliva 7 days post intraperitoneal infection with 10^5 PFU MCMV (Figure 12A). Salivation was virus dose dependent; infection with as little as 400 PFU of MCMV decreased saliva output, while 100 PFU had no effect. Virus levels in the spleen, an indicator of systemic viral load, and the salivary gland were also virus dose dependent yet there were notable differences between the two organs (Figure 12B, 12C). While spleen virus levels increased proportionately with virus dose over most of the range tested, infection with the highest dose of 10^5 PFU led to a substantial increase in virus load. This may be reflective of the inability of host immunity to control splenic viral replication above a certain threshold. In contrast, salivary gland virus levels were relatively insensitive to virus inoculum; virus was easily detected in all animals and increased only slightly over the dose range.

C57BL/6 mice are relatively resistant to MCMV in comparison to NZM2328 mice (91). Therefore, we expected low virus levels and functional salivary glands in infected C57BL/6 mice. Not surprisingly, MCMV replication was effectively undetectable in C57BL/6 spleen by day 7 at all but the highest dose (Figure 12B, right).

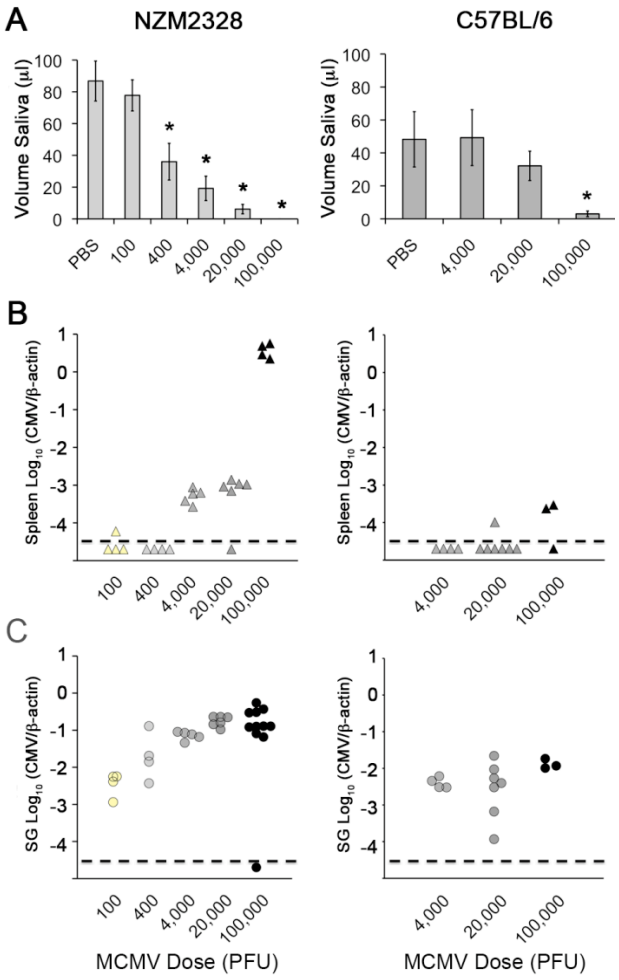

Figure 12. Titrated MCMV infection causes salivary gland dysfunction in both susceptible and resistant mice. A, The secretory response to pilocarpine was determined 7 days post infection of female mice with the indicated doses of MCMV (PFU). Saliva volumes are reported mean ± SEM, *$p < 0.05$ compared to PBS control. B, Spleen virus levels were quantitated by QPCR at day 7. C, Salivary gland (SG) virus levels were quantitated by QPCR at day 7. The limit of detection is denoted by the dotted line.

Despite this, MCMV replicated in the salivary gland in C57BL/6 to levels only slightly lower than NZM2328 highlighting the efficient dissemination and replication of the virus in this organ (Figure 12C). Only the highest virus dose of 10^5 PFU caused secretory dysfunction in C57BL/6 mice (Figure 12A). Therefore, in comparison to C57BL/6 mice, NZM2328 mice were more susceptible to virus-induced dysfunction. The inability to control splenic virus levels to undetectable levels after the first week of infection correlated with secretory dysfunction.

MCMV-induced secretory dysfunction after high dose infection does not require lymphocytes

Loss of secretion in NZM2328 mice was most severe at day 7 post high dose infection. At this point, both innate and adaptive systems are activated and could affect secretion. Because of their prominent role in early MCMV defense, and the influx of NK1.1+ and CD3+ cells into the salivary gland observed at day 7 in NZM2328 (data not shown), we tested the role of NK and T cells in MCMV-induced salivary gland dysfunction at day 7 (Figure 13). First, we found that MCMV continued to cause dysfunction in NZM2328 mice when NK cells were depleted with PK136 mAb. (Figure 13A). Salivary gland virus levels were slightly elevated after NK depletion, confirming a role for these cells in salivary gland virus control even under high dose conditions (Figure 13C, compare to 12C). Next, the role of T and B cells in MCMV-induced secretory dysfunction was tested in NZM2328 mice genetically deficient in Rag1. NZM2328.Rag1 mice secreted normally, while high dose MCMV infection caused near complete loss of secretion at day 7, similar to

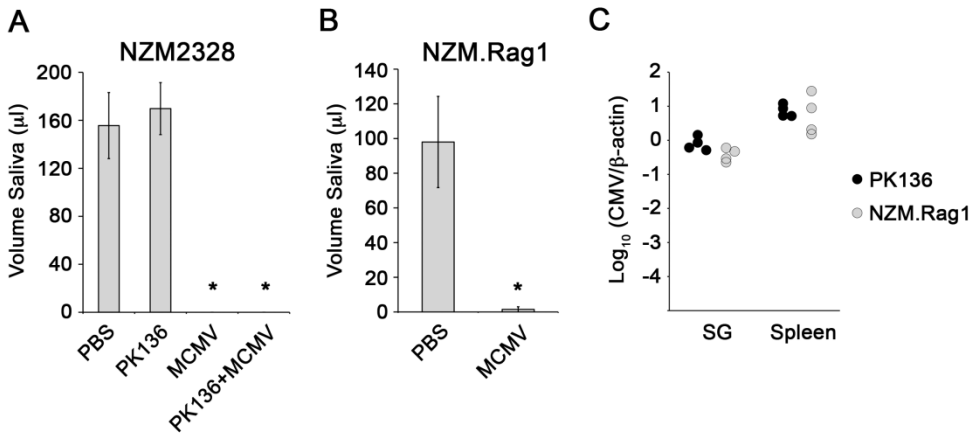

Figure 13. MCMV-induced salivary gland dysfunction does not require lymphocytes after high dose infection. A, The secretory response was determined 7 days post infection of female NZM2328 mice with 10^5 PFU MCMV with or without NK depletion. Saliva volumes are reported mean ± SEM (n=4-5), *$p < 0.05$ compared to treatment with PK136 alone. B, The secretory response to pilocarpine was determined 7 days post infection of female NZM.Rag1 mice with 10^5 PFU MCMV. Saliva volumes are reported mean ± SEM (n=4), *$p < 0.05$ by Student t test. C, Virus levels in salivary gland and spleen were quantitated by QPCR at day 7 of NK depleted NZM2328 (black) and NZM.Rag1-/- (grey) mice. No differences were found compared to NZM2328 virus levels (Figure 12).

NZM2328 (Figure 13B). NZM2328.Rag1 virus levels in the spleen and salivary gland were relatively unchanged compared to NZM2328 mice indicating virus levels are independent of T and B cells after high dose infection (Figure 13C, compare with 12B, 12C). Taken together, these data strongly indicate that under high dose conditions lymphocytes are not causing dysfunction after MCMV infection at day 7.

The relationship between salivary gland virus level and secretory dysfunction was investigated over time. MCMV seeds the gland and establishes high titer replication during the first week of infection. Therefore, it was anticipated that impaired saliva secretion would parallel the increase of virus levels. Instead, we found that NZM2328 mice displayed secretory dysfunction as early as day 2 despite low or undetectable MCMV in the salivary gland after high dose infection (Figure 14, A and B). C57BL/6 were also dysfunctional at day 2 (Figure 15A). Virus levels in the spleen increased from day 2 through 7 (Figure 12B, 14B). Glandular dysfunction was sustained through day 7 (Figure 12A). Thus, early loss of secretion at day 2 was not linked to salivary gland virus levels after initial infection, but did correspond to high virus replication in the spleen.

Secretory dysfunction can occur before inflammatory infiltration of the salivary gland

To better understand the mechanism of salivary gland dysfunction after high dose MCMV infection, histological analysis was performed on submandibular glands (Figure 14C). NZM2328 mice given PBS alone had normal glandular structures: acini which are responsible for saliva production, granulated convoluted tubules (GCTs) which channel and modify saliva, and ducts which deliver saliva to the oral cavity. A few small periductal lymphocytic infiltrates were noted in the PBS group (not shown), which was

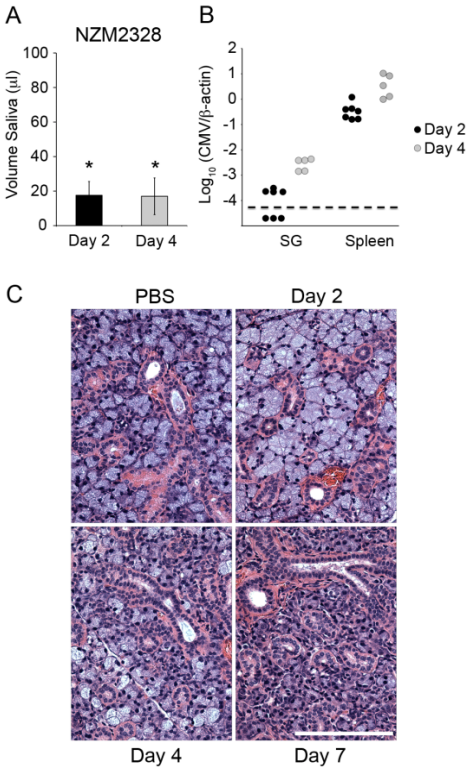

Figure 14. MCMV infection induces secretory dysfunction early, prior to establishment of high level virus replication and inflammation. NZM2328 female mice were infected with 10^5 PFU MCMV. A, The secretory response was determined at day 2 or day 4. Saliva volumes are reported mean ± SEM (n=7-8), *$p < 0.05$ compared to PBS controls (Figure 1). B, Virus levels in salivary gland and spleen were quantitated by QPCR at day 2 (black) and day 4 (grey). C, Representative H&E stained sections of NZM2328 submandibular glands are shown (200x). At day 2, acini appeared enlarged and foamy but free from inflammatory cells. At day 4, some infiltrating leukocytes were found near blood vessels surrounding the ducts while acini in these areas appeared atrophic. At day 7, atrophy of acini was more evident with severe infiltration of mixed leukocytes. White scale bar, 200 μm.

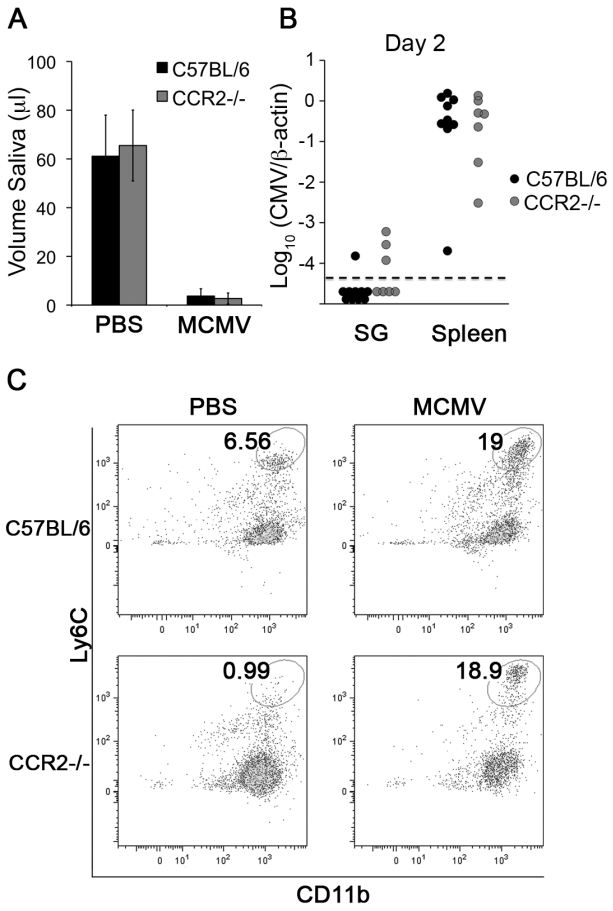

Figure 15. CCR2 is not required for inflammatory monocyte recruitment to salivary glands after MCMV infection. A, The secretory response to pilocarpine was determined 2 days post infection of female mice with 2 x 10^5 PFU MCMV. Saliva volumes are reported mean ± SEM, *$p < 0.05$ compared to PBS control. B, Salivary gland and spleen virus levels were quantitated by QPCR at day 2. C, Salivary gland cells at day 2, gated on live CD45+ cells. The percentage of inflammatory monocytes ($CD11b^+Ly6C^{hi}$) is shown. Panels are representative of 3-5 pooled glands from at least 2 independent experiments.

expected due to the autoimmune genetic background of this strain. At day 2 after high dose infection, when the salivary gland functions at about 20% of normal, no infiltrates were found above background. Acini showed no signs of atrophy or necrosis. We did observe that the acini appeared enlarged and foamy, i.e. lighter in color with prominent vacuoles. These changes were observed across entire regions of tissue, affecting most of the gland. By day 4, small areas of infiltrating leukocytes were observed near blood vessels surrounding the ducts. Acini in these areas began to show signs of atrophy, or shrinking. The GCTs were unchanged. By day 7 after high dose infection, a severe mixed infiltrate was widespread and included both mononuclear and polymorphonuclear cells. Ducts and GCTs remained intact while many acini were atrophic with dense nuclei or necrotic. Cytomegalic inclusions were also found in submandibular acinar cells at this time point, corresponding to high virus level. In summary, dysfunction occurred at day 2 before an inflammatory infiltrate was observed. Later atrophy of acini corresponded to the presence of inflammatory leukocytes in the submandibular gland.

Inflammatory monocytes are recruited to salivary gland early, but are not required for secretory dysfunction

After high dose MCMV infection, loss of secretion is first apparent at day 2. Though the salivary glands appeared free from inflammation at this time histologically, flow cytometric analysis of salivary gland CD45+ cells was performed to characterize immune cells within the gland which may cause secretory dysfunction (Figure 15C and 16C). The major resident leukocyte population of mouse salivary glands is CD11b+

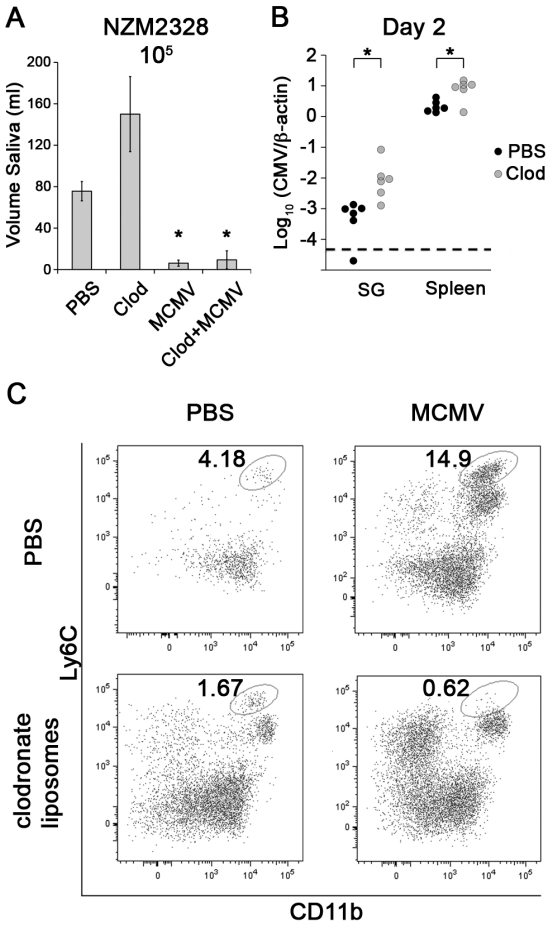

Figure 16. Early recruitment of inflammatory monocytes is not required for secretory dysfunction. NZM2328 female mice were treated with PBS or clodronate liposomes (Clod) i.v. 1 hour before infection with 10^5 PFU MCMV. A, The secretory response was determined at day 2. Saliva volumes are reported mean ± SEM (n=4-11), *p < 0.05 compared to PBS controls or Clod alone. B, Virus levels in salivary gland and spleen were quantitated by QPCR at day 2. *p < 0.05 by Student t test. C, Salivary gland cells at day 2, gated on live CD45+. Percentage inflammatory monocytes ($CD11b^{+}Ly6C^{hi}$) is shown. Panels are representative of 4 pooled glands from at least 2 independent experiments.

macrophages/dendritic cells. After virus infection of NZM2328, recruitment of three major cell types was observed. Accumulation of neutrophils, defined as $CD11b^{hi}Ly6C^{int}SSC^{hi}$, was variable between experiments. The presence of neutrophils may reflect the extent of blood in the organ at the time of harvest, since the blood contained a predominance of these cells at day 2 (data not shown). In contrast to NZM2328, few neutrophils accumulated in salivary glands of C57BL/6 mice. A CD11b- population expressing low Ly6C levels was also recruited to the gland. These cells were lymphocytes based on their high expression of CD45 (not shown). However, these cells had already been ruled out as the cause of secretory dysfunction (Figure 13). Finally, inflammatory monocytes defined as $CD11b+Ly6C^{hi}SSC^{lo}$ were consistently found in infected salivary glands at day 2. This population was a good candidate for the cause of salivary gland dysfunction because MCMV is thought to be delivered to the salivary gland by a monocyte emigrating from blood (121), and inflammatory monocytes can cause tissue damage in other viral infections, i.e. influenza (122). The association of glandular inflammatory monocytes and dysfunction at day 2 was also observed in C57BL/6 mice (Figure 15). Consistent loss of function required a two-fold higher dose of virus in this mouse strain.

To test whether inflammatory monocytes play a role in virus-induced secretory dysfunction, mice deficient in the chemokine receptor CCR2 were utilized. CCR2 plays an important role in targeting monocytes/macrophages to sites of inflammation (123-125), though the role of CCR2 in recruitment to salivary glands has not been reported.

The recruitment of inflammatory monocytes to salivary glands was unaffected by CCR2 deficiency after high dose MCMV infection at day 2 (Figure 15C). Therefore, an alternate means to test the role of these cells in secretory dysfunction was sought.

Clodronate liposome treatment rapidly depletes phagocytic cells, including intravascular monocytes and macrophages (126, 127). NZM2328 mice were treated with clodronate liposomes one hour prior to high dose infection. Depletion of CD11b+Ly6C^{hi} cells was confirmed in the blood after 24 hours (not shown) and in the salivary glands at day 2 immediately after collection of saliva (Figure 16C). Salivary gland resident macrophages were not depleted after clodronate liposome treatment. MCMV levels were elevated in spleen and salivary gland at day 2 after treatment (Figure 16B). Importantly, MCMV infection of clodronate liposome-treated mice led to secretory dysfunction, similar to infection of sham injected mice (Figure 16A). Clodronate liposome treatment alone had no significant effect on secretion. Therefore, monocytes within the salivary gland and elsewhere are not mediating the early loss of secretory function.

Low dose infection reveals secretory function is independent of salivary gland virus level

Infection with low dose (100 PFU) of MCMV effectively targets the infection to the salivary gland while spleen virus levels are undetectable by day 7 (Figure 12). To test the relationship of salivary gland virus levels to dysfunction in another way, NZM2328 mice infected with a low dose of MCMV were followed through day 14, a time of peak salivary gland virus replication. After low dose infection of a separate cohort of NZM2328 mice, we confirmed that secretory function was intact at day 7 (Figure 17A).

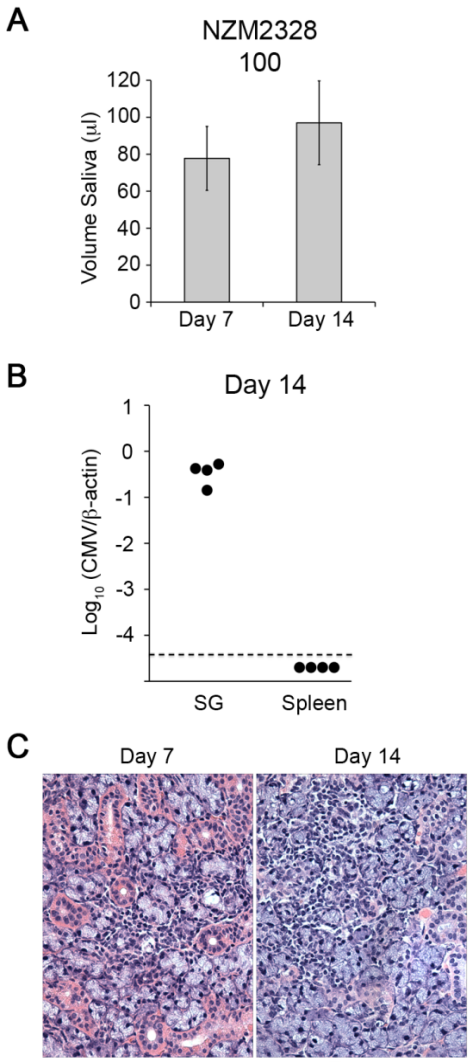

Figure 17. Low dose MCMV infection fails to elicit secretory dysfunction despite high level virus replication within the salivary gland. NZM2328 female mice were infected with 100 PFU MCMV. (A) The secretory response was determined at day 7 and 14 of the same cohort of mice. Saliva volumes are reported mean ± SEM (n=4). (B) Virus levels in salivary gland and spleen were quantitated by QPCR at day 14. (C) Representative H&E stained sections of NZM2328 submandibular glands are shown (200x).

At day 14, the same mice continued to secrete normally yet salivary gland virus levels had increased nearly 100-fold in comparison to day 7 (Figure 12B, 17B). Note that the high salivary gland virus levels at day 14 after low dose infection were similar to those at day 7 after high dose infection, but the functional capacities differed greatly. Again, virus was not detectable in the spleens at day 14 after low dose infection (Figure 17B). Taken together, both high and low dose regimens suggested that secretory function does not correlate with salivary gland virus level but may depend on systemic infection.

After low dose infection, when secretion was normal from 7-14 days, the submandibular gland displayed a different inflammatory profile compared to high dose infection (Figure 17C, compare to 14C). At day 7, low dose infection led to a relatively mild mononuclear infiltrate. The majority of acini appeared normal with basement membranes intact, despite some being surrounded by patches of infiltrating leukocytes. At day 14, the mononuclear infiltrate was more severe and focal in nature. Acini surrounded with lymphocytes were atrophic, but zones of healthy acini were prevalent, corresponding to the functional output. The histological analysis showed that normal secretion can occur in the face of a formidable mononuclear inflammatory response in the gland.

NK cells protect against MCMV-induced secretory dysfunction

NK cells have long been recognized to play a critical role in MCMV control. However, most studies employ high dose regimens to observe the antiviral effects of NK cells. Since salivary gland dysfunction correlated with detectable splenic virus levels after the first week of infection (Figure 12), we hypothesized that NK cells may limit systemic

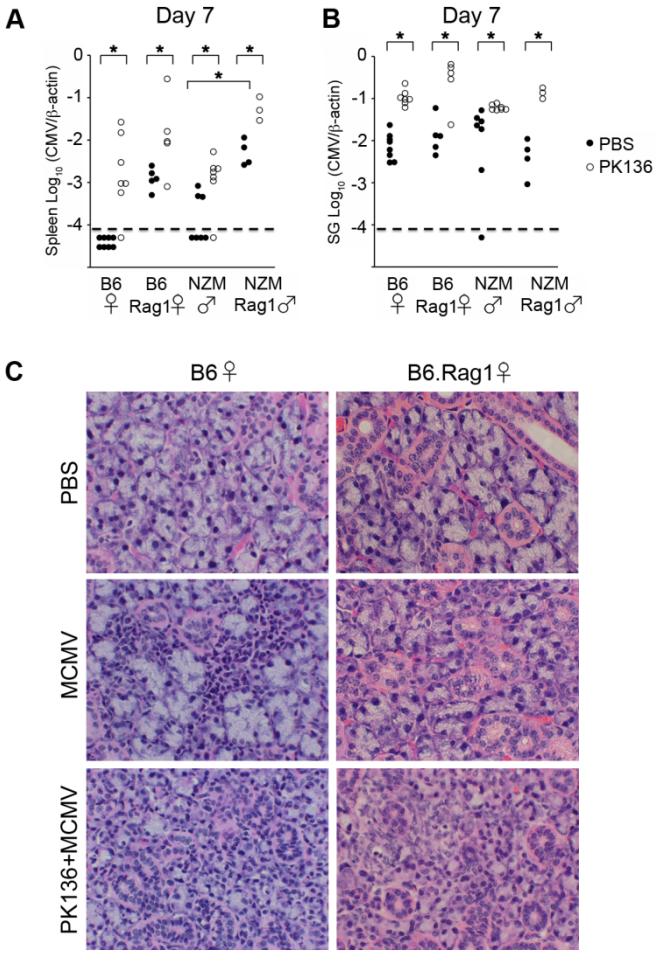

Figure 18. NK cells regulate virus levels and immunopathology after low dose infection. Indicated mice with or without NK depletion were infected with low dose MCMV: 4000 PFU for B6 and B6.Rag1 females, 100 PFU for NZM2328 and NZM.Rag1 males. A, Spleen virus levels were quantitated by QPCR at day 7. B, Salivary gland virus levels were quantitated by QPCR at day 7. The limit of detection is denoted by the dotted line. *$p<0.05$ by ANOVA. C, NK cells protect against widespread acinar atrophy and inflammation after infection. Representative H&E stained submandibular glands. See text and Table 6 for description.

Table 5. Regulation of saliva secretion by NK cells after low dose MCMV infection[a,b]

	B6 ♀	B6.Rag1 ♀	NZM ♂	NZM.Rag1 ♂
PBS	47 ± 10	106 ± 28	123 ± 20	251 ± 27
PK136	66 ± 20			
MCMV	30 ± 11	57 ± 38	62 ± 20	182 ± 43
PK136+MCMV	3 ± 3*	11 ± 7*	38 ± 21*	68 ± 54*

[a]Secretion assessed at day 7 post infection with 4000 PFU in C57BL/6 and 100 PFU in NZM2328.

[b]Values are mean saliva volume (μl) ± SEM, 6-8 animals per group (C57BL/6 and NZM2328) or 3-6 animals per group (B6.Rag1 and NZM.Rag1) combined from 2 experiments.

*$p < 0.05$ compared to PBS group or PK136 group by ANOVA or Kruskal-Wallis test.

viral loads under low dose conditions and thus protect the salivary gland from dysfunction. To test this, we depleted NK cells from various strains prior to low dose infection and measured saliva production at day 7. NK cells contributed to virus control in the spleen and salivary gland of both mouse strains after low dose infection (Figure 18A, 18B). As shown before, the secretion of C57BL/6 mice was not significantly affected at a low dose of 4000 PFU MCMV at day 7 (Table 5). Depletion of C57BL/6 NK cells prior to infection led to a significant drop in secretion compared to the PBS group. PK136 treatment alone did not affect secretion. This phenomenon was not exclusive to C57BL/6 mice since loss of secretion after NK depletion occurred in NZM2328 males administered 100 PFU MCMV as well.

We next examined whether T and B cells also limit virus replication and protect against secretory dysfunction after low dose infection. Rag1 mice had higher virus levels than WT in the spleen at day 7 after low dose infection as expected, but not in the salivary gland (Figure 18A, 18B). Secretion was not impaired in the absence of adaptive immunity (Table 5). Thus, in contrast to a protective NK cell effect, T (including NKT) and B cells did not limit dysfunction at day 7 at low viral dose. Saliva secretion was significantly decreased in NK depleted Rag1 mice also. We conclude that NK cell protection from dysfunction is independent of adaptive immunity and that T and B cells are not mediating dysfunction after low dose infection.

NK cells limit salivary gland pathology after low dose infection

We hypothesized that an NK effect on SG secretory function may be due to their ability to regulate inflammation in the gland following virus infection. To test this, we examined

Table 6. Summary of Histological Observations of Submandibular Glands after Low Dose MCMV Infection[a]

Strain	n	Treatment	Inflammation	Extent	Glandular Changes	Extent
B6	4	PBS	None	0	None	0
	4	MCMV	Mononuclear, some apoptotic, peri-acinar	2.4	Acinar atrophy	1.5
	4	PK136+MCMV	Mononuclear, some apoptotic, peri-acinar	2.3	Acinar and GCT atrophy, necrosis	2.8**
B6.Rag1	3	PBS	None	0	None	0
	3	MCMV	None	0	Acinar atrophy	n.g.
	3	PK136+MCMV	Interstitial PMN	1.2*	Acinar atrophy	n.g.

[a]Assessed at day 7 post infection with 4000 PFU, extent scored blind on a scale of 0-4 by pathologist.

n.g. not given

* $p < 0.05$ or ** $p < 0.0005$ compared to MCMV infection alone, Student t test

SMG sections for inflammation and tissue pathology (Figure 18C, Table 6). After low dose infection, C57BL/6 salivary glands displayed moderate inflammation. Cells were mostly lymphocytic, located in perivascular and periductal areas. Apoptotic lymphocytes were observed in certain clusters. The tissue architecture was largely preserved, though some acini were atrophic, or smaller in size compared to control. While inflammation scores did not change after NK depletion (Table 6), inflammatory cells were distributed throughout the interstitium and in contact with acinar cells in contrast to the clusters of lymphocytes observed in B6 mice without NK depletion (Figure 18C). Notably, NK depletion prior to infection led to drastic glandular changes. The majority of acini were atrophic or necrotic. The extent of atrophy was more severe in NK depleted mice as reflected in the scores in Table 6. Thus, NK cells limited glandular atrophy after infection which correlated with secretory dysfunction at day 7 (Table 5).

To determine whether T and B cells contributed to the observed changes, B6.Rag1 salivary glands were examined. After infection, B6.Rag1 displayed mild acinar atrophy without inflammation. NK cell depletion of B6.Rag1 mice led to severe polymorphonuclear inflammation. Large clusters of PMNs, some apoptotic, were observed in contact with acini. We conclude that NK cells regulated the severity of SG inflammation and atrophy after MCMV infection.

Discussion

Understanding mechanisms of secretory dysfunction is fundamental to the development of effective treatments for xerostomia. This report examines key factors thought to affect secretory dysfunction after acute viral infection in an established mouse model. Administration of high dose MCMV to two immunocompetent mouse strains indicated two distinct phases of SG dysfunction, early at day 2 and later at day 7. We found that inflammatory monocytes and lymphocytes were not required for the observed loss of function. On the contrary, NK cells, but not T or B cells, were protective under low dose conditions. Our results support a novel regulatory role of NK cells in limiting inflammation and tissue damage after viral infection.

A model of MCMV-induced secretory dysfunction is shown in Figure 19. In the early phase at day 2, it seems likely that secretion is inhibited by a serum factor as a result of an inflammatory response elsewhere since 1) MCMV replication was high in the spleen, an indicator of widespread systemic infection; 2) salivary gland virus levels were low or undetectable at this point rendering direct infection of acini an unlikely explanation; and 3) histologically, acinar structure remained normal and the gland was largely free from inflammatory cells yet global changes in acinar size and appearance were observed. While this manuscript was in preparation, another group reached a similar conclusion after studying acute MCMV infection of BALB/c mice and favored liver damage as a possible cause of salivary gland dysfunction (106). Other explanations for dysfunction after acute MCMV infection were tested by Kasman et al. but with negative

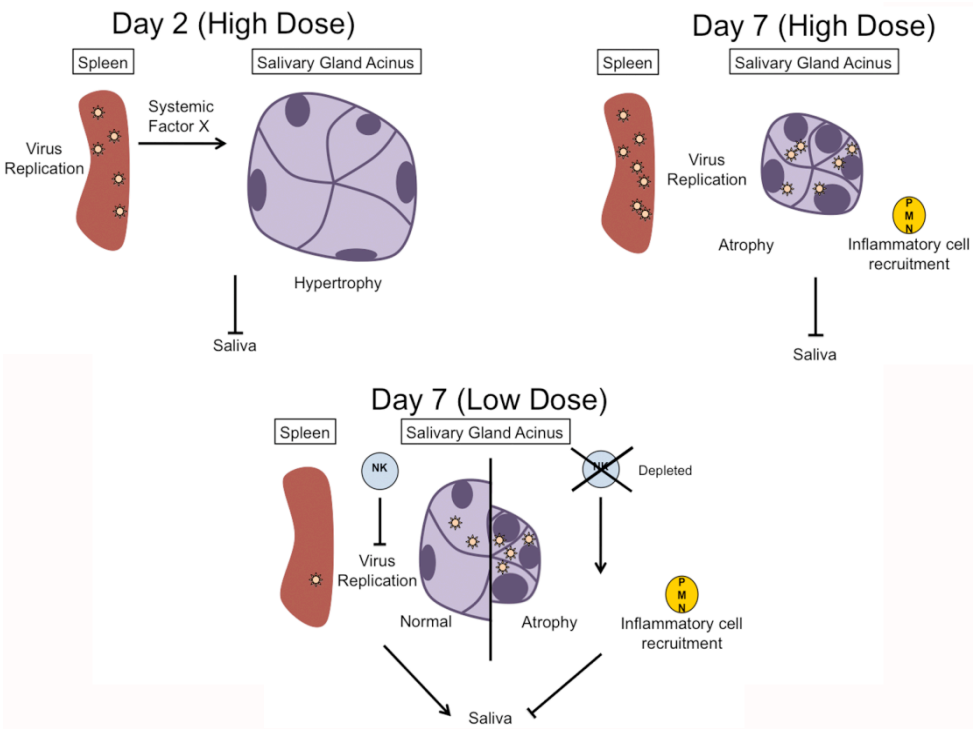

Figure 19. Model for MCMV-induced secretory dysfunction. At day 2 post high dose infection, high virus replication in extraglandular sites may induce a systemic pro-inflammatory response (X) which acts on the salivary gland to cause hypertrophy and inhibition of saliva secretion. The origin of the systemic factor remains to be determined; spleen virus replication serves as a surrogate marker for widespread MCMV infection. At day 7 post high dose infection, spleen virus burden is associated with acinar atrophy, vigorous inflammatory cell recruitment to the salivary gland and secretory dysfunction. NK, T or B cells are not required for dysfunction. Inhibition of secretion may be due to atrophy or the actions of other recruited inflammatory cells, including granulocytes. At day 7 post low dose infection, NK cells limit virus replication which may indirectly lead to decreased systemic responses, prevention of acinar atrophy and inflammation, and preservation of function.

results, i.e. there was no improvement in secretion after splenectomy or hydration and no correlation with changes in aquaporin 5 levels, water channels important in secretion (128). We expand on these results to show that monocytic cell depletion via clodronate liposomes did not rescue secretion after infection at day 2, indicating these cells are not the source of a serum factor. Instead, these cells were protective in the spleen, in agreement with previous findings in other mouse strains (32, 129). The possibility remains that salivary gland resident macrophages not depleted by the liposome treatment or the glandular tissue itself is a source of inflammatory mediators responsible for hypofunction. For example, the few infected macrophages or epithelia may produce type I IFN which acts on neighboring cells. Indeed, the fact that inflammatory monocytes are recruited to the gland at day 2 predicts a change in the local environment. The early loss of secretory function at day 2 prior to atrophy is in line with the non-apoptotic model of hypofunction of SS. In this model, immune-mediated inhibition of secretion initiates atrophy rather than immune-mediated destruction of acini leading to loss of function (96, 116).

The identity of the serum factor(s) responsible for secretory dysfunction remains to be found. The innate response to infection mediated by systemic inflammatory cytokines is an attractive candidate for causing dysfunction. Indeed, a decrease in saliva production has been demonstrated after activation of the innate immune response by repeated systemic administration of TLR3 agonist (130). Secretion was restored once treatment was discontinued, suggesting systemic inflammatory signals were required. The kinetics of induction of innate cytokines such as type I IFN, IL-6, and TNF in serum

after MCMV infection have been characterized (70, 71) and immediately precede the secretory defect observed at day 2. It is hypothesized that a serum factor disrupts the normal secretion signaling pathway within acini, a carefully orchestrated process initiated by activation of muscarinic receptors, generation of IP3 and release of calcium from intracellular stores, and modulated by key small molecules such as cyclic ADP ribose and NO among others (93, 131).

By day 7 post high dose infection, loss of secretory function was most severe. In this later phase, the salivary gland was heavily infiltrated with inflammatory cells and many acini were atrophic or necrotic (Figure 19, upper right). Interestingly, day 7 MCMV-induced SG dysfunction was lymphocyte independent. In addition, dysfunction was independent of salivary gland virus levels since normal secretion was observed in SG with high virus (Figure 17). These data suggest that early control of systemic virus replication prevented dysfunction. Once innate defenses were overwhelmed, such as after high dose infection or in a genetically susceptible host, a damaging systemic inflammatory response ensued. Systemic cytokines above a certain threshold may initiate widespread acinar hypertrophy as noted at day 2 post high dose infection. Over the next few days, salivary gland cytokines and chemokines in response to infection may lead to recruitment of inflammatory cells. Atrophy was observed with the entry of inflammatory cells at day 4-7. This was most evident in comparison of salivary gland histology at day 7 after high and low dose infection. Severe mixed inflammation and widespread acinar atrophy was characteristic of high dose infection, while low dose infection had relatively mild, but focal mononuclear infiltrate and preserved tissue architecture. We cannot

determine whether acinar atrophy at day 7 is a downstream consequence of early systemic factors or due to the actions of inflammatory cells such as macrophages or PMNs. In summary, early control of virus replication "sets the stage" to limit glandular inflammation, damage and loss of function.

Accordingly, we show that NK cells are critical for protection against MCMV-induced loss of secretory function after low dose infection (Figure 19, bottom). We envision NK cells mediating their effect by restraining MCMV during acute infection and thus limiting a damaging systemic inflammatory response, as documented in sera of infected mice (132). The timing of NK cell virus control may be critical for protection from organ damage. NK cells are innate immune responders while MCMV-specific T cells require 4-5 days to develop (70). The antiviral effect of adaptive immunity in the spleen was not sufficient to protect the gland, since Rag1 mice did not succumb to secretory dysfunction. Only when NK cells were depleted did Rag1-deficient animals lose secretory function. This finding informs us that tissue damage was independent of T and B cells since atrophy of acini and associated dysfunction was observed in NK-depleted Rag1 mice.

It is interesting that although NK-mediated MCMV resistance is generally less effective in NZM2328 than B6 mice, NK cells in both strains were needed to support secretory function following low dose MCMV infection. These results suggest that NK cells may regulate potential damage inflicted during an inflammatory response by cytokine crosstalk with other cells. In this regard, it has been suggested that NK cell cytokine responses can be protective in other disease models. NK cell IFNγ in the lung

was shown to limit interstitial fibrosis 21 days after bleomycin treatment (133) and IL-22 produced by NK cells prevented weight loss in DSS-induced colitis (134) and increased survival after *C. rodentium* infection (135). A recent study further demonstrated that NK cells produce IL-10 after infection with various systemic pathogens, which in turn diminished IL-12 production by dendritic cells (120). This immunoregulatory circuit could potentially explain how NK-mediated protection may restrict inflammation and further limit SG dysfunction (136).

This report highlights the importance of the innate immune system in controlling virus-induced salivary gland dysfunction and later organ damage. Classic immune players such as monocytes and lymphocytes were dispensable for virus-induced dysfunction, directing us toward alternative mechanisms. In immunocompetent animals, it is clear that high systemic viral load is associated with dysfunction in our model. Therefore, anti-viral drugs may be an effective treatment of virus-induced xerostomia. In point of fact, the prevalence of DILS has significantly decreased among cohorts of HIV patients since the introduction of highly active antiretroviral treatment (HAART) (6, 137).

A common characteristic of the viruses associated with sicca syndromes is their ability to infect for life, either through persistent infection (HIV, HCV) or in a latent/reactivating state (EBV). Direct inflammatory signals in response to viral products may be a causative agent for secretory dysfunction, including times of viral persistence and reactivation. Thus, keeping virus replication at bay is critical. In the case of MCMV, our previous study of NZM2328 mice showed that a subset of animals displayed

secretory dysfunction six months after infection despite the fact that all mice exhibited severe focal sialadenitis (117). Although virus was undetectable by PCR in most of these animals, formal reactivation studies have not been carried out. The ability of the host to both restrict virus replication and limit innate inflammatory responses to reactivating virus will likely influence the susceptibility to virus-induced dysfunction.

In conclusion, MCMV infection initiated two stages of early dysfunction: the first stage occurred with intact glandular architecture and was independent of infiltrating inflammatory monocytes; the second stage was characterized by glandular atrophy and independent of lymphocytes. These observations support the view that salivary gland dysfunction does not necessarily require immune cell-mediated destruction and point to other systemic inflammatory mediators that affect the homeostasis of secretion. Importantly, we show a novel regulatory role for NK cells in preventing tissue damage and secretory dysfunction mediated by innate immunity after MCMV infection. It remains to be seen whether NK cell regulation occurs via a simple dampening of viral load or through another immunoregulatory pathway. MCMV infection serves as a useful model of virus-induced secretory dysfunction and will be helpful in dissecting the role of NK cells in regulating a pathogenic innate immune response.

Chapter IV.

Conclusions

MCMV infection of NZM2328 mice is a model for human SS

This study establishes a novel mouse model of human SS. Viral infection of autoimmune NZM2328 mice induces many SS disease features, including focal inflammation of the exocrine glands, autoantibodies to glandular proteins, and progressive loss of secretory function. In line with human SS, there is a sex bias in that NZM2328 females develop more severe focal lesions in both the salivary and lacrimal glands and are susceptible to loss of function in comparison to males. The strength of this model lies in the reproducible ability of MCMV to induce severe organ-targeted chronic inflammation. Unlike other models with specific gene or glandular defects, focal sialadenitis occurs in immunocompetent animals after viral infection and thus closely mimics a physiological situation. The organized areas of CD4 T cells and B cells are similar to those found in human SS patients, and it will be interesting to see whether these structures produce B cell lymphoma, a fatal complication in advanced cases of SS. MCMV infection of NZM2328 mice represents a good model to define the requirements for focal inflammation.

Virus-induced chronic inflammation is controlled by genetic factors. Only when infection proceeds in lupus-prone animals is the potential for MCMV-induced lesions revealed. In support, genetically related NZM2758 or NZW mice are relatively protected from spontaneous kidney disease and from virus-induced focal sialadenitis (Figure 19

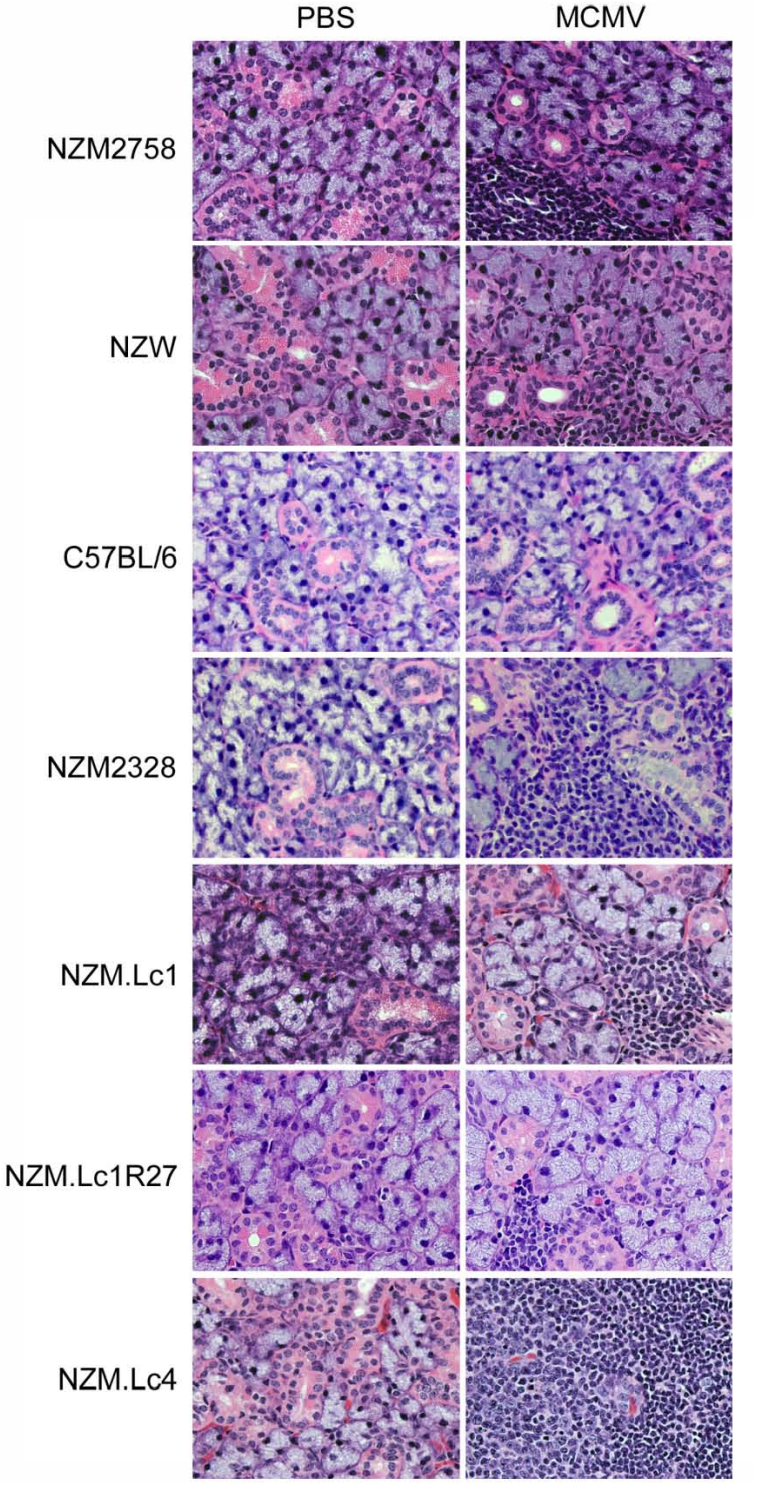

Figure 19. Genetic control of focal sialadenitis after MCMV infection. Female mice were infected with 10^5 PFU. H&E stained glands at day 105-130 are shown (400x).

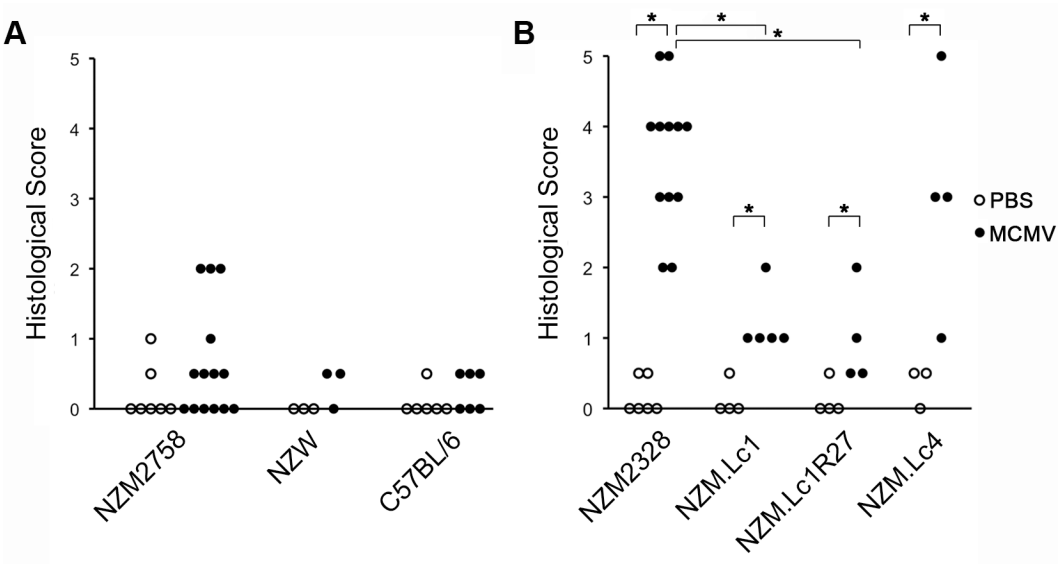

Figure 20. Genetic control of focal sialadenitis after MCMV infection. Indicated female mice were infected with 10^5 PFU. At day 105-130, submandibular gland sections were stained with H&E and scored blindly for severity of focal inflammation. *$p < 0.05$ by Student t test.

and 20). Furthermore, preliminary studies suggest that the Chr 1 genetic interval that controls progression to spontaneous kidney damage also controls the severity of focal sialadenitis after infection (Figure 19 and 20). SS and SLE may share a common predisposing factor, a premise supported by the frequent association of these diseases in human patients.

Focal sialadenitis and secretory dysfunction are distinct disease features. Loss of function occurs in a subset of infected NZM2328 females after day 100, but follow up studies reveal that there is no correlation between the severity of focal inflammation and saliva output late after infection (Figure 21). Furthermore, the majority of infected NZM2328 females were functional after day 100 despite having severe lesions within the gland (Figure 22). These observations agree with the NOD model of SS, where histological changes are not associated with impaired secretory function over time (34). Further evidence that focal inflammation and dysfunction are separate disease processes is provided by study of mice resistant to MCMV-induced sialadenitis. Low secretors were found among all MCMV-infected groups regardless of the presence of focal inflammation (Figure 22). Thus, while MCMV infection may influence secretion in some capacity during latent infection, virus-induced focal sialadenitis and secretory dysfunction are not linked. These studies do not exclude a role for infiltrating lymphocytes in hypofunction in SS-like disease, but show that focal lesions do not necessarily result in organ damage or dysfunction.

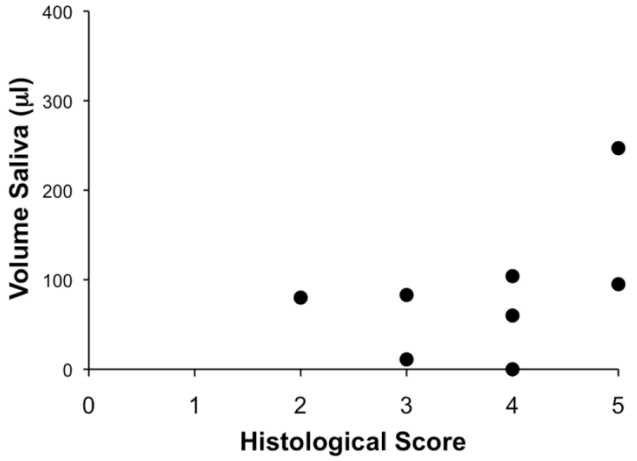

Figure 21. No correlation between focal sialadenitis score and saliva output at day 120 post MCMV infection. NZM2328 females infected with MCMV from Figure A were tested for secretory function 1 day before sacrifice. Severity of focal sialadenitis versus saliva output is plotted.

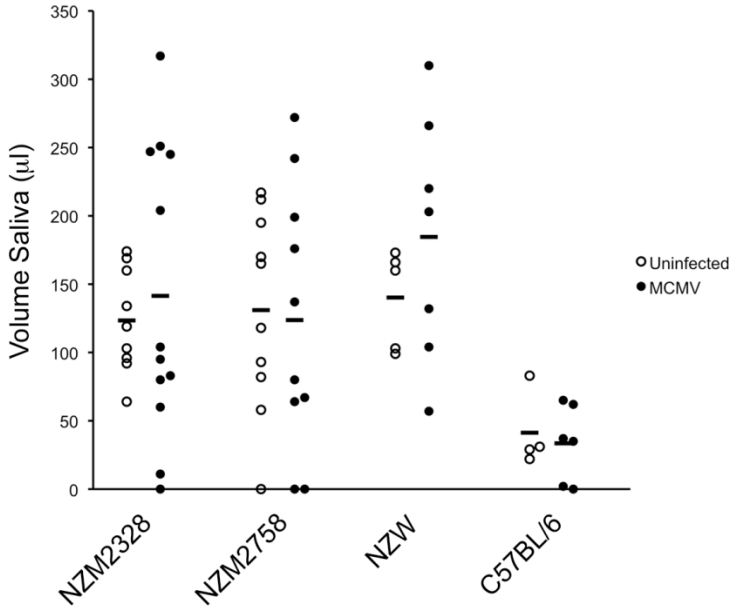

Figure 22. Secretory function of MCMV-infected mice at day 120. Indicated female mice were infected with 10^5 PFU. Note NZ strains receive 0.5 mg/kg pilocarpine, while C57BL/6 receive 1 mg/kg pilocarpine.

Acute MCMV infection is a model for virus-induced xerostomia

Certain human viruses are associated with xerostomia. Here, we establish the first animal model of virus-induced xerostomia and examine the mechanism. The choice of MCMV is clinically relevant due to the high prevalence of HCMV and because the pathogenesis of infection is similar between mouse and human CMV. Viral infection caused rapid loss of secretory function in both NZM2328 and C57BL/6 mice. Investigation of the mechanism of secretory dysfunction early after viral infection led to several key findings: 1) MCMV impairs saliva secretion independently of salivary gland virus levels; 2) lymphocytes or circulating monocytes are not required for secretory dysfunction; and 3) loss of secretion can occur before glandular inflammation, suggesting a soluble mediator is responsible. Since prototypical C57BL/6 mice are susceptible to MCMV-induced xerostomia, the wealth of knowledge surrounding MCMV infection in this strain and genetic knockouts on this genetic background will facilitate the identification of the soluble factor(s) leading to dysfunction.

It is remarkable that MCMV causes dyfunction in animals without NK or T cells. These cells are thought to mediate cytotoxicity of infected cells in general. In the salivary glands, it is known that MCMV replication is refractory to immune control by CD8 T cells but NK cells and CD4 T cells do limit virus levels partly through delivery of IFNγ. As we observe widespread atrophy of acinar epithelium after infection, it is clear that lymphocyte-independent modes of cell killing must be considered in future studies.

Importantly, we show that NK cells are protective against MCMV-induced acinar atrophy and loss of secretions. Infection with an exceedingly low virus inoculum revealed

that NK cells are critical in limiting viral replication under these conditions. In both resistant C57BL/6 and susceptible NZM2328 mice, NK cells are essential to prevent damaging inflammatory responses and dysfunction of the salivary gland.

Future Directions

1. Virus or self? Antigen specificity of salivary gland-infiltrating lymphocytes during chronic inflammation

It will be important to determine whether the focal lesions are a result of a dysregulated antiviral response or viral induction of autoimmunity. It is likely that a combination of both contributes to chronic inflammation based on studies completed thus far. First, MCMV is sialotropic and persistent. In the previous model of MCMV infection of C57BL/6 mice, the virus and immune system reach a balance where latency within salivary glands is actively enforced by lymphocytes for the lifetime of the host. C57BL/6 mice eventually resolve salivary gland inflammation. In contrast to this model, examination of a large group of NZM2328 females post day 100 revealed the presence of salivary gland MCMV in 1/3 of the animals (Figure 23) and resolution of inflammation does not occur. Therefore, reactivating or persistent virus may drive the development of chronic sialadenitis depending on genetic background. In addition, evidence that the virus induces an autoimmune response in NZM2328, but not NZM2758 animals is demonstrated in Figure 24. NZM2758 are resistant to MCMV-induced focal sialadenitis, control salivary gland virus to undetectable levels (data not shown), and do not develop many autoantibodies after infection. In contrast, focal sialadenitis in NZM2328 is associated with low-level virus persistence and an array of autoantibody responses to glandular antigens.

To address antigen specificity:

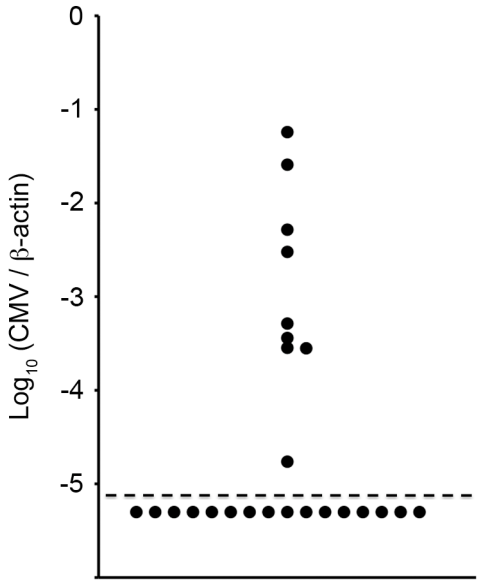

Figure 23. Salivary gland MCMV levels in NZM2328 females day 100-150. Mice were infected with 10^5 PFU MCMV. Detection limit denoted by dotted line. Frequency of detection was 36% (9/25).

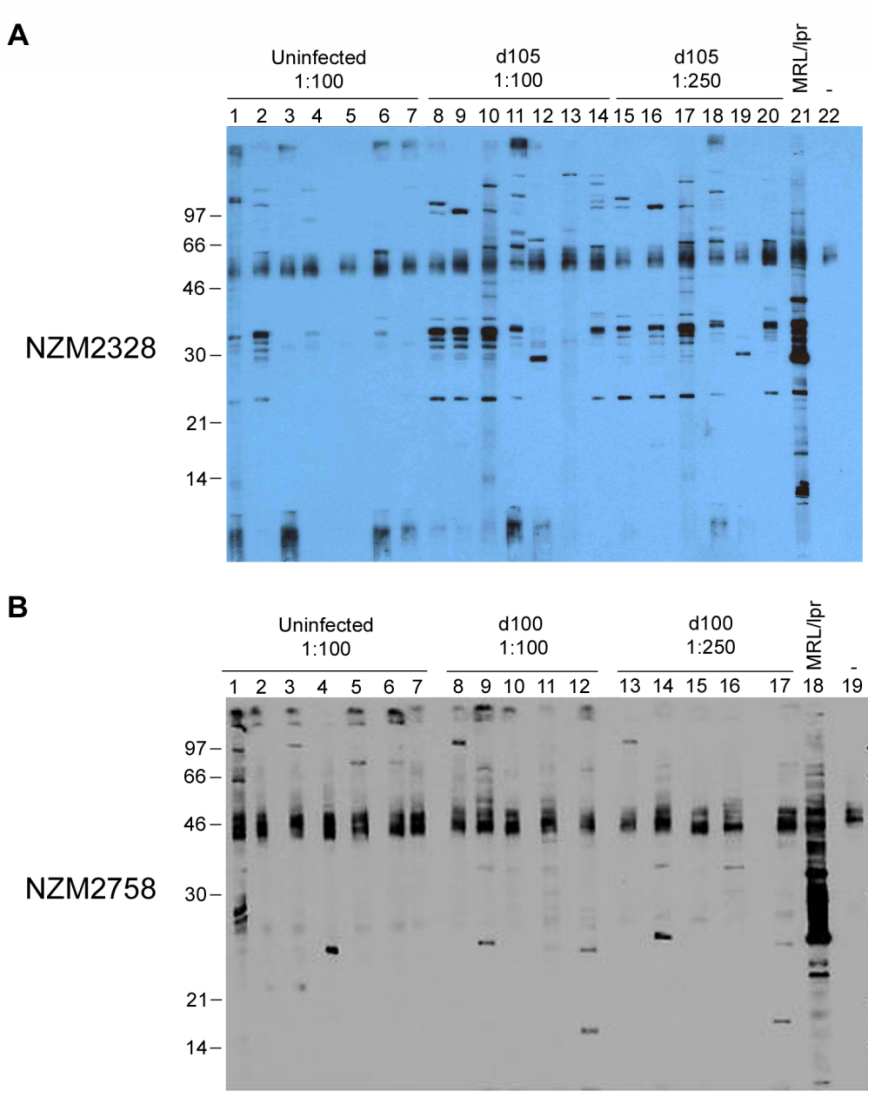

Figure 24. MCMV induction of autoantibodies to salivary gland proteins in NZM2328, but not NZM2758 mice. Serum was collected from (A) NZM2328 and (B) NZM2758 females at day 105 or 100 respectively, or uninfected age-matched controls. Sera were used to probe uninfected NZM2328 salivary gland homogenate by Western blot. Sizes in kD are indicated at right. MRL/lpr sera, positive control, - secondary antibody alone.

i. Test the role of persistent viral antigen in maintenance of focal lesions by inhibiting viral replication. The antiviral drug ganciclovir is effective against acute MCMV replication in the spleen, liver and salivary gland of mice (138). Alternatively, Koszinowski and colleagues have engineered a recombinant MCMV that expresses a dominant-negative small capsid protein which severely limits *in vivo* viral replication upon doxycycline administration (70, 139). Because MCMV establishes latency in the mouse strains examined previously, the antiviral effects of both of these approaches during the late times examined here have not yet been tested and would need to be established first.

ii. Characterize T cell reactivities to viral antigens and salivary gland proteins. Draining lymph node cells from infected or uninfected NZM2328 mice can be tested *ex vivo* in antigen presentation assays. Salivary gland homogenates from infected or uninfected NZM2328 can provide the source of antigen. Proliferation or intracellular IFNγ production would indicate functional reactivity.

2. Identification and testing of candidate inflammatory mediators in virus-induced dysfunction

MCMV-induced secretory dysfunction occurs as early as day 2 post infection. One way to support a role for systemic inflammatory mediators is to transfer serum from an infected mouse to an uninfected mouse. Inflammatory cytokines in serum such as type I IFN, IL-6, and TNFa peak at day 1.5 (70, 71). These candidates can be specifically tested by infecting genetic knockouts and evaluating saliva production at day 2. To generate

more candidates, cytokines present in sera and salivary gland extracts at day 2 can be defined with a multiplex array. Comparison of uninfected and infected mice at this early timepoint would target proteins related to dysfunction since severe histopathological changes have not yet begun. Identifying the molecules responsible for a decrease in acinar function will provide a starting point for the development of targeted therapies, especially for xerostomia patients who do not respond to pilocarpine treatment.

3. Linking innate immune control and prevention of MCMV-induced immunopathology

We show that NK cells are critical in prevention of atrophy and secretory dysfunction of the salivary gland after MCMV infection. This model gives us the opportunity to understand how NK cells are regulating immunopathology after viral infection. It is clear that loss of secretion is a function of early viral load. NK cells may restrict virus replication early, limit the systemic response at day 2 after infection and thus prevent early loss of secretion. To address this, NK cells can be depleted before low dose infection of B6 mice and function assessed at day 2. Levels of cytokines in sera and salivary gland extracts can be measured to identify candidates. In addition, experiments using the conditionally replicating MCMV have shown that lowering viral titers by treatment with doxycycline decreases the proinflammatory cytokines in the serum the first few days after infection (70). If the only role of NK cells is to lower virus burden, doxycycline treatment would prevent secretory dysfunction in the presence or absence of NK cells. On the other hand, NK cells may be important in other ways, such as providing

anti-inflammatory cytokines or tissue protective factors. NK cell regulation of a damaging innate response that is independent of adaptive immunity is an emerging concept in NK cell biology that will be important to examine.

NK cell-mediated protection from glandular damage at day 7 may have a lasting impact. We have extended our functional studies to 30 and 60 days post infection, using low or high dose infection (Figure 25 and 26). Under high dose conditions, NZM2328 mice mount a prolonged vigorous immune response to the virus since histologically and functionally, acini do not recover until day 60. Under low dose conditions however, secretory function is not affected over the 55 day time course, even though the virus replicates to similar levels in the gland. It will be interesting to characterize the inflammation at these late times after low dose infection. Furthermore, NK depletion before low dose infection may lead to the long-term effects observed under high dose conditions. Other long-term consequences of early control of virus replication are currently being examined by other groups. A recent study indicates that NK cell expression of Ly49H, an activating receptor important in early MCMV control, can limit the duration and antiviral effectiveness of T cell responses and is linked with MCMV persistence in the salivary gland (140). Viral persistence in the study contrasts with our results since SG MCMV was not detected by 8 weeks after infection in C57BL/6 mice (Figure 4). This difference may be due to differences between K181 and Smith MCMV strains used in the two studies. Nevertheless, the recent work highlights the power of NK cells in shaping T cell responses and thus contributing to viral persistence. It will be interesting to examine whether NK cells modulate immunopathology in the salivary

gland at these late times as well. The NK cell is poised to be a central player in regulation of acute and chronic inflammation.

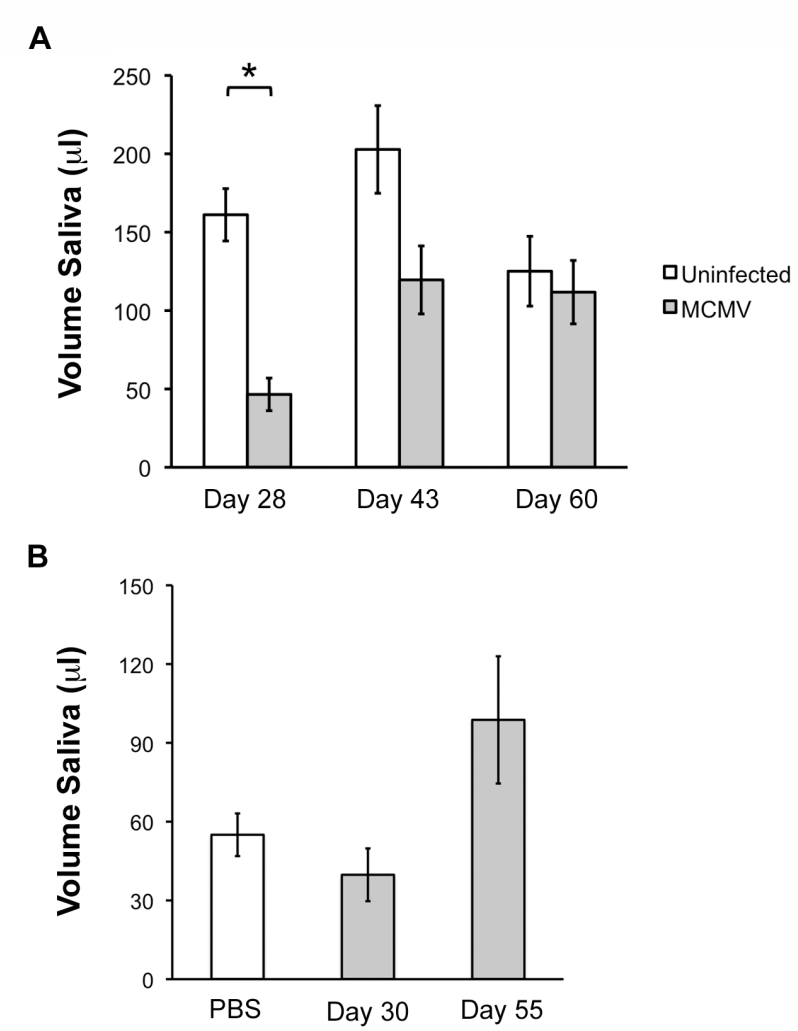

Figure 25. Prolonged secretory dysfunction in NZM2328 mice after high dose, but not low dose infection. Females were infected with (A) 10^5 PFU or (B) 100 PFU MCMV. At the indicated day post infection, secretory function was evaluated. Uninfected or PBS controls are age-matched to the infected groups. Different pilocarpine stock solutions were used in A and B, which may explain variability in controls. *$p < 0.0001$ by Mann-Whitney test.

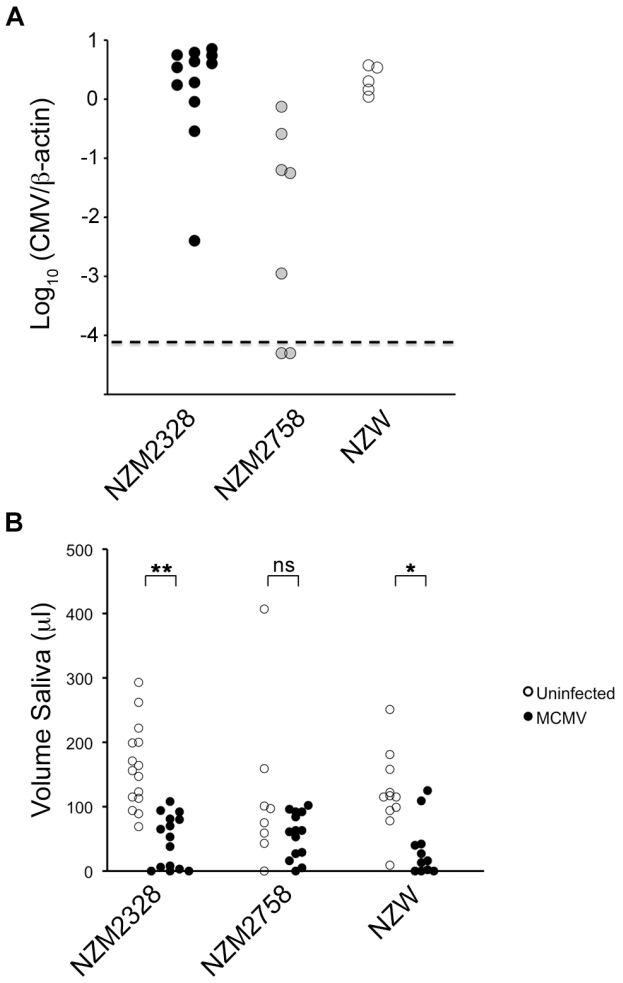

Figure 26. Genetic control of salivary gland virus and secretory function at day 28. Female mice were infected with 10^5 PFU MCMV. A, Salivary gland MCMV levels. B, secretory function. NZM2328 data are taken from Figures X and A to provide a comparison. **$p < 0.0001$, *$p < 0.01$ by Mann Whitney test. The difference between infected and control groups differed between NZM2328 and NZM2758 ($p = 0.017$) by two-way ANOVA with the rank-transform method (141).

Materials and Methods

Mice

NZM2328 breeders were maintained at the University of Virginia. NZM2328.Rag1-/- were a speed congenic created by M. McDuffie at the University of Virginia. Briefly, the Rag1 mutation from B6.129S7-*Rag1*^{tm1Mom}/J was introduced to the NZM2328 strain. Mice were backcrossed to NZM2328 for 3 generations to achieve complete background selection based on genome-wide analysis. Genotyping for the Rag1 mutation was performed as described by The Jackson Laboratory. A deficiency in splenic T and B cells was confirmed by staining for CD3 and CD19 by flow cytometry. C57BL/6J, B6.MRL-*Fas*^{lpr} (B6-*lpr*) and B6.CCR2-/- (B6.129S4-*Ccr2*^{tm1Ifc}/J) were obtained from The Jackson Laboratory. All animals were bred and maintained under specific pathogen-free conditions. Mice were 8-18 weeks of age, prior to spontaneous kidney disease in NZM2328. Unless noted, female mice were used, due to sex related differences in secretory function. Animal studies were approved and conducted in accordance with Animal Care and Use Committee oversight.

MCMV and virus assays

MCMV (Smith Strain, ATCC, Rockville, MD) Stock 1 was passaged twice in 6-week-old BALB/c females and titered on MEFs. MCMV Stock 2 and other stocks were serially passaged in weanling BALB/c mice and titered on 3T3 cell monolayers. Experimental mice were intraperitoneally infected with MCMV in 0.2 ml sterile PBS. SG (submandibular plus sublingual glands) homogenate virus titers were determined by

standard plaque assay on MEFs as described (142). Salivary and lacrimal gland virus levels were determined by quantitative real-time PCR (QPCR) as described previously (142). All sample measurements were performed in triplicate. Results are reported as the number of MCMV genomes detected per number of endogenous (β-actin) genomes.

Clinical and histological assessment

Submandibular, parotid, sublingual, and lacrimal glands were fixed with 10% phosphate-buffered formaldehyde. Paraffin embedded tissue sections (5 μm) were stained with H&E or Trichrome in the University of Virginia Research Histology Core. Glandular inflammation in one representative section from each mouse was assessed by the following criteria 1) Diffuse infiltration of mixed cell types (0 = no inflammatory cell infiltration, 1 = diffuse inflammatory cell infiltration less than 2500 / mm^2, 2 = diffuse inflammatory cell infiltration less than 10,000 / mm^2, 3 = diffuse inflammatory cell infiltration more than 10,000 / mm^2) 2) Mononuclear cell infiltration in periductal areas (0 = no focal mononuclear cell infiltration around ducts, 1 = one periductal mononuclear cell focus / 4 mm^2, 2 = two periductal mononuclear cell foci / 4 mm^2, 3 = more than three periductal mononuclear cell foci / 4 mm^2, 4 = confluent infiltration with glandular destruction). Tissues were scored blind by Y. Ohyama and V.C. Selected tissue sections were similarly evaluated by experienced pathologists H. Bagavant (Department of Medicine, University of Virginia) and K. Tung (Department of Pathology, University of Virginia). In low dose studies, the extent of inflammation and atrophy of salivary gland cells was scored 0-4 in a blinded fashion by K. Tung.

Kidneys were scored blind on a scale of 0-5 by H. Bagavant. Acute GN scores were based on severity of glomerular hypercellularity, mesangial matrix expansion, focal necrosis, and epithelial cell crescents. Chronic GN scores were based on the severity of glomerulosclerosis, tubular atrophy, and interstitial fibrosis. Screening for severe proteinuria (≥300 mg/dL on consecutive screens) in female NZM2328 mice (≥ 8 wks.) was performed biweekly with Multistix 10 SG (Bayer Corporation).

For immunohistochemistry, frozen SMG sections were stained with biotinylated monoclonal antibodies GK1.5, 53-6.7, and RA3-6B2 (anti-CD4, anti-CD8, and anti-B220, PharMingen, San Diego, CA), and biotinylated 5a-8 (anti-Thy1.2, Caltag, Burlingame, CA). After washing, Vectastain® ABC reagent and DAB/Ni Substrate Kit (Vector) were used per manufacturer's instructions.

Salivary gland functional assessment

Salivary secretion was measured after induction by i.p. injection of pilocarpine (MP Biomedicals) into unanaesthetized mice essentially as described (98). NZM2328 and NZM.Rag1 received 0.5 mg/kg, while C57BL/6 and B6.CCR2-/- received 1 mg/kg. Briefly, saliva was collected continuously from the oral cavity by vacuum suction from 2-27 min. after pilocarpine induction. This time period coincides with the peak response. Saliva volumes were measured by micropipette and expressed as total volume saliva produced in 25 minutes.

ELISAs and Western blotting

Anti-Ro and anti-La autoantibodies were measured by ELISA using recombinant murine Ro60, Ro52 and La absorbed onto Immulon-4 (Dynatech Inc.) as described previously (143). Western blot analysis was also performed as described previously (143). Briefly, salivary and lacrimal gland extracts from uninfected adult mice were used as Western blot substrates for sera collected from MCMV infected or control mice.

Isolation of salivary gland leukocytes

Submandibular glands were carefully excised, removed of all periglandular lymph nodes, and placed in 3% NCS in DMEM on ice. Glands were pooled from 2-4 mice, minced, and digested with 0.9 mg/ml collagenase Type IV (Sigma) in a 37° water bath for 15 min with periodic agitation. EDTA in PBS was then added for a final concentration of 0.05 M for an additional 5 min. Digested tissue was pelleted by centrifugation and resuspended in cold 3% NCS in DMEM with 5 mM EDTA. Tissue was mashed through a 70 mm nylon cell strainer on ice. Single cell suspension was washed once with 3% NCS in PBS prior to plating for flow cytometry.

Antibodies and flow cytometry

2.4G2 and PK136 mAbs were purified from spent cell-free hybridoma supernatants (Lymphocyte Culture Center, Department of Anatomy and Cell Biology, University of Virginia). The following antibodies were used in flow analysis: CD45-APC-Alexa Fluor 750, CD11b-Alexa Fluor 647, DX5-FITC (eBioscience), Ly6C-FITC, NK1.1-PE, CD3e-

APC, CD19-APC-Cy7 (BD Biosciences) and anti-mouse NKp46-PE (R&D Systems). Cell surface FcRs were blocked with 2.4G2 prior to staining with indicated mAbs on ice. Dead cells were excluded from analysis by either 7-AAD staining (Sigma) or with LIVE/DEAD fixable violet dye (Invitrogen). Fixed cells were run on a FACScan or FACSCanto II (BD Biosciences) and analyzed with FlowJo (version 8.0, Tree Star).

In vivo treatment protocols

To deplete NK cells, NZM2328 mice received 350 mg PK136 mAb i.p. 2 days prior to infection. C57BL/6 mice received 200 mg PK136. PBS was injected as control. Flow cytometry of splenocytes at day 7 showed >95% depletion of $DX5^{+}CD3^{-}$ after high dose infection or >67% depletion of $NKp46^{+}CD3^{-}$ cells after low dose infection. To deplete monocytes/macrophages, liposomes containing dichloromethylenediphosphonic acid, Cl_2MDP (Sigma) were prepared as described (126). 0.2 ml of the liposome suspension or PBS was injected i.v. 1 hour prior to infection of NZM2328 mice. Flow cytometry of PBMC 24 hours post injection showed >90% depletion of $CD11b+Ly6C^{hi}$ monocytes.

Statistics

Results were analyzed with GraphPad Prism software. The non-parametric Kruskal-Wallis and Dunn's multiple comparison test was used unless indicated. One-way ANOVA and Tukey's multiple comparison test was used on transformed values when assumptions could be fulfilled. Two-tailed Student t test was used for comparison of two groups. $p < 0.05$ was considered significant.

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