ACTIVATION OF INNATE IMMUNITY BY SMALL RIBONUCLEOPROTEINS

By

KINDRA MICHELE KELLY-SCUMPIA

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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By

Kindra Michele Kelly-Scumpia

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Systemic lupus erythramatous (SLE) is an autoimmune disease that affects 1 in 4000 people in the United States, predominantly women of child bearing age. Autoantibodies against protein-nucleic acid complexes such as small nuclear ribonucleoproteins (snRNPs) are prevalent in this disease and other autoimmune diseases and include the common autoantibodies anti-U1 snRNP and anti-double stranded DNA. The U1 snRNP consists of the proteins U1-70K, A, B'/B, C, D1/2/3, E, F, and G and a single U1 RNA molecule and is reactive with both anti-Sm and anti-RNP autoantibodies. We studied why these protein-nucleic acid complexes are such frequent targets in SLE and hypothesized that the RNA molecules within these complexes may become dissociated from the proteins they associate with, stimulating the immune system through activation of one or more toll like receptors (TLRs), leading to type I interferon production (IFN-I). We focused our studies on the U1 RNA molecule that is a component of U1 snRNP. We show that U1 RNA is capable of stimulating a variety of cells types to produce IFN-I *in vitro*. We found U1 RNA stimulates via TLR7, a receptor for guanidine and uridine rich single stranded (ss) RNA, that signals through the adapter protein myeloid differentiation factor 88 (MyD88). Treatment of mice with U1 RNA also caused activation of B cells, T cells, and dendritic cells (DCs) *in vivo*. U1 RNA was capable of driving an antigen specific immune

response in both B and T cells similarly to the classic adjuvant, aluminum hydroxide (alum). Finally, we analyzed how U1 RNA may become immunostimulatory *in vivo*. We found snRNPs from normal cells were not able to induce IFN-I because both RNA and proteins remain intact in the complex. However, snRNPs isolated from cells undergoing necrosis become immunostimulatory because many of the associated proteins are missing from the complex, thus allowing the immunostimulatory RNA to become exposed. These findings have led to a better understanding of the pathogenesis of SLE-related autoantibody development.

CHAPTER 1
INTRODUCTION

Systemic Lupus Erythrematous

Systemic lupus erythematosus (SLE) or lupus is a chronic autoimmune disease of unknown etiology that can affect multiple organ systems including the skin, joints, kidneys, and nervous system. The Lupus Foundation of America estimates that approximately 1.5 million Americans have a form of lupus. Ninety percent of diagnosed cases are women and 80% of those are between the ages of 15-45. The prevalence of SLE varies between ethnic groups (1). SLE is about four times more prevalent in individuals of African, Asian, or Latin ancestry than in individuals of European ancestry. Some people believe that SLE or lupus gets its name from the characteristic reddish rash (malar or butterfly rash) that resembles a wolf's face.

SLE can present with dermatological, musculoskeletal, hematological, neurological, renal, or other manifestations. It should be noted that SLE is a syndrome not a disease and that different patients may have very different clinical manifestations.

Dermatological manifestations are found in about 65% of SLE patients at some point in the disease process with as many as 30-50% of patients presenting with the classic photosensitive malar rash. Some patients may also present with a discoid lupus rash (red, scaly dense patches that leave scars). Patients most often seek assistance for the musculoskeletal manifestations most commonly presenting as arthralgias, or joint pain. The arthritis, unlike that in rheumatoid arthritis, is classically non-erosive and non-deforming. Anemia may develop in as many as half of SLE patients and may be due either to chronic inflammation or to autoimmune hemolytic anemia mediated by anti-RBC antibodies. Some SLE patients may present with seizures, psychosis, or other neurological problems.

One of the most dangerous manifestations is kidney disease, which can lead to end stage renal failure. Now because of earlier recognition and improved management, renal failure is less common. Lupus nephritis is due to the deposition of immune complexes (IC) along the glomerular basement membrane and is manifested as hematuria and proteinuria. ICs are clusters of interlocking epitopes and antibodies. Normally, after an antigen-antibody reaction, the circulating ICs are cleared in the spleen or liver by binding to the FcγRIII of resident phagocytic cells. However, if ICs continue to circulate, either due to excessive formation or impaired mechanisms of clearance, they may cause disease when deposited in different organs including: blood vessels (vasculitis), lungs, skin, joints (arthritis), or kidneys (glomerulonephritis). The complexes activate the complement system causing the release of chemokines attracting polymorphonuclear leukocytes (PMNs) resulting in inflammation and organ damage.

The American College of Rheumatology (ACR) has established eleven criteria for the classification of lupus (2). The criteria include: 1. malar rash, 2. discoid lupus rash, 3. photosensitivity, 4. oral ulcers, 5. arthritis, 6. renal disorder, 7. neurologic disorder, 8. serositis, 9. hematologic disorder, 10. anti-nuclear antibody, 11. immunologic disorder or autoantibody production. Patients who have any four of the eleven criteria are considered to have SLE.

Autoantibodies in SLE

The discovery of specific autoantibodies for SLE and other systemic autoimmune diseases was a major advance in the diagnosis of these diseases. Antinuclear antibodies are found in virtually 100% of SLE patients, and 80-90% of SLE patients have a positive anti-nuclear antibody (ANA). Thirteen percent of these patients do not have a diagnosis while 84% are diagnosed with a specific disease (3). The most common diagnoses for patients with a positive ANA is SLE (18.8%) and autoimmune thyroid disease (10.5%) (3). Other disorders that exhibit

positive ANA include connective tissue disorders (4), systemic sclerosis, Sjogren's syndrome, rheumatoid arthritis, and Raynaud's phenomenon (5).

SLE Specific Autoantibodies

A number of specific autoantibodies have been identified that are found only in SLE. These include anti-dsDNA, anti-Sm, anti-RNA helicase A, anti-PCNA, and anti-ribosomal P (6-11).

The most sensitive autoantibody found in SLE is anti-double-stranded DNA (dsDNA) (12, 13). Between 30-70% of SLE patients are found to have dsDNA antibodies at some point during the disease and these dsDNA antibodies are 95% specific for SLE making it a valuable disease marker (6). Some would argue that dsDNA antibodies are nearly 100% specific (depending on the immunoassay performed). Anti-dsDNA is associated with active disease as well as nephritis (14).

Another virtually pathognomonic autoautibody of SLE is anti-Sm. These autoantibodies are produced by 7-30% of SLE patients and are highly associated with another autoantibody, anti-RNP, which although is found in 20-40% of SLE patients is not specific for the disease (8, 15). Antibodies to Sm react with the 6S Sm core particle, which consists of proteins designated B'/B, D, E, F, and G. The Sm core particle has been shown to form a ring structure that the U1 RNA molecule passes through based on charge (16). The Sm core particle has been found to be associated with U1 snRNP as well as with U2 snRNP, U5 snRNP, and U4/U6 snRNP. In addition, each U snRNP complex contains a unique uridine-rich (U) RNA species (U1, U2, U5, U4/U6) and proteins specific to that particular snRNP. All of the snRNP complexes, which include the Sm core particle, are involved in splicing of heterogeneous nuclear RNA into mRNA which will be translated into protein. Although not specific for SLE, anti-snRNP antibodies are

found in 20-40% SLE patients. Table 1-1 lists common autoantibodies, their antigen, RNA components, and associated disease.

Anti-proliferating cell nuclear antigen (PCNA) antibodies, though uncommon, are very specific for SLE. They are found in 1 to 5% of patients (7, 10, 17). Expression of this autoantibody is associated with nephritis. PCNA is an auxillary factor for DNA polymerase.

Anti-ribosomal antibodies (reactive with the P0, P1, and P2 proteins) have been described to be highly specific for the diagnosis of SLE with a prevalence of about 15% (10, 18). Ribosomes are the molecular factory involved in protein synthesis. Anti-ribosomal P autoantibodies have been associated with neuropsychiatric involvement in SLE (19).

Other autoantibodies are found in SLE as well as other diseases. These include anti-Ro (SS-A), anti-La (SS-B), anti-RNA polymerase II, anti-Su, and anti-Ku. Anti-Ro (SS-A) antibodies are found in 10-50% of SLE patients while anti-La (SS-B) antibodies are found in 10-20% of SLE patients. Both of these autoantibodies are found at a very high frequency in Sjogren syndrome and have been associated with clinical manifestations including sicca syndrome and subacute cutaneous lupus.

Autoantibodies Specific for Other Autoimmune Diseases

It is unknown why certain autoantibodies are found only in certain diseases. SLE is not the only autoimmune disease associated with specific autoantibodies. Anti-topoisomerase I, fibrillarin, and RNA polymerases I/III are autoantibodies specific for systemic sclerosis while anti-tRNA synthesase antibodies are specific for myositis. The autoantigens mentioned above are linked by an important common factor: they are mainly proteins that associate with either RNA or DNA (Table 1-1).

Autoantibodies Are Generated Selectively Against Nucleic Acid-Protein Complexes

Autoantibodies in SLE and certain other systemic autoimmune diseases (Sjogren's syndrome, scleroderma, and polymyositis) are generated selectively against nucleic acid-protein complexes. The autoantibody production in SLE is strongly focused on a relatively small number of cellular antigens, including chromatin (DNA-protein), Ro60 (associated with the Y1-5 small cytoplasmic RNAs) (20) and La (associated with RNA polymerase III precursor small RNAs), ribosomal P (rRNA), and the Sm and RNP antigens, associated with the U series of small nuclear RNAs (U1, U2, U4-6, and U5) (21). Most of these nucleic acids serve an essential role in the body.

The U snRNPs contain small nuclear (sn) RNA and proteins and involved in RNA splicing (22). The RNAs were originally called U snRNAs due to high uridine content. These include the U1, U2, U4/U5, and U6 as well as other less abundant U snRNPs (8). U1, U2, U4, and U5 are transcribed by RNA polymerase II and receive a tri-m_7G-cap which serves as a nuclear export signal.

Splicing is the process of removal of the non-coding gene regions (introns) that separate the coding regions (exons) for effective translation of messenger RNAs in the into proteins. U snRNPs and other nuclear proteins make up the spliceosome, the complex responsible for the removal of the introns and the re-ligation of exons. The initial phase involves the recognition and pairing of the 5' end splice site of U1 RNA by U1 snRNP. Upon cleavage of the splice site, the intron loops to form an intron lariat. This requires the binding of U2 snRNP to the branch region through interactions with U2AF (U2 snRNP auxiliary factor). Next, the cleavage occurs at the 3' splice site with the release of the lariat and rejoining of the adjacent exons (8). The U4/U5/U6 snRNPs join the assembling spliceosome. It is unclear how the U4/U5/U6 snRNPs are recruited, but this process may be mediated through protein-protein interactions and/or base

pairing interactions between U2 snRNA and U6 snRNA. The U5 snRNP interacts with sequences at the 5' and 3' splice sites. The first rearrangement is probably the displacement of U1 snRNP from the 5' splice site and formation of a U6 snRNA interaction. It is known that U1 snRNP is only weakly associated with fully formed spliceosomes.

The U1 RNA molecule contains four regions with extensive duplex RNA, stem-loops I-IV. Stem-loops I and II interact directly with the U1-70K and U1-A proteins, respectively, via RNA recognition motifs (23). Proteins reportedly do not cover stem-loops III and IV. The RNA-dependent association of U1-C with the U1 snRNP is mediated through interactions with the B'/B components of the Sm core particle (24) which binds to U1 in a region of single-stranded RNA located between stem-loop III and IV (Figure 1-1). In addition to the U1 snRNP, other snRNPs, each with a unique uridine-rich (U) RNA species as well as unique proteins, carry the Sm core particle (Table 1-2). In the cytoplasm, snRNAs interact with Sm proteins, D, E, F, and G followed by the addition of Sm B protein which completes the Sm core particle. After, the Sm ring has assembled around the Sm specific sequence of snRNP, typically AUUUGUGG the m_7G is hyper-methylated to 2,2,7-trimethylguanosine (m_3G or TMG). The completed snRNP core is transported into the nucleus via the protein importin β where addition of proteins specific to the particular snRNP (U1, U2, U4, U5) occurs. The U snRNP specific proteins are listed in Table 1-2.

Y RNAs are small cytoplasmic RNAs that are components of the Ro ribonucleoprotein complex. Although, this complex is largely found in the cytoplasm, a fraction of the Ro60 protein is nuclear (25). Human cells contain four distinct Y RNAs (Y1, Y3, Y4, and Y5). Other eukaryotes contain different numbers of Y RNA homologues. Ro RNP has been found to be ubiquitous in particular organisms. Ro 60 has been found to associate with defective 5S

ribosomal RNA precursors (rRNA). It is speculated to play a role in the discard pathway for defective 5S ribosomal RNA precursors (20). It has been proposed that the Ro60 protein may function as an "RNA chaperone", facilitating the folding of 5S rRNA precursors into the proper conformation (by binding transiently) and assisting in the degradation of abnormal RNAs (by binding more strongly) (26, 27). Y RNAs fold into a conserved secondary structure that contains at least three stem structures (regions of dsRNA). The Y RNA component is thought to regulate the access of other RNAs to Ro and therefore assist in the novel discard pathway (28). Ro60 antigen binds to the stem of the molecule (26) (Figure 1-2).

Anti-La associates transiently with precursors of several small RNAs synthesized by RNA polymerase III (29), and is a transcription termination factor for RNA polymerase II (30).

There are a number of additional common RNA-protein autoantigens. These include tRNA sythetase (associated with tRNA), fibrillarin (associated with U3, U8 and U22 RNA), To/Th (associated with 7-2, 8-2 RNA) and SRP (associated with 7SL RNA). Table 1-1 lists common autoantibodies and their associated nucleic acid components.

Apoptosis and Necrosis in SLE: A Source of Endogenous Adjuvants

Apoptosis, or programmed cell death and is a highly conserved mechanism and that was first described by Currie in 1972 (31). A key feature of apoptosis includes changes in the morphology to the cell. A series of biochemical events occurs during apoptosis including blebbing, cell membrane changes (such as loss of membrane asymmetry), cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. This results in the cell being engulfed by macrophages and neighboring cells in a non-inflammatory manner (32) by a number of different receptors, including CD14 (33), the vitronectin receptor $\alpha_v\beta_3$ (34), CD36 (35), the complement receptor (36), the phosphatidylserine receptor (37), and other

scavenger receptors. There are two main apoptotic pathways, the death receptor pathway (extrinsic) and the mitochondrial pathway (intrinsic). The death receptor pathway can be initiated by the binding of death inducing ligands to cell surface receptors namely TNF-TNFR1 (38), FasL-Fas , and TRAIL-DR4 or DR5 (39). Intrinsic apoptosis pathways can be induced by pathologic stimuli such as ultraviolet and gamma irradiation, viral infection, and toxic compounds.

Necrosis has traditionally been considered an unregulated form of cell death and is quite different from apoptosis. More recent data suggests that necrosis may be a normal physiological and programmed event and may be considered a form of programmed cell death like apoptosis. Necrosis can be induced by various bacterial and viral infections (including bacterial toxins) (40), components of the immune system (such as complement) (41), activated natural killer cells (42), and peritoneal macrophages (43). It is also suggested that members of the TNF receptor family (TNF, FAS, and TRAIL), can initiate necrosis as well as apoptosis (44). Different from apoptosis, during necrosis cells first swell, the plasma membrane collapses and cells are rapidly lysed provoking an inflammatory response. The balance between necrosis and apoptosis may be involved in the development of some diseases.

It has been suggested that defects in apoptosis may expose endogenous molecules allowing for an immune response in SLE. There are a number of reports implicating apoptotic cells as the possible source or autoantigens in SLE (45-48). An accumulation of large amounts of apoptotic cells in various tissues has been observed in SLE patients (49, 50). In addition, cells from SLE patients show a reduced phagocytic capability (51, 52). Evidence for this reduced activity is also found in mice. Large numbers of apoptotic cells or defects in the clearance of apoptotic cells (as seen in mice deficient in C1q, complement receptor 2, serum amyloid protein,

DNase I, CD31 or Mer) can lead to lupus-like manifestations including ANA production and glomerulonephritis (53-56). When phagocytosis of apoptotic cells is inhibited or defective, the plasma membranes can become permealized and cells undergo what is termed secondary necrosis (57).

It is well established that lupus autoantigens are redistributed in apoptotic cells such that they become clustered and concentrated within small surface blebs and apoptotic bodies (45, 46, 48, 58-62). The nuclear autoantigens become concentrated as a rim around the condensing chromatin in early apoptosis and remain there as apoptosis progresses. Many of these autoantigens are also structurally modified during apoptosis through cleavage, phosphorylation and novel complex formation (e.g. ubiquitination). The U1 snRNP undergoes four total modifications during apoptosis (60, 62).

The first modification involves the cleavage of the U1-70K protein. It has been found that the 70K protein is cleaved at position 338DGPD341 by caspase-3 resulting in a two-protein fragment: a N-terminal 40kDa fragment and a C-terminal 22kDa fragment. The N-terminal fragment remains associated with the U1 snRNP complex. The second modification involves cleavage of the U1 snRNA molecule. Specifically, the first 5-6 nucleotides including the 2,2,7-trimethylguanosine (TMG) cap is cleaved from the RNA molecule. The cleavage of U1 snRNA is inhibited by the presence of caspase inhibitors, indicating that an apoptotically activated ribonuclease is responsible for the specific modifications of the U1 snRNA during apoptosis. Proteolytic cleavage of the Sm-F protein during apoptosis is the third modification. The last modification involved in U1 snRNP modifications during apoptosis involves the association of phosphorylated SR proteins with the U1 snRNP complex in apoptotic cells. SR proteins, serine-arginine family of RNA splicing factors, are critical regulators of constitutive messenger RNA

splicing. In addition, the U1 snRNP complex has been shown to translocate from its normal subcellular localization to apoptotic bodies near the surface of cells undergoing apoptosis (62). This redistribution might assist in exposing the modified components of the U1 snRNP complex to the immune system when the clearance of apoptotic cell remnants is some how disturbed.

Little is known about modifications to autoantigens during necrosis. There is evidence of a few modifications, however. These include the cleavage of DNA topoisomerase I and PARP into unique fragments (63) and the disappearance of the cleaved U1-70 kd protein (64).

It has been shown that dendritic cells preferentially phagocytose apoptotic blebs and bodies via both "eat-me signals" and FcγR-mediated mechanisms. Specifically, autoantibodies to histone (using a H2B-GFP fusion protein) displayed an increase in DC phagocytosis of opsonized apoptotic cells leading to enhancement of autoantigen presentation and T cell proliferation. If the chromatin fragmentation was blocked however, in apoptotic cells, the enhancement of phagocytosis was also blocked (65).

It is possible that a defect in the clearance of apoptotic cells in addition to an increase in number of apoptotic cells could lead to secondary necrosis of the cells and the release of molecules that can act as danger signals. This would leave autoantigens easily accessible and could be the source of initiation for an autoimmune reaction.

The Immune System

The immune system can be simplistically divided into two components, the innate and the adaptive immune systems, although these components are closely linked. The innate immune system is the first line of defense for a host and therefore comes before the adaptive immune response (66). In the innate immune system there are a number of cellular receptors and secreted proteins that recognize common molecular patterns associated with a microbial infection.

Through recognition of microbes or microbial products, the innate immune system is our first line of defense and is mediated largely by the release of secreted proteins and cytokines. The overall goal of this response is to decrease the pathogen's ability to cause disease. It does this by delaying the microorganism's growth and invasion and allowing the host responses to eventually eliminate the bacterial infection or by protecting the host until an adequate acquired immune response (adaptive immunity) can be mounted. These responses include increased phagocyte activation (by both neutrophils and macrophages), the production of free radicals (superoxide and nitric oxide), and activation of antigen presenting cells (APCs) including DCs, B-cells, and macrophages.

In the past ten years, the major discovery of pattern recognition receptors (PRRs) present on cells that recognize common pathogen-associated molecular patterns (PAMPs) and initiate an immune response towards these pathogens (67, 68). Toll-like receptors (TLRs) are a specific type of PRR originally discovered in the early 1990 as Toll receptors in Drosophila, that recognize molecules unique to bacteria, viruses, parasites, or fungi such as lipopolysaccharide (gram negative bacteria), peptidoglycan (gram positive bacteria), and dsRNA (produced during viral infections). The specific pattern of PRRs activated on antigen presenting cells (APCs) controls the activation of APCs to upregulate the appropriate costimulatory molecules and to produce the cytokines needed to polarize the appropriate helper T cell response. Therefore, TLRs serve as a link between the innate and adaptive immune system. Table 1-3 shows the known TLRs and their ligands.

Toll-like receptors (TLRs) listed in Table 1-3, consist of an extracellular domain with leucine-rich repeats, a C-terminal flanking region, a membrane-spanning domain, and a cytoplasmic Toll/IL-1 receptor homology domain or TIR, which mediates signaling (69). TLR

3, 7, 8, and 9 are located in endosomes and therefore do not possess an extracellular domain (70). The TIR domain interacts with one or more adapter proteins, including MyD88, TIRAP/Mal (71, 72), TRAM, and TICAM-1/TRIF (73, 74). The adapter protein MyD88 is a key element of the signaling pathway for all TLRs with the exception of TLR3 (originally it was thought to signal via MyD88) (69, 75). Signal transduction through MyD88 recruits IL-1 receptor associated kinase (IRAK-4) activating the transcription factors NFκB and MAP kinase.

Although most TLRs are dependent on MyD88 for signaling, TLR3 and TLR4 are unique in their ability to signal via a MyD88-independent pathways (74, 76-78). MyD88-independent signaling is dependent on the adapter protein TICAM-1/TRIF (73, 74) and activates the transcription factor interferon regulatory factor 3 (IRF-3) (79). This pathway is important because it induces the production of IFNβ (74, 78, 80). IFN-I can also be induced through engagement of TLRs by nucleic acid ligands through MyD88-dependent which involves signaling through the transcription factor IRF7. This includes certain single-stranded (ss) RNA and methylated CpG DNA sequences the ligands for TLR7/8 and TLR 9 respectively.

Furthermore, there are additional intracellular detectors of nucleic acids (such as viral RNA) that can induce IFN-I. These include the cytoplasmic helicase protein retinoic-acid-inducible protein I (RIG-I), the melanoma-differentiation-associated gene 5 (MDA-5), as well as the dsRNA dependent protein kinase (PKR) (81-83). The signaling for these cytoplasmic receptors is similar to the TLR pathways as many of the same kinases and IRFs are used.

Although, TLRs have evolved to recognize components of foreign antigens, such as TLR3 binding dsRNA from viruses, LPS (component of bacterial cell wall) binding TLR4, ssRNA from viruses binding TLR7 in mice and TLR8 in humans, and unmethylated CpG DNA binding TLR9, under certain circumstances TLRs have been found to also recognize self-nucleic

acids. It has been shown that chromatin-IgG complexes can activate rheumatoid factor B cells through dual interactions with the antigen receptor (surface IgM specific for IgG2a^{a}) and TLR9 (84). Additionally, mammals were found to have evolved mechanisms that protect naïve B cells from becoming inappropriately activated by TLR9 signaling (85). These studies have important implications for autoimmunity because they suggest mechanisms that may lead to the preferential immune responses to nucleic acid-protein antigens in SLE.

Dendritic Cells in Immunity

In the 1970s, the dendritic cell (DC) was described as a novel APC population in the mouse spleen (86-88). These studies originated from research attempting to explain the requirement for non-lymphocytes in generating the T cell dependent antibody responses. DCs from different tissues express varying cell membrane markers and have multiple functions. Many different classification schemes of DCs have been used to try to define the unique abilities of their subpopulations of DCs. The predominant classification schemes that exist are based on maturity (immature versus mature DCs) and morphology (conventional versus plasmacytoid).

Conventional DCs

Despite phenotypic and functional differences, most conventional DCs have several features in common. First, DCs originate from bone marrow stem cells or circulating monocytes and migrate via the blood stream to almost all tissues, where they develop into immature DCs (89). In this immature state, DCs continuously sample the environment and readily take up antigen through receptor-and nonreceptor-mediated mechanisms to process and present these antigens to T-helper cells (90, 91). In response to antigens and danger signals such as TLR ligands, DCs upregulate MHC Class II molecules and costimulatory (CD80 and CD86) molecules, and then migrate through the lymphatic system to the T cell-rich areas of lymphoid

organs, where they can stimulate naïve or memory $CD4^+$ T cells (90, 91). This response can be induced through activation of TLRs on DCs, but the maturation of DCs by bacteria and viruses is intimately regulated by the production of IFN-I (92). IFN-I promotes the differentiation of human monocytes into DCs that have enhanced T cell stimulatory capacity. In addition, IFN-I upregulated the expression of the costimulatory molecules MHC class I and II, CD40, CD80 and CD86, and led to an increased ability to stimulate T cell proliferation by DCs (92).

In mice, conventional DCs express CD11c and constitutively express MHCII (unlike monocytes). At rest, conventional DCs express CD80 and express variable levels of CD86. Costimulatory markers are upregulated in response to stimuli such as TLR ligands from foreign antigens.

Plasmacytoid DCs

Plasmacytoid DCs (pDCs) were first identified in humans by their plasmacytoid morphology and their ability to produce large amounts of IFN-I in response to viral infection (93-97). In mice, pDCs can be classified by their unique cell surface phenotype, characterized by the expression of the granulocyte marker Gr-1, the B cell marker B220, the DC marker CD11c (98) and the pDC markers 120G8 (99)and PDCA-1 (100), both of which may be interferon inducible proteins.

pDCs express a number of TLRs including TLRs 1,6,7,9, and 10. Their signature response to infection or engagement of TLR7/8 or TLR9 is the ability to produce large amounts of IFN-I (93, 94). However, their ability to produce IFN-I has been shown to drive conventional DC maturation and IL-12 production, which can lead to the indirect stimulation of T cells by pDCs. Although, PDCs are thought to be the predominant producer of IFN-I (producing

1000-fold higher than other cells), non-plasmacytoid dendritic cells or conventional dendritic cells have been found to produce IFN-I in response to certain infections (101).

SLE and Increased Type I Interferon

The type I interferon (IFN-I) system contains a number of components including the inducers of IFN-I, the IFN-I genes and proteins, the cells producing IFN-I and the cells that respond to IFN-I. Humans have a large IFN-I gene family. This includes 13 genes encoding IFN-α, one gene encoding IFN-β and one gene encoding IFN-ω (102, 103). The genes and their products are very similar in function and structure. IFN-I are induced by viruses, double-stranded (ds) or single-stranded (ss) RNA, or certain foreign DNA sequences. IFN-I modulates immunity and autoimmunity by promoting dendritic cell maturation, T cell survival, antibody production, and plasma cell differentiated (104-107). All of the IFN-I proteins share one common receptor known as the IFNα/β receptor. Ubiquitously expressed, this heterodimeric receptor is composed of an α (IFNAR1) and a β (IFNAR2) chain (108). Both chains are required for signal transduction. Dimerization of the chains occurs upon ligand (IFN-β) binding resulting in auto- and trans-phosphorylation of two receptor-associated Janus protein tyrosine kinases (Tyk2 and Jak1) (109). STAT1 and STAT2 are subsequently recruited and phosphorylated resulting in the translocation of STAT1/STAT2 heterodimers into the nucleus where it associates with IFN regulatory factor 9 (IRF-9) forming the heterotrimeric complex IFN-stimulated gene factor 3 (ISGF3) (103). The transcription of hundreds of IFN-I inducible genes is initiated upon ISGF3 binding to the upstream consensus IFN-stimulated response elements (ISRE) (110).

In 1982, it was reported that SLE patients exhibit an increase in serum interferon-α (111). Increased IFN-I or expression of IFN-I regulated genes has been associated with disease activity,

renal involvement, and the presence of autoantibodies against dsDNA and the Sm/RNP antigens, components of the U1 snRNP (112, 113). An "interferon signature" has been described by a number of researchers. By both microarrays and real-time PCR the overexpression of numerous IFN-I inducible genes have been found to be upregulated, including Mx1, 2,5-oligoadenlyate synthetase, IRF-7, Ly6E, as well as others (114, 115). Some of these .IFN-I induced genes have been shown to correlate with disease activity (113, 116, 117) and expression of these genes has been found to reflect total IFN-I production (113).

The major IFN-I producing cells are pDCs, which represent ~0.5% of peripheral blood mononuclear cells (PBMCs) (118). Although it would seem that since SLE patients have increased IFN-I their PDC number would also be increased, the number of circulating PDCs is drastically reduced (by 70 fold) in SLE patients (119). Some reports indicate that the reduced PDC number is due to the treatment of the disease (i.e. steroids) (120), while others have found this not to be the case (113). There is evidence, however, that PDCs are recruited into tissues (inflammatory and non-inflammatory skin) of SLE patients (121, 122) and this could be the reason for the decreased PDCs in circulation.

In general, it is believed that PDCs are responsible for the increase in IFN-I in patients with SLE. However, recent evidence suggests that a subset of monocytes from SLE patients is capable of producing IFN-I (Zhuang H, unpublished data). Although their number is reduced, the PDCs isolated from SLE patients are still functional and are capable of producing 5-10 pg of IFN-α per cell after activation (123).

Adjuvants

Adjuvants have been used to increase vaccine efficacy since the early 1920's. A variety of adjuvants have been discovered that function through different mechanisms. Early vaccines

consisted of live, attenuated intact microorganisms or heat-killed intact organisms. Aluminum hydroxide (alum) is the only approved vaccine for humans in the United States and enhances immune responses by binding antigen and causing the slow-release, thus prolonging antigen presentation. Alum drives predominantly a T_H2 type response. Many TLR agonists have been used as adjuvants including lipopolysaccharide (124), CpG DNA (125, 126), muramyl dipeptides (127, 128), monophosphoryl lipid A (129), and imiquimod (130, 131). TLRs, like oil emulsion adjuvants effectively stimulate both cellular and humoral immune responses. Currently, CpG DNA is the most promising of the immunostimulatory adjuvants and is undergoing human trials. It induces predominantly T_H1 type immune responses (132, 133).

Conclusion

Macrophages, neutrophils, and DCs make up the phagocytic cells of the innate immune system, and play an integral role in the first line of defense against pathogens. The innate system is nonspecific, in the traditional sense in that similar immune responses can be produced against a variety of pathogens, including the production or various cytokines and chemokines. IFN-I is a primary cytokine produced in response to viral and bacterial infections and heightens the responsiveness of virtually every cell in the body. Phagocytes use oxygen and nitrogen free radicals to slow the rate of pathogen growth, while antigen presenting cells engulf pathogens, processing them to peptide fragments, and present the fragments to T cells, allowing the body to develop a more specific response towards the specific infecting pathogen.

In some cases, autoimmunity may result from defects in the clearance of pathogens or cross-reactivity between pathogen related molecules that have similar structures to host proteins, leading to molecular mimicry. Evidence for both of these theories has been shown in both animal models of autoimmunity and human autoimmunity. In mice, a defect in Fas signaling as well as a defect in C1q a component of the complement cascade leads to spontaneous lupus. In

SLE, there are many reports of increased circulating apoptotic cells and decreased in phagocytosis of apoptotic cells. Due to an increase in apoptosis and a decrease in the clearance of apoptotic cells there is speculation that these cells act as reservoirs for autoantigens. These autoreactive cells can then cause a cascade where the immune dampening effects of regulatory systems cannot overcome the autoreactivity leading to organ specific or systemic autoimmunity. Nucleic acid associated proteins are prevalent autoantigens, example: the U1 snRNP molecule that contains U1 RNA, which becomes modified during apoptosis/ necrosis, we speculate that the nucleic acid associated with the autoantigens becomes exposed and then can act as an endogenous adjuvant via TLRs, inducing IFN-I and driving an immune response towards the self antigen.

The underlying hypothesis for this project was that the small RNAs associated with Sm and snRNP (U1, U2, U4-6, an U5) as well as with Ro (Y1-Y5) might promote autoimmune responses to the corresponding snRNPs by stimulating signaling via TLRs and inducing IFN-I production, as well as DC maturation and/or the activation of autoreactive B cells specific for the protein components of the snRNPs, ultimately leading to autoantibody production. We examined whether RNA associated with small ribonucleoproteins can stimulate innate immunity (IFN-I) and what normally prevents endogenous snRNAs from activating IFN-I. This information may help to explain the high frequency of autoantibodies to RNA-associated proteins in autoimmune diseases.

Table 1-1. Common autoantibodies, their RNA components, and their associated disease

Antigen	RNA Component	Disease
Sm	U1, U2, U4/U6, U5 RNA	SLE
RNP	U1 RNA	Several
Ribosomal P	rRNA	SLE
Ro60 (SSA)	Y1-5 RNA	Sjogren/SLE
La (SSB)	RNAP III precursors	Sjogren/SLE
Fibrillarin	U3, U8, U22 RNA	SSc
To/Th	7-2, 8-2 RNA	SSc
tRNA synthetases	tRNA	PM
SRP	7SL RNA	PM
RNA Polymerase II	mRNA	SSc

SLE – systemic lupus erythematosus, SSc – systemic sclerosis

Table 1-2. Protein and RNA components of the major U snRNPs

SnRNP particle	*RNA*	*Shared proteins*	*Unique proteins*	*Autoantibodies*
U1 snRNP	U1	Sm core particle*	70K, A, C	Sm, nRNP
U2 snRNP	U2	Sm core particle*	A', B"	Sm, A', B"
U5 snRNP	U5	Sm core particle*	8 proteins	Sm, 100K, 102K, 200K (doublet)
U4/U6 snRNP	U4 and U6	Sm core particle*	150K	Sm, 150K

* proteins B'/B, D (D1, D2, D3), E, F, and G form the 6S Sm core particle, which is shared by all of the U snRNPs listed

Table 1-3. List of Toll-like receptors and their known ligands

Receptor	Ligand
TLR1	Functionally associate with TLR2; responds to triacyl lipopeptides
TLR2	Lipoproteins/lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from Gram-positive bacteria
TLR3	dsRNA from viruses; synthetic dsRNA polyinosinic-polycytidylic acid
TLR4	Lipopolysaccharide (LPS)
TLR5	Flagellin
TLR6	Functionally associate with TLR2 responds to diacyl lipopeptides
TLR7	ssRNA rich in guanadine and uridine (in mice), imidazoquinolines
TLR8	ssRNA rich in guanadines and uridines (in humans), imidazoquinolines
TLR9	Unmethylated DNA (CpG)
TLR10	Unknown but may participate in human asthma pathogenesis
TLR11	Uropathogenic bacteria

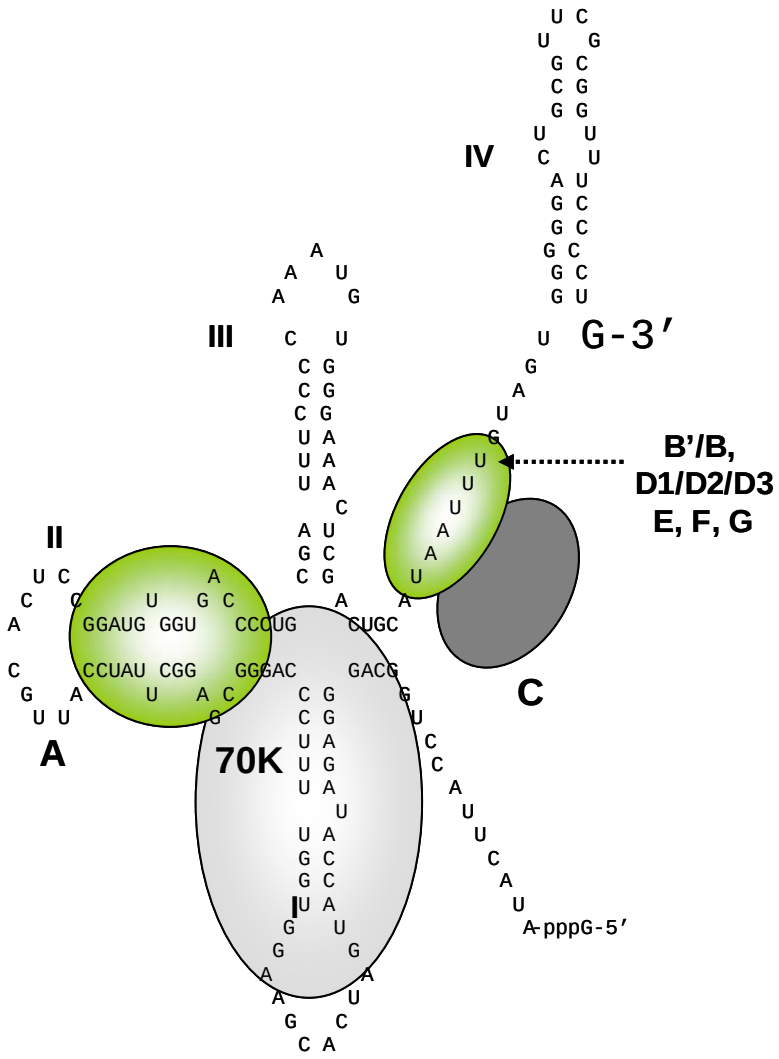

Figure 1-1. Structure of U1 snRNP. U1 RNA contains regions of highly structured RNA (dsRNA), stem-loops I-IV. Stem-loops I and II interact directly with the U1-70K and U1-A proteins, respectively. No proteins cover stem-loops III and IV. U1-C interacts with the U1 snRNP through interactions with the B'/B components of the Sm core particle (proteins B'B, D, E, F, G) which binds to U1 in a region of ssRNA located between stem-loop III and IV.

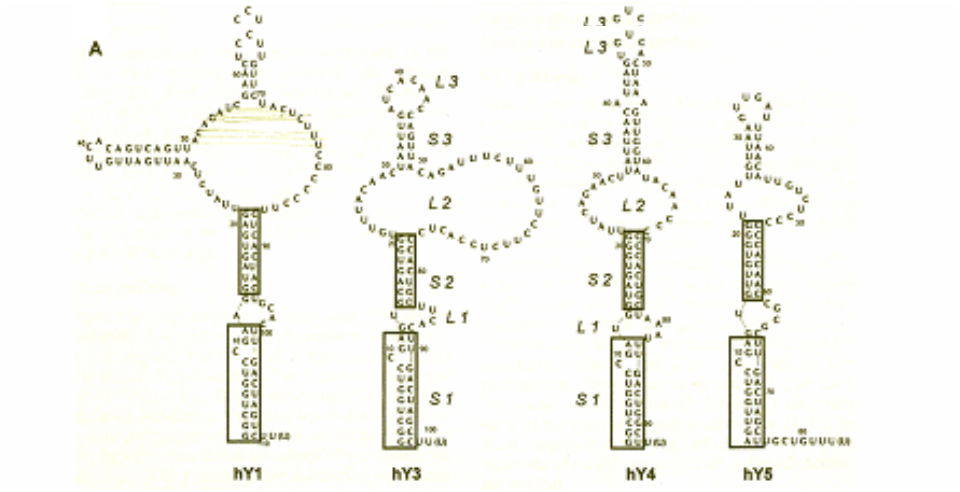

Figure 1-2. Structure of human Y RNA. Shown above are the 4 different human Y RNA. Notice the regions of highly structured RNA (dsRNA). Ro binds to the stem (boxes above) of Y RNA.

CHAPTER 2
ENDOGENOUS ADJUVANT ACTIVITY OF THE RNA COMPONENTS OF LUPUS AUTOANTIGENS SM/RNP AND RO60

Introduction

Many patients with systemic lupus erythematosus develop autoantibodies to ubiquitous host cellular molecules such as the Sm/RNP and Ro60 (SS-A) ribonucleoproteins, which contain the small RNAs U1-U6 and Y1-Y5, respectively (8, 21, 134). These small, highly structured, RNAs have extensive regions of double-stranded (ds) and single-stranded (ss) RNA, portions of which mediate interactions with proteins (8, 23, 135). Duplex RNA and certain ssRNA sequences are signatures of viral infection, the presence of which is sensed by intracellular dsRNA binding proteins such as protein kinase R (PKR) (136, 137), retinoic acid inducible gene I (RIG-I), and melanoma differentiation associated gene 5 (mda-5) (81, 138). The Toll-like receptors (TLR) 3 (139) and TLR7/TLR8 also recognize dsRNA and certain ssRNAs, respectively (96, 140, 141). Binding of RNA to these TLRs activates NFκB and mitogen-activated protein kinases (MAPKs), leading to production of inflammatory cytokines such as IL-12, IL-6, TNFα and Type I interferon (IFN-I) (96, 139-141). TLR ligand-stimulated IFN-I production is mediated through signaling pathways involving the adapter proteins MyD88 (142) and TRIF (72, 73). PKR is a downstream component in both of these pathways (72), but also can be activated by dsRNA independently of TLRs, MyD88, or TRIF (101). Like TLR9 (a sensor for DNA containing unmethylated CpG motifs) (143), TLR7, TLR8, and TLR3 are located inside of endosomes (144). Thus, in contrast to the induction of IFN-I by cytoplasmic dsRNA via PKR, RIG-1, or mda-5, the recognition of dsRNA or ssRNA by TLR3 or TLR7/8, respectively, occurs extracytoplasmically within endosomes.

Peripheral blood mononuclear cells from a subset of lupus patients with autoantibodies against the Sm/RNP and Ro60 small ribonucleoproteins express high levels of IFN-I, resulting in increased expression of mRNA for a group of IFN-I inducible genes (113). In this study, we asked whether the highly structured RNA components (U1 and Y1-5) of Sm/RNP and Ro small ribonucleoproteins, when provided extracellularly into the endosomal pathway, can initiate an "anti-viral" response (101, 145) leading to dendritic cell maturation and IFN-I production, as also occurs following endosomal uptake of immunostimulatory DNA adjuvants (143).

Materials and Methods

Reagents

Polyinosinic-polycytidlylic acid sodium salt (PIC), lipopolysaccharide (LPS, from *Salmonella minnesota*), chloroquine, bafilomycin A1, and 2-aminopurine were purchased from Sigma Chemical Company (St. Louis, MO). CpG oligodeoxynucleotide (ODN) #1826 and #2006 were from Coley Pharmaceutical Group Inc. (Wellesley, Massachusetts), and loxoribine was from Invivogen (San Diego, CA).

Synthesis of U1 RNA Stem-Loop II

U1 RNA stem-loop II, r[CAG GGC GAG GCU UAU CCA UUG CAC UCC GGA UGU GCU GAC CCC UG], was synthesized by Dharmacon using 2-ACE chemistry. The RNA was resuspended in RNase-free (diethyl pyrocarbonate treated) water. The RNA was annealed at 80°C for 5 minutes and cooled to room temperature.

Purification of U1 and hY RNAs

U1 RNA was purified from K562 (human erythroleukemia) cells as described (146). Cell extract was added to protein A-Sepharose beads coated with anti-U1-70K mAb 2.73 (147) or high titer monospecific human anti-RNP or anti-Ro60 sera. After overnight incubation, the beads were collected by centrifugation and washed three times. RNA was extracted using phenol-

chloroform, ethanol precipitated, resuspended in water, and quantified by spectrophotometry ($A_{260\ nm}$). Purity was ascertained by A_{260}/A_{280} ratio and by gel electrophoresis with ethidium bromide staining (146).

Stimulation of Cells

RAW264.7 (murine macrophage) and HEK293 (human embryonic kidney) cell lines from the American Type Culture Collection (ATCC, Rockville, MD) were maintained in DMEM + 10% fetal bovine serum (Cellgro, Herdon,VA) supplemented with L-glutamine and penicillin/streptomycin plus 10 mM HEPES (complete DMEM) at 37°C in the presence of 5% CO_2. Cells were plated at 10^6 per well in 6-well plates and 5 X 10^5 per well in 12-well plates 24 hours before adding fresh medium containing U1 RNA, Y1-5 RNA, or TLR ligands. In some cases, cells were pretreated with 10 mM 2-aminopurine or 100 nM bafilomycin A1 30 minutes before adding U1 RNA or TLR ligands or co-incubated with 100 nM chloroquine.

Spleen cells from B6, B6.PKR-/-(137), or B6.MyD88-/- mice were treated with 0.15 M NH_4Cl, 10 mM $KHCO_3$, 2 mM EDTA to lyse erythrocytes and cultured for 6 hours at 2 X 10^6/ml in complete DMEM with or without 10 μg /ml U1 RNA.

Dendritic cells were cultured from bone marrow as described (148). Briefly, bone marrow was harvested from tibiae and femurs of BALB/cJ mice, erythrocytes were lysed, and 10^6 bone marrow cells/well were cultured in RPMI 1640 + 10% fetal bovine serum (Cellgro), L-glutamine and penicillin/streptomycin at 37°C. GM-CSF (10 ng/mL; Biosource International, Camarillo, CA) or Flt-3 ligand (Flt3-L, 100 ng/mL; R&D Systems, Minneapolis, MN) was added to generate MDCs or PDCs, respectively (148, 149). For MDCs, medium was changed and fresh GM-CSF was added every 48 hours. On day 6, the cells were resuspended in medium without GM-CSF and cultured with or without U1 RNA (10 μg /ml) for 6 hours. PDCs were cultured for

6 days after which they were stimulated with or without U1 RNA for 6 hours before measuring mRNA expression. GM-CSF dendritic cells were 85-90% $CD11c^+$/MHC class II^+ and Flt3-L dendritic cells were ~50% $PDCA\text{-}1^+$ by flow cytometry. In some experiments, GM-CSF dendritic cells were cultured for 24 hours in the presence or absence of U1 RNA before measuring IFNβ and IL-12 in culture supernatants by ELISA.

Flow Cytometry

Anti-I-A^d- I-E^d-FITC, anti-CD86-PE, anti-CD11c-APC, and 7-amino-actinomycin D (7-AAD) were from BD Pharmingen (San Diego, Ca) and anti-PDC antigen-1 (PDCA-1)-PE antibody from Miltenyi Biotech (Auburn, Ca). Isotype controls were used to evaluate background fluorescence. Dendritic cells were pre-incubated for 10 minutes with 2 μg of anti-CD16 in 10 μl of staining buffer (4% bovine serum albumin Hank's azide buffer; 4% HAB) to block Fc binding. Primary antibodies were then added at pre-titrated amounts and incubated for 20 minutes, followed by washing in 1% HAB. The cells were washed and resuspended in 1% HAB supplemented with 1% 7-AAD. Data were acquired on a FACSCalibur (BD Biosciences) and analyzed with Cell Quest Pro (BD Biosciences) software. At least 20,000 events per sample were acquired and analyzed using 7-AAD to exclude dead cells.

Real-Time RT-PCR

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and treated with DNase (1 unit/μg, Invitrogen) at room temperature for 15 minutes. The RNA was reverse transcribed using Superscript II First Strand cDNA Synthesis (Invitrogen). Real-time PCR was performed with an Opticon 2 continuous fluorescence detector (MJ Research, Waltham, MA) using SYBR green core reagents and Ampli-Taq gold DNA polymerase from Perkin Elmer-Applied Biosystems (Foster City, CA). Gene expression was normalized to β-actin. Primers were designed using the

Primer3 Output program **(**Table 2-1**)**. Optimal reaction concentrations were determined over four logs of linear amplification. Forty-five cycles of PCR were performed in duplicate for each primer. The fold change in gene transcript quantity compared with β-actin was measured using the comparative ($2^{-\Delta\Delta Ct}$) method.

As total IFN-I consists of 14 or more different isoforms of IFNα plus IFNβ and IFNω, ELISAs may vary depending on the specificity of the detection antibodies (150). Measuring the expression of IFN-I inducible genes such as Mx1 by real-time PCR reliably estimates total IFN-I levels in mice (151) and humans (152).

TLR Expression

The expression of TLR3, 4, 7, 8, and 9 in resting and TLR ligand-stimulated HEK293 cells was assessed by RT-PCR using the primers indicated in Table 2-1. Reactions run without reverse transcriptase were negative in all cases, verifying the absence of genomic DNA contamination. Reactions run without adding cDNA also were negative.

Immunoblotting

RAW264.7 cells were solubilized in Laemmli buffer and cleared by centrifugation. Proteins were resolved on 7.5-10% SDS-polyacrylamide gels and transferred to nitrocellulose paper using a semi-dry transfer system (Bio-Rad, Hercules, CA). The membranes were blocked with 5% powdered milk, 20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20. Blots were incubated for 18 hours at 4°C with primary antibody (1:1000 phospho-PKR or eIF2-α; Cell Signaling, Beverly, MA) in the manufacturer's dilution buffer. Blots were washed 3 times with 20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20 before adding horseradish peroxidase labeled goat anti-rabbit secondary antibody (1:1000 for two hours). Enhanced

chemiluminescense (ECL, Amersham Biosciences, Piscataway, NJ) was used for detection. The constitutively expressed eIF-2α protein served as a loading control.

IFNβ and IL-12 p40 ELISA

Microtiter plates (Nunc, Amino F96, Roskilde, Denmark) were pre-coated overnight with 2 μg/mL anti-IFNβ antibody (US Biological, Swampscott, Massachusetts) or 4 μg/mL anti-IL-12 (BD PharMingen) in 0.1 M Na_2HPO_4/NaH_2PO_4 pH 9 and then blocked with RPMI containing 10% fetal bovine serum for 2 hours at 22°C. Following incubation with culture supernatants (1:4 dilution) overnight, polyclonal rabbit anti-mouse IFNβ or biotinylated rat anti-mouse IL-12 antibodies (1:1000 dilution in 0.5% BSA NET/NP40) (PBL Biomedical Laboratories and BD Pharmingen respectively) were added to the wells. Alkaline phosphatase-conjugated mouse anti-rabbit IgG antibodies (Sigma-Aldrich) or neutralite avidin-AP (Southern Biotechnology Associates, Birmingham, AL) were added followed by alkaline phosphatase substrate (Sigma-Aldrich). IFNβ and IL-12 concentrations were determined using standard curves of IFNβ (PBL) and IL-12 (BD Biosciences) from 0.1-100 ng/ml. Absorbance at 405 nm was determined using a VERSAmax™ microplate reader and Softmax Pro® version 4.3 (Molecular Devices, Sunnyvale, CA).

Results

Autoantibodies to the Ro60 and Sm/RNP ribonucleoprotein particles, containing Y1-5 and U1 RNAs, respectively, are associated with increased levels of IFN-I (113). We investigated whether the highly structured RNA components of these ribonucleoproteins activate RNA sensing systems mediating IFN-I production in response to viral infection. U1 RNA and the Y1-5 RNAs were affinity purified using an anti-RNP mAb and monospecific human anti-Ro serum, respectively. As shown previously (153), U1 RNA migrated as a single band on 7%

polyacrylamide-6M urea gels, whereas there were multiple bands corresponding to the human Y RNA species, the most abundant of which was Y1 (*data not shown*).

U1 and Y RNAs Induce Expression of IFN-I in Murine and Human Cell Lines

The stimulation of IFN-I production by U1 RNA was initially examined in the murine macrophage cell line RAW264.7. Purified U1 RNA enhanced expression of the IFN-inducible genes Mx1 **(**Fig. 2-1A**)** and IRF-7 (not shown) by 6-12 hours. IL-6 **(**Fig. 2-1B**)** and TNFα (not shown) gene expression also increased. A similar pattern was seen when RAW 264.7 cells were stimulated with the prototypical TLR3 ligand poly (I:C) (PIC, not shown).

HEK293, a human embryonic kidney cell line, is frequently used to study TLR function because it lacks TLRs and is readily transfected (154, 155). HEK293 cells exhibited a ~10 and 25-fold increase in Mx1 expression after stimulation with U1 RNA or Y1-5 RNA, respectively ($P = 0.02$, $P = 0.003$ by unpaired t-test for U1 and Y1-5 RNA, respectively) **(**Fig. 2-1C**)**. Unexpectedly, TLR3 ligand (PIC), TLR7 ligand (loxoribine), and TLR9 ligand (CpG ODN # 2006), but not TLR4 ligand (LPS), also enhanced Mx1 expression 5-25-fold **(**Fig. 2-1C**)**. As expected, IFNα also induced Mx1 expression (not shown).

To further evaluate the unexpected stimulation of Mx1 gene expression in HEK293 cells by TLR ligands, the expression of TLR3, 4, 7, 8, and 9 was examined by RT-PCR **(**Fig. 2-1D**)**. Resting HEK293 cells expressed TLR3, 4, 7, and 8 mRNA at low levels **(**Fig. 2-1D**)**. The expression of some or all of these TLRs increased when the cells were cultured with PIC, LPS, loxoribine, or CpG DNA or with U1 RNA or synthetic stem-loop II of U1 RNA **(**Fig. 2-1D**)**, strongly suggesting that the low levels of constitutive TLR expression by this cell line can be enhanced by TLR-mediated signaling. Interestingly, the TLR4 ligand LPS did not induce Mx1 expression above background at 6 hours **(**Fig. 2-1C**)** or 24 hours (not shown), suggesting that

TLR4 expression in this cell line was not linked to signaling pathways leading to IFN-I production and that the induction of IFN-I (Mx1 gene expression) by U1 RNA was not due to endotoxin contamination **(**Fig. 2-1C**)**. The U1 RNA did not contain detectable endotoxin by *Limulus* assay, further supporting the latter conclusion. Moreover, the stimulatory activity of U1 RNA was abolished by pre-treating the RNA for 30 minutes with ribonuclease A (RNase), as determined by measurement of IFNβ and IFNα5 (Fig. 2-2A). Thus IFN-I expression was stimulated by U1 RNA and not by a contaminant, such as LPS.

Together, the data demonstrate that U1 and Y1-5 small RNAs, components of the Sm/RNP and Ro autoantigens, respectively, stimulate the production of IFN-I in cultured human and murine cell lines. To examine whether primary APCs also were responsive to self-RNAs, bone marrow-derived dendritic cells were generated in the presence of GM-CSF (myeloid dendritic cells) or Flt3-L (plasmacytoid dendritic cells). Murine myeloid and plasmacytoid dendritic cells express an array of Toll-like receptors and undergo maturation in response to TLR ligands (156). As shown in Fig. 2-2B, incubation with U1 RNA enhanced Mx1 expression in MDCs ($p = 0.001$, unpaired t-test); there was a similar trend in PDCs ($p = 0.06$). U1 RNA also stimulated the secretion of both IFNβ and IL-12 by GM-CSF dendritic cells (ELISA, $p = 0.04$; $p = 0.03$ for IFNβ and IL-12, Mann-Whitney) **(**Fig. 2-2C**)**.

Surface markers characteristic of dendritic cell maturation also increased upon stimulation with U1 RNA. The percentage of $CD11c^{+}CD86^{high}$ GM-CSF DCs increased from a mean of 3.2% to 36% ($p = 0.03$, Mann Whitney) (Fig. 2-2D, data representative of four experiments) and $CD11c^{+}$MHC-II^{high} DCs exhibited a less dramatic increase from 22% to 28% (not shown). The enhanced production of IFN-I and IL-12 along with the increased expression of CD86 and class

II by murine bone marrow derived DCs suggest that U1 RNA promoted the maturation of GM-CSF bone marrow derived dendritic cells.

U1 RNA Stem-Loop II Stimulates IFN-I Production

To examine whether portions of the U1 RNA molecule containing tracts of dsRNA stimulate IFN-I production, the second stem-loop was synthesized. Stem-loop II is 32 base-pairs long and self-anneals to form a dsRNA structure **(**Fig. 2-3A**)** covered by the U1-A protein in the intact snRNP particle. Stem-loop II stimulated the expression of the IFN-inducible genes Mx1 **(**Fig. 2-3B**)** and IRF7 **(**Fig. 2-3C**)**. However, it was less potent than the intact U1 RNA molecule, suggesting that other portions of the U1 RNA molecule are also immunostimulatory. Since viral RNA can stimulate IFN-I either through extracellular/endosomal or intracellular/cytoplasmic pathways, we investigated whether immunostimulation by U1 RNA is mediated by TLRs that interact with ds (TLR3) or ss (TLR7 and 8) RNAs or the cytoplasmic RNA sensor PKR.

PKR Mediates U1 RNA Induced IFN-I Production

The immunostimulatory activity of U1 RNA was strongly inhibited by a prototypical PKR inhibitor (2-aminopurine) as indicated by reduced expression of U1-stimulated IFNβ, Mx1, TNFα, and IL-6 **(**Fig. 2-4A**)**. TLR3 ligand (PIC)-induced Mx1, IL-6, and TNF-α expression also was inhibited by 2-aminopurine, but somewhat less potently **(**Fig. 2-4A**)**. In addition, phosphorylation of PKR in RAW264.7 cells was increased in the presence of U1 RNA or PIC (1 hour incubation) when compared to untreated cells **(**Fig. 2-4B**)**.

The role of PKR also was examined in PKR -/- mice **(**Fig. 2-4C**)**. In PKR -/- spleen cells, U1 RNA-induced Mx1 and IL-6 expression was decreased to < 10% of the wild type value **(**Fig. 2-4C**)**. The results were similar using U1 RNA stem-loop II. Mx1 and IL-6 expression induced by LPS (TLR4 ligand) and loxoribine (TLR7 ligand) was decreased to ~50% of control values in

PKR -/- mice. In contrast, Mx1 and IL-6 expression induced by PIC was similar (or even increased) in PKR -/- mice vs. controls, suggesting that TLR3 signaling was less dependent on PKR than signaling initiated by U1 RNA or loxoribine. These data indicate that the intracellular dsRNA sensing protein PKR is required for the immunostimulatory activity of U1 RNA.

Role of Toll-Like Receptors

Although PKR activation typically is thought to require intracellular delivery of dsRNA, U1 RNA was stimulatory when administered extracellularly **(**Figs. 2-1 through 2-4**)**. To assess whether the activity of U1 RNA required endosomal uptake we examined the effects of chloroquine, a non-specific inhibitor of endosomal acidification, and bafilomycin A, a specific inhibitor of the endosomal proton translocating ATPase (157). Although chloroquine (100 nM) had no effect, U1 RNA-stimulated Mx1 expression was abrogated by bafilomycin (100 nM or 250 nM) ($p = 0.0001$, unpaired t-test) **(**Fig. 2-5A**)**, strongly suggesting that U1 RNA must reach a late endosomal compartment in order to stimulate IFN-I production. In contrast, Mx1 expression induced by the TLR3 ligand PIC was reproducibly enhanced somewhat by chloroquine and was unaffected by bafilomycin **(**Fig. 2-5B**)**. As expected, TLR9 ligand mediated Mx1 expression was strongly inhibited by chloroquine **(**Fig. 2-5C**)**, indicating that at a dose of 100 nM, chloroquine has different effects on Mx1 expression stimulated by U1 RNA (no effect), TLR3 ligand (enhancement), and TLR9 ligand (inhibition). These experiments suggest that U1 RNA must be taken up into endosomes in order to stimulate IFN-I production and that the induction of IFN-I does not involve interactions with TLR3 or TLR9. To evaluate whether U1 RNA interacts with an endosomal TLR, the ability of U1 RNA to induce IFN-I production was examined in MyD88 deficient cells.

TLR7, 8, 9, and 3 all are thought to interact with their ligands inside of endosomes (144). TLR3 signals through the adapter proteins MyD88 and TRIF (TICAM-1), but IFN-I production

is TRIF-mediated. In contrast TLR7, 8, and 9 signal exclusively via MyD88 (144). Spleen cells from MyD88-/- mice were incubated with U1 RNA **(**Fig. 2-5D**)**. IFNβ expression induced by either U1 RNA or the TLR7 ligand loxoribine was reduced to ~2% of control values in MyD88-/- spleen cells **(**Fig. 2-5D, left**)**. In contrast, as expected there was little effect on TRIF-dependent IFNβ expression stimulated by PIC (95% of control value in MyD88-/- spleen cells). TNFα expression induced by U1 RNA or loxoribine also was decreased in MyD88-/- spleen cells (to 31% and 10% of wild type control values, respectively) **(**Fig. 2-5D, right**)**. TNFα expression induced by PIC was reduced as well (21% of control value). The data indicate that an endosomal TLR utilizing the MyD88 signaling pathway mediates the immunostimulatory activity of U1 RNA, further arguing against involvement of TLR3, which stimulates IFN-I production via a MyD88-independent, TRIF-dependent pathway (73).

Discussion

Autoantibody production in SLE is focused on a small subset of cellular antigens including the ribonucleoproteins Ro60 (SSA), La (SSB), and Sm/RNP(21). The U1 snRNP consists of proteins U1-70K, A, B'/B, C, D1/2/3, E, F, and G and a single U1 RNA molecule containing four regions of duplex RNA (stem-loops I-IV) interspersed with single-stranded segments (8, 134). The Y1-5 RNAs also contain extensive duplex as well as single-stranded RNA (20).

It is not known why the most prominent autoantigens in SLE are nucleic acid-protein complexes. As U1 RNA is an abundant, highly stable cellular RNA associated with a common lupus autoantigen, we investigated the possibility that this and other RNA components of autoantigens might promote autoimmunity by acting as "endogenous adjuvants". Viral nucleic acids can be detected because they have a different structure than cellular nucleic acids or an abnormal location. Duplex (ds) RNA, a structure common to the replication of many ssRNA

viruses, is detected by cytoplasmic sensors (e.g. PKR, RIG-1, and mda-5) (81, 136-138) as well as TLR3 (139). In contrast, GU-rich ssRNA is sensed by TLR7 and TLR8 (96, 140, 141). TLR3 (158), TLR7 and TLR8 (70, 159) are endosomal whereas recognition of dsRNA by PKR, RIG-1, and mda-5 requires entry into the cytoplasm (101).

Our findings indicate that the U1 and Y1-5 RNA components of the Sm/RNP and Ro60 autoantigens stimulate innate immunity via an endosomal TLR. Unexpectedly, the cytoplasmic dsRNA sensor PKR also was involved. The fact that a specific inhibitor of endosomal acidification (bafilomycin) as well as the PKR inhibitor 2-aminopurine both abrogated the immunostimulatory effect of U1 RNA suggests that the TLR and PKR are essential components of the same pathway, culminating in dendritic cell activation and the production of IFN-I and other cytokines. As these outcomes are hallmarks of the adjuvant effect, our data suggest that ribonucleoproteins may be common targets of autoimmunity because they carry endogenous adjuvant in the form of immunostimulatory RNA.

Others have found that siRNA and mRNA may be immunostimulatory as well (95, 160, 161). Thus, the association of self-antigens with a variety of immunostimulatory cellular RNAs may render this class of autoantigens more immunogenic. It remains to be determined how widespread this phenomenon may be, but in preliminary studies, we have found that tRNA (associated with autoantigenic aminoacyl tRNA synthetases) also is immunostimulatory (K Kelly-Scumpia, unpublished data). RNA modifications, such as the trimethylguanosine cap structure of the U RNAs, could further influence the immunostimulatory properties. The importance of accessibility of the RNA also remains to be fully investigated. Much of the U1 RNA molecule is encased by proteins in the intact U1 snRNP. However, misfolding of the RNA may occur under physiological or pathological conditions. Interestingly, the Ro60 autoantigen

plays a role in the recognition of misfolded RNAs including U snRNPs (28, 162). Moreover, a lupus-like syndrome develops in mice lacking Ro60 (163), suggesting that abnormal exposure of potentially immunostimulatory RNAs could promote autoimmunity.

We show here that U1 RNA exerts its adjuvant effect through a pathway involving both an endosomal TLR and PKR. A central role of PKR is supported by several lines of evidence: 1) IFN-I production stimulated by U1 RNA was abrogated by 2-aminopurine, a prototypical but non-specific PKR inhibitor (164, 165) 2) U1 RNA stimulated PKR phosphorylation, and 3) spleen cells from PKR-/- mice produced little IFN-I in response to extracellular U1 RNA or stem-loop II, suggesting that cytoplasmic recognition of U1 RNA may not be essential.

PKR recognizes intracellular dsRNA intermediates during viral infection via a ~70 amino acid dsRNA binding motif (83), resulting in its autophosphorylation and dimerization (166, 167) followed by phosphorylation of eIF2, which blocks protein synthesis (137, 168, 169). However, PKR also can be activated independently of dsRNA. LPS-induced upregulation of co-stimulatory molecules via TLR4 is strongly inhibited by 2-aminopurine (170) and the induction of IL-6 and IL-12 by LPS is defective in PKR-/- cells (137). Moreover, the induction of IFNα by TLR7/8 and TLR9 ligands is PKR dependent (150). It is likely that U1 RNA activates PKR via this latter pathway.

In addition to PKR activation, endosomal acidification was required for U1 RNA stimulated IFN-I, IL-6, and TNFα production. Interestingly, at a chloroquine dose of 100 nM, which abolished IFN-I production in response to TLR9 ligand, there was little effect on U1 RNA-stimulated IFN-I production. In contrast, it was completely blocked by a specific inhibitor of endosomal acidification, bafilomycin A, demonstrating that U1 RNA must reach a late endosomal-lysosomal compartment to activate innate immunity and suggesting that U1 RNA is

not recognized by TLR9. Notably, TLR3 ligand-stimulated IFN-I production was resistant to bafilomycin, suggesting that U1 RNA did not signal via this endosomal receptor (158). These data implicate other endosomal TLRs such as TLR7/8 in the recognition of U1 RNA (70, 159, 171).

The induction of IFN-I by TLR3 ligands is MyD88 independent and TRIF dependent, whereas TLR7/8 and TLR9 signaling is MyD88 dependent and TRIF independent (144). The decreased U1 RNA-stimulated IFN-I production by MyD88-/- cells provides further evidence that U1 RNA was not recognized primarily by TLR3. Thus, although it has been reported that synthetic U1 RNA stimulates IL-6 and IL-8 production via TLR3 (172), our data suggest that U1 RNA of cellular origin does not substantially stimulate TLR3 signaling.

Stimulation of IFN-I by TLR3 ligand was relatively insensitive to the absence of PKR **(**Fig. 2-4**)**, was not inhibited by bafilomycin **(**Fig. 2-5B**)**, and was MyD88 independent **(**Fig. 2-5D**)** whereas stimulation by U1 RNA was dependent on PKR, endosomal acidification, and MyD88, strongly implicating one of the endosomal TLRs. TLR7 and 8 both recognize single-stranded RNAs rich in G-U sequences (96, 140). In human cells, TLR8 is thought to be the main receptor, whereas in the mouse, TLR7 is more important (140). As TLR8 signaling is thought to be defective in mice (144, 173), our data are most consistent with TLR7. Although we cannot formally exclude TLR9, its ligand is CpG DNA (132, 143) and stimulation by CpG DNA was strongly inhibited by chloroquine, whereas stimulation by U1 RNA was not. We conclude that extracellular U1 RNA was recognized by TLR7 in acidic endosomal compartments followed by PKR activation, leading to IFN-I, TNFα, and IL-6 production.

Our data add to the evidence that TLR signaling is involved in autoimmunity. Recently, it was reported that in immune responses to chromatin, the interaction of cellular CpG sequences

with TLR9 acts in concert with antigen receptor engagement to promote B cell activation and could play a critical role in generating anti-DNA antibodies (84, 174). We propose here a mechanism involving the activation of dendritic cells by immunostimulatory self-RNAs such as U1 and Y1-5, which may be exposed during endosomal degradation of apoptotic cells (175) or in the course of UV irradiation (162), a known precipitating factor in lupus flares. Their interaction with an endosomal TLR stimulates both cytokine production and dendritic cell maturation **(**Fig. 2-2B-D**)**, suggesting that the exposed small RNA components of these autoantigens serve as endogenous adjuvants. In view of the importance of self-antigen presentation by immature dendritic cells for immune tolerance (176), the stimulation of dendritic cell maturation by self-RNA molecules in the presence of self-antigen could help explain why ribonucleoproteins are such common targets of autoimmunity in lupus and other systemic autoimmune diseases.

Table 2-1. List of PCR primers used in these studies

Gene	Forward Primer	Reverse Primer
Mx-1 (murine)	GATCCGACTTCACTTCCAGATGG	CATCTCAGTGGTAGTCAACCC
Mx1 (human)	CACGAAGAGGCAGCGGGATCG	CCTTGCCTCTCCACTTATCTTCGT
IRF-7 (murine)	TGCTGTTTGGAGACTGGCTAT	TCCAAGCTCCCGGCTAAGT
TLR3 (murine)	TCCGCCCTCTTCGTAACTTG	AACCCCGACTGGGATTTCAT
TLR4 (murine)	GAGGCAGCAGGTGGAATTGT	TGCTCAGGATTCGAGGCTTT
TLR3 (human)	CTTTGGGATGCTGTGTGCAT	TTGGCGGCTGGTAATCTTCT
TLR4 (human)	GCTAAGGTTGCCGCTTTCAC	ACCGTCTGACCGAGCAGTTT
IL-6 (murine)	CACGGCCTTCCCTACTTCAC	TCAGAATTGCCATTGCACAAC
TNFα (human)	GCCCGACTATCTCGACTTTG	GGTTGAGGGTGTCTGAAGGA
IFNβ (murine)	TGGGAGGATTCTGCATTACC	CAGCATCTGCTGGTGGAAGA
IFNβ (human)	AGCTCCAAGAAAGGACGAACAT	ACACTGTCTGCTGGTGGAGT
IL-6 (human)	AAAGAGGCACTGGCAGAAAA	GCTCTGGCTTGTTCCTCACT
Actin (murine)	CCCACACTGTGCCCATCTAC	CGCTCGGTCAGGATCTTCAT
Actin (human)	CACTGTGAAGACCTGTACGCCAACACA	CTTCTGCATCCTGTCGGCAAT
TLR3	ATTGGGTCTGGGAACATTTCTCTTC	GTGAGATTTAAACATTCCTCTTCGC
TLR4	CTGCAATGGATCAAGGACCA	TCCCACTCCAGGTAAGTGTT
TLR7	AGTGTCTAAAGAACCTGG	CTTGGCCTTACAGAAATG
TLR8	CAGAATAGCAGGCGTAACACATCA	AATGTCACAGGTGCATTCAAAGGG
TLR9	TTATGGACTTCCTGCTGGAGGTGC	CTGCGTTTTGTCGAAGACCA

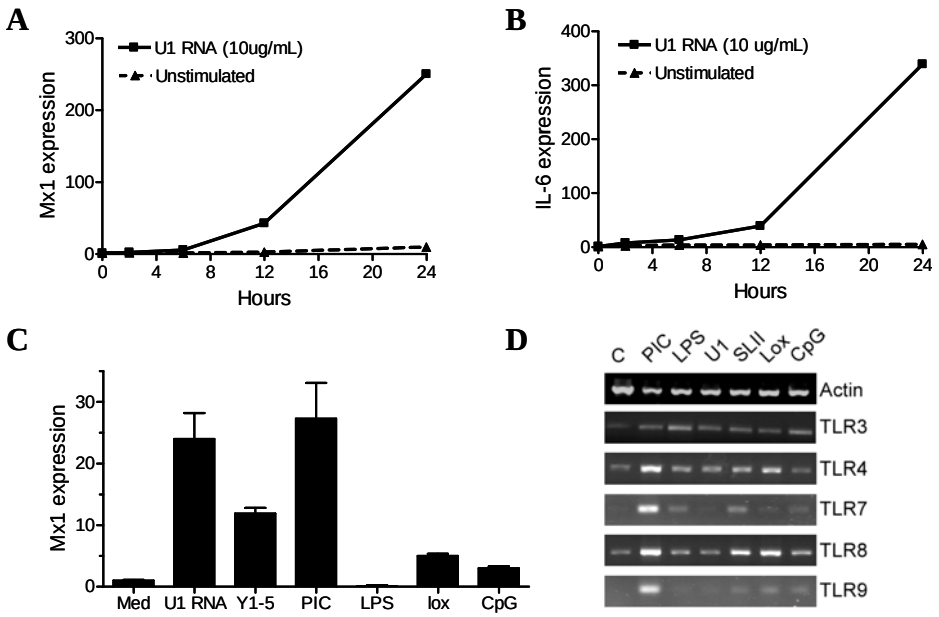

Figure 2-1. U1 RNA induces IFN-I and IL-6 expression. A-B) RAW264.7 cells. RAW264.7 cells were cultured with U1 RNA (10 μg/ml) and expression of A) Mx1 and B) IL-6 was measured by real-time PCR normalized to β-actin after 0-24 hours (n = 3 experiments). C) HEK293 cells cells were cultured for 6 hours with medium alone (Med) or with U1 RNA (10 μg/ml), Y1-5 RNA (10 μg/ml), PIC (50 μg/ml), LPS (100 ng/ml), loxoribine (100 nM), or CpG ODN #2006 (10 μg/ml) before measuring Mx1 gene expression (n = 3 experiments). Mx1 expression induced by LPS was also tested at 24 hours (not shown). D) TLR expression by HEK293 cells. HEK293 cells were cultured for 6 hours with PIC, LPS, U1 RNA, U1 RNA stem loop II (S-LII, 10 μg/ml), loxoribine, or CPG ODN #2006. RNA was extracted and expression of TLR3, TLR4, TLR7, TLR8, TLR9, and β-actin was examined by RT-PCR (n = 3 experiments).

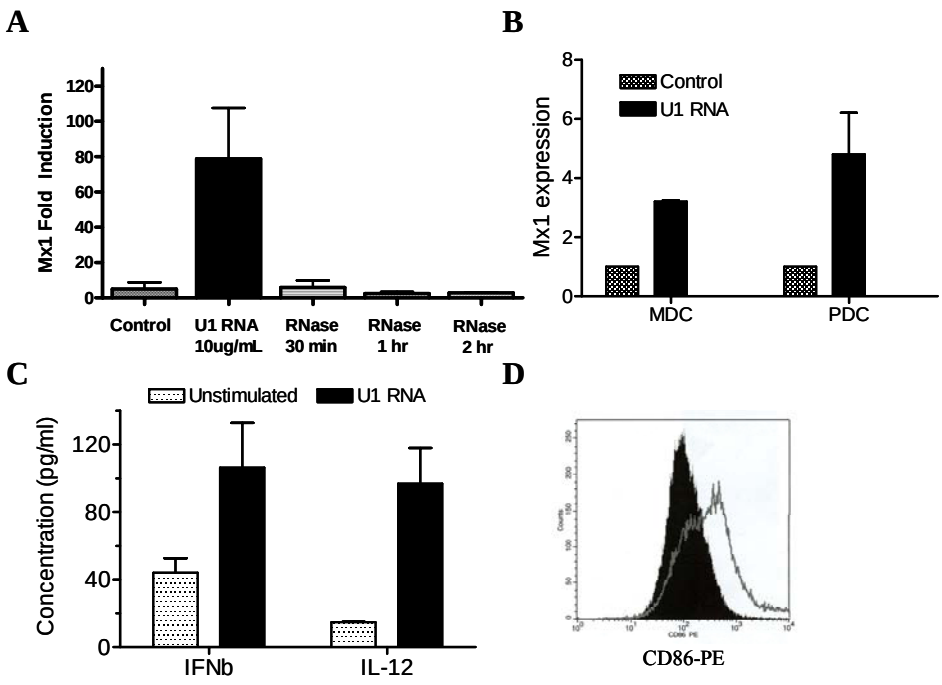

Figure 2-2. U1 RNA promotes dendritic cell maturation. A) RNase sensitivity of U1 RNA stimulated IFN-I production. RAW 264.7 cells were cultured for 6 hours with medium alone (Control), U1 RNA 10 μg/ml, or ribonuclease A treated (0.5 μg/ml for 0.5, 1, or 2 hours) U1 RNA 10 μg/ml. IFNβ (left) and IFNα5 (right) expression was assessed by real-time PCR (n = 3 experiments). B) U1 RNA stimulates IFN-I in murine bone marrow derived dendritic cells. Dendritic cells generated from bone marrow cells were treated for 6 days with GM-CSF or Flt-3L and incubated for 6 hours with U1 RNA (10 μg/ml) or medium alone. Mx1 expression was measured by real-time PCR (n = 3 experiments). C) IFNβ and IL-12 production by U1 RNA stimulated bone marrow derived MDCs. Bone marrow derived dendritic cells (grown in the presence of GM-CSF) were cultured with U1 RNA (10 μg/ml) or medium alone (unstimulated) for 24 hours before testing culture supernatants by ELISA for IFNβ and IL-12 (n = 4 experiments). D) Flow cytometry of bone marrow-derived dendritic cells incubated with U1 RNA. GM-CSF dendritic cells were incubated with U1 RNA (10 μg/ml) for 24 hours before staining with anti-CD11c and anti-CD86 antibodies. Untreated $CD11c^+$ cells were 3.2% $CD86^+$ whereas U1 RNA treated $CD11c^+$ cells were 36% $CD86^+$ (representative of 4 experiments).

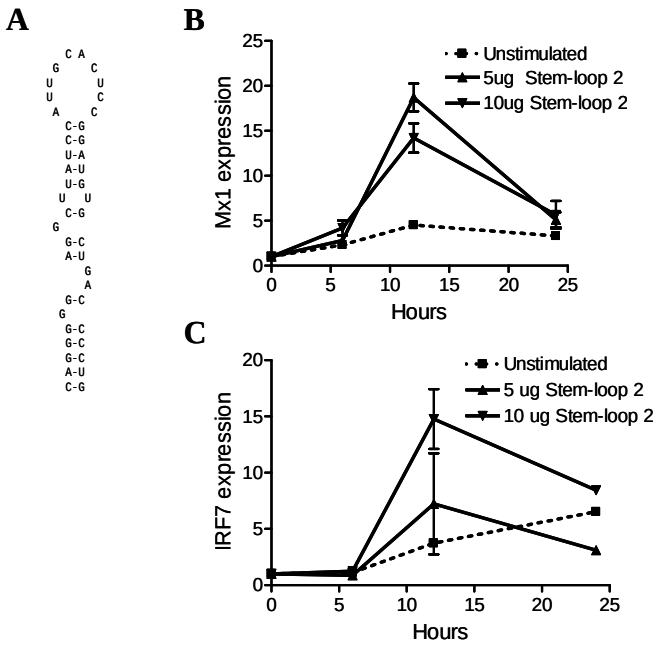

Figure 2-3. Stem-loop II of U1 RNA stimulates IFN-I expression. A) Structure of stem-loop II. B-C) Stimulation of Mx1 and IRF-7 expression by stem-loop II. RAW264.7 cells were cultured with stem-loop 2 (5 or 10 μg/ml) for 0-24 hours and Mx1 and IRF-7 expression was determined by real-time PCR (normalized to β-actin expression, n = 3 experiments).

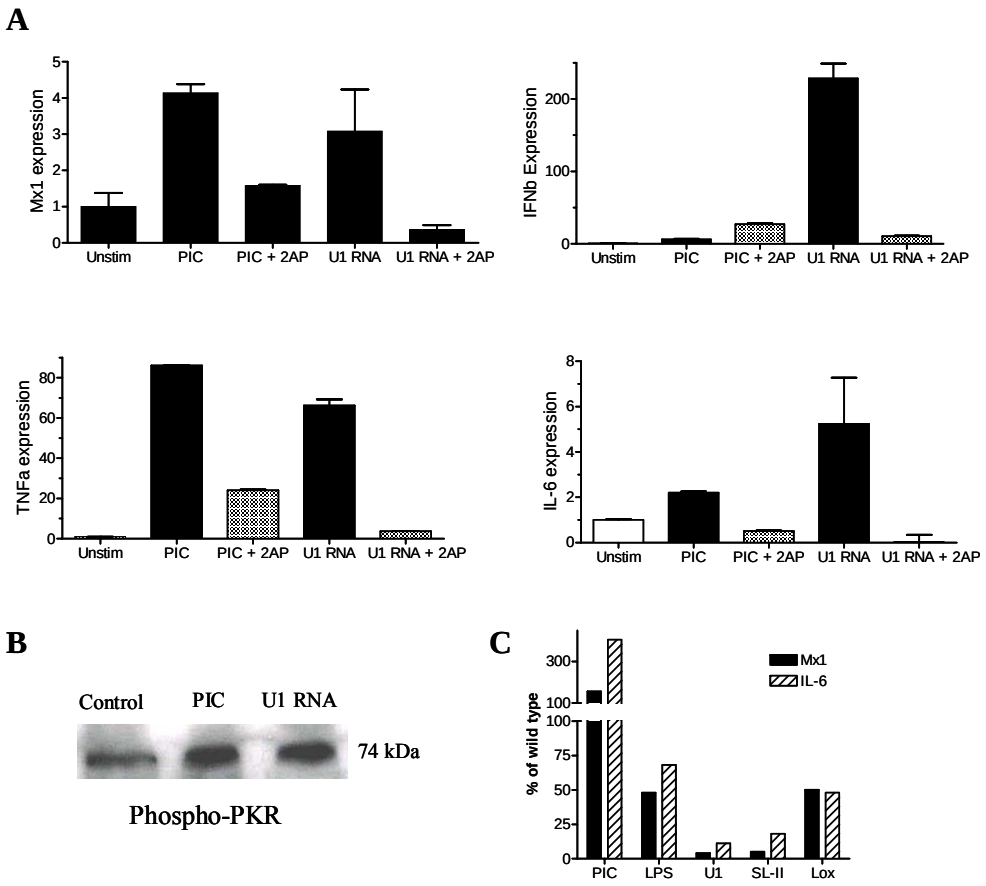

Figure 2-4. U1 RNA-inducible IFN-I production is PKR dependent. A) Effect of 2-aminopurine. HEK293 cells were cultured for 6 hours with U1 RNA (10 μg/ml) or poly (I:C) (50 μg/ml) with or without 30 minutes pre-treatment with 10 mM 2-aminopurine and the expression of IFNβ, Mx1, TNFα, and IL-6 was determined by real-time PCR (n = 3 experiments). B) Phosphorylation of PKR is stimulated by U1 RNA. RAW264.7 cells were cultured in medium alone (Control) or in the presence of U1 RNA (10 μg/ml) for 1 hour after which cell extract was resolved by SDS-PAGE and transferred to nitrocellulose. Membrane was probed with anti-phospho-PKR antibodies (1:1000, 18 hours). C) PKR deficient spleen cells. Spleen cells were isolated from PKR-/- mice and wild type controls and cultured with PIC (50 μg/ml), LPS (100 ng/ml), U1 RNA (10 μg/ml), U1 RNA stem-loop II (SL-II, 10 μg/ml), or loxoribine (Lox) for 6 hours before determining Mx1 and IL-6 expression (% of wild type level) (n = 3 experiments).

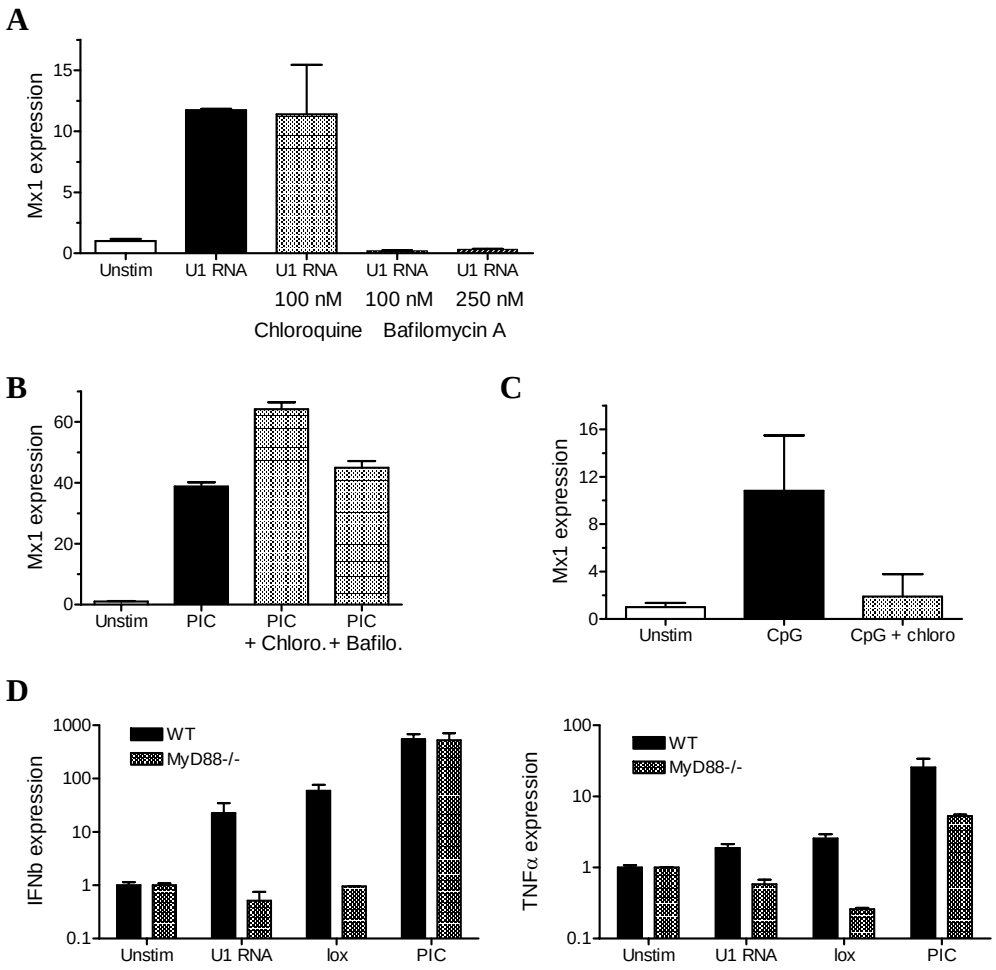

Figure 2-5. U1 RNA-inducible IFN-I production requires endosomal acidification and TLR signaling. A-B) Effect of bafilomycin A and chloroquine on IFN production stimulated by 1 μg/ml of U1 RNA or 50 μg/ml of poly (I:C) (n = 3). HEK293 cells were cultured with U1 RNA or PIC for 6 hours in the presence or absence of bafilomycin A (100 or 250 nM) or chloroquine (100 nM) or with medium alone (Control). Mx1 gene expression was measured by real-time PCR (n = 3 experiments). C) Chloroquine inhibits TLR9 signaling. RAW264.7 cells were cultured with medium alone (unstimulated) or CpG ODN #1826 (10 μg/ml) for 6 hours in the presence or absence of 100 nM chloroquine. Mx1 expression was determined by real-time PCR (n = 3 experiments). D) U1 RNA stimulation is MyD88 dependent. MyD88-/- or wild type spleen cells were cultured with U1 RNA (10 μg/ml) for 6 hours followed by measurement of IFNβ and TNFα expression by real-time PCR (n = 3 experiments).

CHAPTER 3
IN VIVO ADJUVANT ACTIVITY OF THE RNA COMPONENT OF THE SM/RNP LUPUS AUTOANTIGEN

Introduction

Adjuvants have been used to increase vaccine efficacy since the early 1920's (177, 178) and act through a variety of different mechanisms. Aluminum hydroxide (alum) is a commonly used adjuvant in both human and animal vaccines that drives predominantly a T_H2 type response and acts by delaying the release of antigen and enhancing uptake by antigen presenting cells (APCs). A newer adjuvant currently in clinical trials consists of oligodeoxyribonucleotides containing unmethylated CpG motifs (CpG ODNs) (173). CpG ODNs activate immune cells through toll like receptor (TLR) 9 and potently induce T_H1 immune responses as evidenced by IFNγ and IL-12 production and the development of cytotoxic T cell responses (133, 143). Recently, it has become evident that certain endogenous molecules can be immunostimulatory and could drive pathological immune responses to self-antigens in systemic autoimmune diseases (172, 174, 179, 180).

Systemic lupus erythematosus (SLE) is commonly associated with autoantibodies against endogenous DNA-protein (nucleosomes) and RNA-protein (small ribonucleoproteins) complexes (146). In experimental systems, antibody-DNA complexes drive autoreactive B cell responses by simultaneously engaging the B cell receptor and activating toll-like receptor 9 (TLR9) via unmethylated CpG motifs contained within the cellular DNA (174). U1 RNA is associated with a common lupus autoantigen, the U1 small nuclear ribonucleoprotein (snRNP) or Sm-RNP antigen. Purified U1 RNA or synthetic oligoribonucleotides derived from U1 RNA can activate the IFNα/β pathway via the TLR7/8-MyD88 pathway in dendritic cells and splenocytes (172, 179, 180). However, whether U1RNA has adjuvant activity *in vivo* is currently unknown.

We show here that U1 RNA acts as an adjuvant *in vivo*, providing further evidence that U1 and other cellular RNAs may promote autoimmunity by enhancing pathological immune responses to the proteins they associate with.

Materials and Methods

Purification of U1 RNA

U1 RNA was purified from K562 (human erythroleukemia) cells as described (146). Cell extract was added to protein A-Sepharose beads coated with anti-U1-70K mAb 2.73 (147). After overnight incubation, the beads were collected by centrifugation and washed three times. RNA was extracted using phenol-chloroform, followed by ethanol precipitation, resuspension in PBS, and quantification by spectrophotometry ($A_{260\ nm}$). Purity was ascertained by A_{260}/A_{280} ratio and agarose gel electrophoresis followed by ethidium bromide staining. The purified U1 RNA was verified to be free of detectable endotoxin using the *Limulus* assay (BioWhittaker, Walkersville, MD).

Mice

All studies were approved by the Institutional Animal Care and Use Committee of the Veteran Affairs Hospital and/or the University of Florida (Gainesville, FL). Eight to ten-week-old C57BL/6 and C3H/HeJ mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in a conventional facility in barrier cages. B6.Tg(TcraTcrb)425Cbn or OVA323-337 specific MHC class II restricted T cell transgenic (OT-II) mice were a gift of Dr. Laurence Morel (University of Florida). Blood was obtained from the tail vein before the mice were injected subcutaneously with 100 μg of 4-hydroxy-3-nitrophenyl acetyl (NP)17-19-conjugated keyhole limpet hemocyanin (KLH) (NP-KLH, Biosearch Technologies, Novato, CA) precipitated in alum (Pierce, Rockford, IL), or mixed with U1 RNA (50 μg) or PBS. Blood was collected from the tail vein at various intervals following immunization and by cardiac puncture at the time of

sacrifice (days 10 or 16). Spleen and lymph node (LN) cells also were isolated at that time.

Anti-NP ELISA

Pre- and post-immune sera were tested for IgM and IgG anti-NP antibodies by ELISA. Microtiter plates were coated with NP_{19}-conjugated bovine serum albumin (BSA, Biosearch Technologies, Menlo Park, CA). Sera were serially diluted 1:2 and incubated for 1 hour at room temperature. Anti-NP IgM and IgG antibodies were detected using alkaline phosphatase conjugated goat anti-mouse IgM or IgG antibodies (1:1000, BD Biosciences, San Jose, CA) followed by phosphatase substrate (Sigma-Aldrich, St. Louis, MO). Optical density was converted to concentration based on standard curves with high titer sera from C57BL/6 mice immunized with NP-KLH using a four-parameter logistic equation (Softmax Pro 3.1 software; Molecular Devices Corporation, Sunnyvale, CA).

T Cell Proliferation Assay

OT-II mice were immunized subcutaneously in the abdomen with 100 μg $OVA_{323-337}$ peptide (GenScript Corp, Piscataway, NJ) absorbed onto alum or mixed with PBS or U1 RNA (50 μg) in 100 μL final volume. Spleens and lymph nodes (inguinal, axillary and cervical) were harvested 8 days after immunization. Single cell suspensions were made by disrupting the tissue with a 0.7 μm cell strainer and red blood cells were lysed using ACK lysis buffer (BioWhittaker). $CD4^+$ cells were positively selected with anti-CD4 (L3T4) microbeads using LS columns and a Midi magnet (Miltenyi Biotech, Auburn, CA). Negatively selected cells from the spleen of a PBS treated mouse were irradiated (3,000 R) and used as antigen presenting cells (APCs). The positive fraction (containing >95% $CD4^+$ T cells) was eluted with 5 mL MACS buffer (0.5% BSA, 2 mM EDTA in PBS). The $CD4^+$ T cells and APCs were counted and 25,000 $CD4^+$ T cells were plated with 250,000 irradiated non-T cells as APCs in a round bottom 96-well plate (Falcon, Becton Dickinson, San Jose, CA) in RPMI 1640 medium supplemented with 10% fetal

bovine serum, penicillin/streptomycin, L-glutamine, and HEPES. Ovalbumin peptide ($OVA_{323-337}$) or BSA (control antigen) was added at a concentration of 10 μg/mL in a total volume of 200 μL. After 48 hours, cells were labeled with [^{3}H]-thymidine and incorporation was determined in quadruplicate 18 hours later using a liquid scintillation counter.

Footpad Injection of U1RNA and Lymph Node Single-Cell Suspensions

Following induction of anesthesia with 1% isofluorane, mice were injected in the left hind footpad with 15 μg of U1RNA in 50 μl PBS using an insulin syringe and 29-gauge needle (BD Biosciences, Franklin Lakes, NJ). In some experiments, U1 RNA was treated with RNase A (0.1 μg RNase A/15 μg of U1 RNA; Gentra Systems, Minneapolis, MN) at 37°C for 2 hours prior to footpad injection. In other experiments, U1 RNA was injected along with an immunoregulatory DNA sequence (IRS) specific for TLR7 (IRS 661: 5'-TGCTTGCAAGCTTGCAAGCA-3') or a control inactive oligonucleotide (ODN) (5'-TCCTGCAGGTTAAGT-3') at a concentration of 5.6 μM (180) (Invitrogen, Carlsbad, CA). Eighteen hours later, mice were euthanized, and bilateral popliteal lymph nodes were harvested. Lymph nodes were placed into 60 X 15 mm tissue-culture dishes (Falcon) containing 2 mL PBS on ice. The lymph nodes were gently disrupted with two 30-gauge needles. The cell suspension was filtered through a 70-μm mesh filter (Falcon) into a 15-ml conical tube. The cells were centrifuged at 1200 rpm for 10 min at 4°C. The supernatant was aspirated, and the cells were resuspended in 5 ml PBS and counted using a hemocytometer. Cells (0.2 to 1 X 10^6) were dispensed into 5 ml polystyrene round-bottom tubes and stained for flow cytometry as described below.

Flow Cytometry

Cells were washed twice with flow cytometry buffer (PBS with 1% BSA, 1 mM EDTA, and 0.1% sodium azide) and then resuspended for 15 minutes in 4% BSA flow cytometry buffer containing anti-CD16 antibodies (Fc block, BD-PharMingen, San Diego, CA) to block Fc

receptors. Popliteal lymph node cells were stained for DCs using fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse MHC class II antibody, rat anti-mouse CD86 antibody conjugated to R-phycoerythrin (PE) or biotin, and allophycocyanin (APC)-conjugated hamster anti-mouse CD11c antibody (all from BD-PharMingen). When anti-CD86-biotin was used, cells were subsequently incubated with 1:500 streptavidin-peridinin chlorophyll protein Cy 5.5 (PerCP-Cy5.5). T cells were stained using anti-CD4-FITC or APC and anti-CD69-PE. B cells were stained using anti-B220 conjugated to FITC or APC-AlexaFluor750 (eBioscience, San Diego, Ca), anti-CD69-PE, and anti-CD19-conjugated to APC or Pacific Blue. Cells were washed once in flow cytometry buffer and resuspended in the same buffer containing either 1% 7-amino-actinomycin D (7-AAD; BD-PharMingen) or 0.1% Sytox Blue (Molecular Probes; Invitrogen, Carlsbad, Ca). Cells were incubated for 15 min at room temperature and analyzed using a FACSCalibur or LSR II flow cytometer (Becton Dickinson). During analysis, debris and dead cells (7-AAD$^+$ or Sytox Blue$^+$ cells) were excluded through gating. DCs were identified using anti-CD11c and anti-MHC class II antibodies, and DC maturation assessed by determining the relative levels of MHC class II and CD86 expression.

Statistics

Statistical analysis was performed using SigmaStat 3.0 (Systat, San Jose, CA). Each data set was tested for variance and normality. Based on the results, the appropriate parametric or nonparametric tests were applied. For comparison between two groups we used a paired t-test (parametric) or the Mann Whitney U test (nonparametric). For comparison between three or more groups either ANOVA (with Tukey's post test comparison) or the Kruskal Wallis test (with Dunn's post test comparison) was used. A p value < 0.05 was considered significant.

Results

Footpad Injection with U1 RNA Increases DC Number and Maturation in the Draining Lymph Nodes

Footpad injection is a valuable model to study local inflammatory events and has been utilized to study the *in vivo* effects of various TLR agonists on DC maturation and migration to the draining popliteal lymph nodes (100, 181). We used this model to determine the effects of U1 RNA on early cellular recruitment, maturation, and activation. Cellularity in the popliteal lymph nodes was examined 24 hours after footpad injection of U1 RNA, comparing the responses in the ipsilateral and contralateral lymph nodes. U1 RNA increased the size of the ipsilateral compared to the contralateral popliteal LNs (*data not shown*), as well as the total cellularity **(**Fig. 3-1A: p = 0. 016**)**. Using flow cytometry, we found an increased percentage **(**Fig. 3-1B: p = 0.004**)** and absolute number **(**Fig. 3-1C: p = 0.005**)** of mature $CD11c^{+}MHCII^{high}CD86^{high}$ DCs in the ipsilateral vs. contralateral lymph nodes of mice injected with U1 RNA, indicating that U1 RNA potently activated DCs *in vivo*. When the mouse was injected with PBS alone in the footpad, total cellularity, percentage of mature $CD11c^{+}MHCII^{high}CD86^{high}$ DCs, and absolute DC number did not increase (*data not shown*).

Footpad Injection with U1 RNA Increases Activation of CD4+ T Cells and CD19+B220+ B cells in the Draining Lymph Nodes

We next determined if U1 RNA could activate $CD4^{+}$ T cells or $CD19^{+}B220^{+}$ B cells in the draining popliteal lymph nodes. Indeed, U1 RNA greatly increased the percentage of $CD4^{+}CD69^{+}$ T cells **(**Fig. 3-1D: p = 0.009**)** and $CD19^{+}B220^{+}CD69^{+}$ B cells **(**Fig. 3-1E: p = 0.001**)** in the ipsilateral compared with the contralateral popliteal lymph nodes. Again, we found that injection with PBS alone did not increase CD69 expression on either B or T cells (*data not shown).*

RNA Mediates the Stimulation

To ensure that the U1 RNA, and not a contaminant, was responsible for the migration and maturation of DCs as well as the activation of B and T lymphocytes, U1 RNA was pretreated with RNase A for 2 hours at 37°C prior to being injected into the footpad. RNase treatment of the U1 RNA caused a loss in adjuvant activity, as determined by a failure to increase total cellularity or DC maturation (Fig. 3-2A and 3-2B). Additionally, the RNase treated U1 RNA failed to increase the percentage of activated T or B cells in the ipsilateral lymph node compared to the contralateral lymph node (Fig. 3-2C and 3-2D). These data indicate that the early *in vivo* adjuvant activity of U1 RNA is dependent on RNA.

U1 RNA Activation of DCs, B and T Cells is TLR7 Dependent

We next determined the mechanism of U1 RNA action in the activation of DCs, B cells and T cells. Previous studies have implicated TLR7 as the ligand for U1 RNA(179, 180, 182). We employed a novel oligonucleotide based TLR-specific inhibitor (IRS 661), which was described previously (180).

Similar to Uchida *et al.* (181), we compared the difference in U1 RNA-mediated activation between the ipsilateral and contralateral LNs following co-administration with IRS 661 or a control ODN. When U1 RNA was co-injected with IRS 661, total cellularity and activation of DCs, B and T cells was inhibited significantly (Fig. 3-3A-D). In contrast, when U1 RNA is co-injected with an inactive control ODN the stimulation was similar to that seen with U1 RNA alone. IRS 661 also inhibited stimulation by the TLR7 agonist R848 (*data not* shown). These data strongly suggest that U1 RNA exerted its immunostimulatory effect via TLR7.

CD4+ T Cell Proliferation in Response to Specific Antigens.

Antigen specificity of the immune response was evaluated by determining whether $CD4^+$ T cells from mice immunized with NP-KLH plus either alum or U1 RNA could proliferate specifically to the carrier antigen KLH. There was a trend toward increased T cell proliferation in mice in which the antigen was co-administered with U1 RNA or alum vs. those receiving PBS alone. However, this did not reach statistical significance, probably due to the small number of antigen specific T cells (*data not shown*). To overcome this problem we immunized OVA specific, MHC class II restricted, T cell receptor transgenic (OT-II) mice with 100 μg $OVA_{323-339}$ peptide in the presence of PBS, alum, or U1 RNA.

Eight days after immunization, draining inguinal, axillary, and cervical lymph nodes as well as spleens were harvested. $CD4^+$ T cells were isolated using anti-CD4 conjugated magnetic beads and flow cytometry was used to determine numbers of antigen specific T cells in the draining LNs. A T cell proliferation assay was performed to measure the antigen-specific OVA response. The total number of $CD4^+$ T cells in the LNs was increased 3-fold in both the alum and U1 RNA treated mice compared with PBS treated controls (no adjuvant) (Fig. 3-4A: $p = 0.015$). The percentage of OVA specific T cells ($V\alpha2^+$ $V\beta5^+$ T cells) was increased by about 10% in the adjuvant (alum or U1 RNA) treated mice compared with the non-adjuvant (PBS) treated mice **(**Fig. 3-4B: $p = 0.05$**)**, however *post hoc* analysis did not reveal a significant difference between the three groups.

To determine whether U1 RNA enhanced antigen-specific T cell proliferation in mice immunized with $OVA_{323-339}$, cells from the draining LNs were cultured with OVA peptide followed by measurement of [^{3}H]-thymidine incorporation **(**Fig. 3-4C**)**. There was a significant difference among the groups ($p < 0.001$) and using Tukey's multiple comparison analysis we

found a significant difference between mice immunized with OVA peptide in alum ($p < 0.001$) or U1 RNA ($p = 0.002$) versus PBS. There was also increased $CD4^+$ T cell proliferation in the spleens from mice immunized with $OVA_{323-339}$ plus alum or U1 RNA versus PBS ($p = 0.04$, *post-hoc*: $p < 0.05$, PBS vs. U1 RNA) (*data not shown*). The data indicate that U1 RNA induced OVA peptide-specific $CD4^+$ T cell proliferation as efficiently as a standard adjuvant, alum. There was no $CD4^+$ T cell proliferation in response to a non-specific antigen BSA (*data not shown*), indicating the response was specific to OVA.

Effect of U1 RNA on Antigen Specific Antibody Responses

To determine whether U1 RNA can act as an *in vivo* adjuvant capable of driving adaptive immune functions, we examined the appearance of antigen specific antibody responses following immunization and determined the kinetics of the appearance of NP-specific antibodies following immunization with NP-KLH in either alum, U1 RNA (50 μg), or sterile PBS. Mice were bled on days 4, 8, 12 16, and 20. As shown in Figure 3-5A, anti-NP IgM antibody levels (ELISA) peaked at day 7 and then gradually declined. Anti-NP IgG responses (ELISA) peaked at around day 16 **(**Fig. 3-5B**)**. These results were consistent with the kinetics of a primary immune response.

In view of the kinetics shown in Figure 4-5, we also compared serum IgM and IgG subclass anti-NP antibody levels at days 7 and 16 **(**Fig. 3-6**)**. When NP-KLH was co-administered with either alum or U1RNA, there was enhanced production of anti-NP IgM in comparison with PBS ($p = 0.007$; Dunn's multiple comparison test: $p < 0.05$, for both U1 RNA and alum compared with PBS). This was also the case for IgG at day 16 (total IgG levels, *data not shown*, $p = 0.015$). As expected, alum promoted class switching to all IgG isotypes, but enhanced IgG1 antibody responses most efficiently ($p = 0.005$, *post-hoc*: $p < 0.05$ for both U1

RNA and alum compared with PBS). U1 RNA potently induced IgG2a antibody responses ($p = 0.013$, *post-hoc*: $p < 0.05$ for U1 RNA compared with PBS only). There was a trend toward enhanced anti-NP IgG2b production by both U1 RNA and alum, but it did not quite reach statistical significance ($p = 0.05$).

Discussion

Autoantibody production in SLE is focused on a small subset of cellular antigens, consisting mainly of deoxyribonucleoproteins (e.g. chromatin) and ribonucleoproteins such as the Ro60 (SS-A), La (SS-B), and Sm/RNP antigens (146). The U1 snRNP, reactive with anti-Sm and anti-RNP antibodies, consists of proteins U1-70K, A, B'/B, C, D1/2/3, E, F, and G and a single U1 RNA molecule containing four regions of duplex RNA (stem-loops I-IV) interspersed with single-stranded RNA segments (8, 134). The Y1-5 small cytoplasmic ribonucleoproteins, reactive with anti-Ro60 autoantibodies, also contain extensive duplex as well as single-stranded RNA (20). Although there is evidence that U1 RNA and other cellular RNAs can activate immune cells *in vitro* (95, 172, 179, 180, 183-185), it has not been shown that they can initiate innate immune responses or augment adaptive immunity *in vivo*.

Our studies demonstrate that U1 RNA does possess adjuvant activity *in vivo*. Using the footpad model of local inflammation, U1 RNA caused activation (enhanced MHC class II and CD86 expression) and migration of DCs into the draining lymph nodes. It also induced the activation of both T and B cells (increased expression of the early activation marker CD69). These results were similar to those obtained with other immunostimulatory adjuvants, many of which are TLR agonists (100). TLR agonists that have been used as adjuvants include lipopolysaccharide (124), CpG DNA (125, 126), muramyl dipeptides (127, 128), monophosphoryl lipid A (129), and imiquimod (130, 131).

The U1 RNA responses were dependent on the RNA component and were not due to contaminants since there was no activation if the U1 RNA was pretreated with RNase prior to being injected into the footpad. Moreover, when C3H/HeJ (TLR4 mutant) mice were immunized with NP-KLH plus U1 RNA, antigen specific antibody responses remained intact (*data not shown*), excluding the possibility that the adjuvant effect was mediated by contaminating endotoxin. Consistent with previous *in vitro* studies suggested that U1 RNA is likely to exert its immunostimulatory effects via TLR7 and protein kinase R (PKR) (179, 184, 185), the immunostimulatory activity of U1 RNA could be blocked using a specific inhibitor of TLR7 (IRS 661).

U1 RNA and alum both augmented OVA-specific T cell proliferation and enhanced humoral responses to the NP hapten (following immunization) comparably, suggesting that this endogenous RNA molecule was as potent as a "standard" vaccine adjuvant. Interestingly, U1 RNA was a relatively more potent inducer of IgG2a than IgG1 antibodies **(**Fig. 3-6**)**, the isotype of the majority of anti-Sm, RNP, and dsDNA autoantibodies produced in murine lupus. IgG2a responses are dependent on the cytokines IFNγ (186) and IFNα/β (187), and we have shown previously that U1 RNA is a good inducer of both IL-12 and IFNα/β (179). Thus, the enhancement of T_H1 cytokine production by U1 RNA may help explain why anti-Sm/RNP responses in murine lupus are predominated by IgG2a autoantibodies.

Since U1 RNA is normally complexed with proteins intracellularly, an important and at present largely unexplored question is whether the intact U1 snRNP is immunostimulatory or if U1 RNA is a "latent" adjuvant, which becomes available only after it is released from or exposed within the RNA-protein particle. Although others have shown that immune complexes from patients who are anti-RNP positive can induce plasmacytoid DCs to produce IFNα/β (180), the

physical form of the antigen in those immune complexes was not characterized, and it remains possible that dissociation or alteration of the structure of the proteins contained in the particle may be necessary to unmask U1 RNA's adjuvant activity. In addition, it has been shown that anti-RNP positive serum IgG combined with apoptotic cells enhances IFNα/β production (188), but again the actual physical state of the U1 snRNP antigen associated with those cells has not been defined. Further studies will be necessary to determine the conditions under which U1 RNA or snRNPs are immunostimulatory and to further define the mechanisms involved. A better understanding of how the RNA components of self-antigens activate DCs and other cell types could help explain why ribonucleoproteins are such common targets of autoimmunity in lupus and other systemic autoimmune diseases.

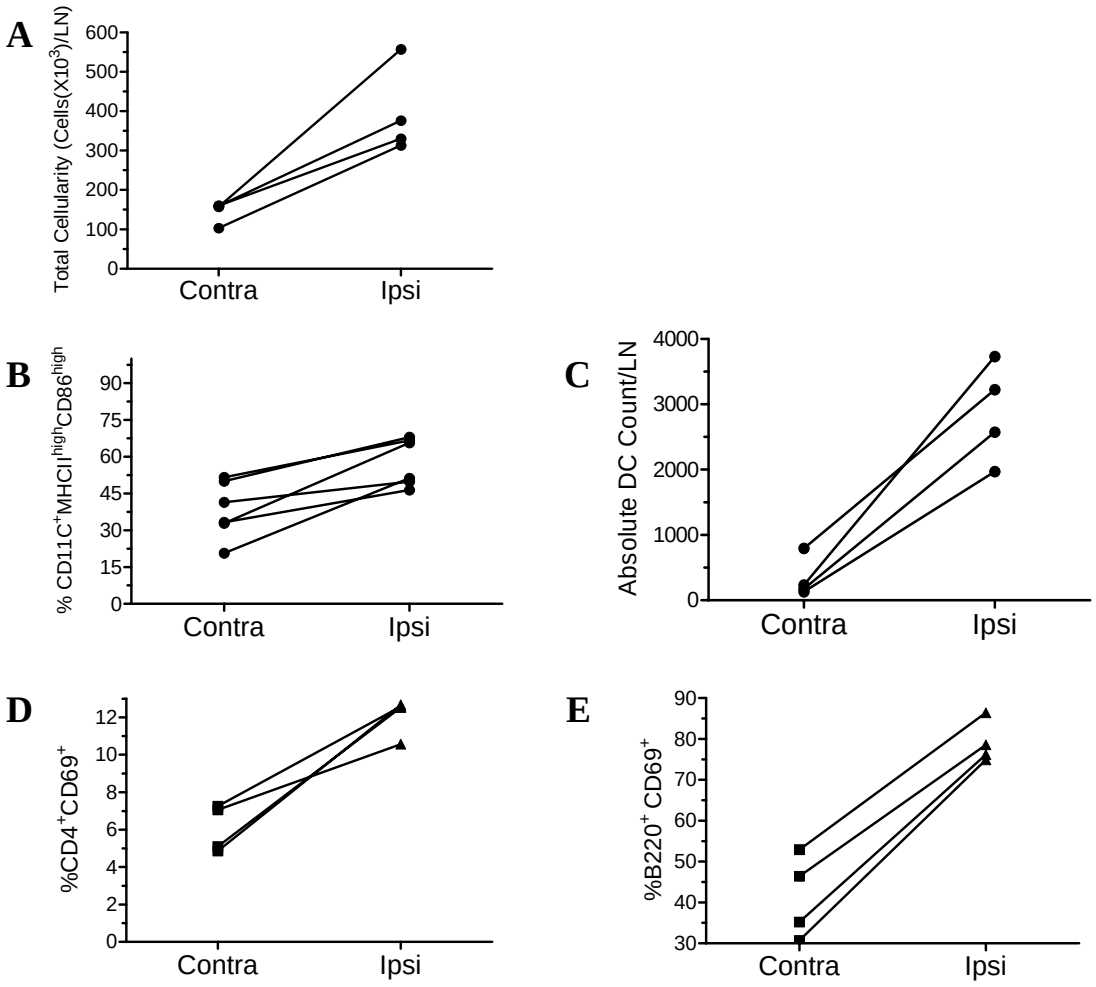

Figure 3-1. U1 RNA activates immune cells. U1 RNA (15 μg) was injected into the hind footpads of C57BL/6 mice. Ipsi- and contra-lateral popliteal lymph nodes were excised and cells were analyzed by flow cytometry. A) Total cellularity of the ipsilateral versus contralateral LNs (4 mice) were compared ($p = 0.016$, paired t-test). B) Cells were first gated based on forward and side scatter to eliminate debris. Dead cells were gated out using 7AAD. Cells were then gated on the $CD11c^+MHC\ II^+$ cell population and the percentage of $CD86^{high}MHCII^{high}$ activated DCs in the ipsilateral vs. contralateral draining lymph nodes was determined (6 mice, $p = 0.004$, paired t-test). C) Absolute number of DCs ($CD11c^+$ $MHCII^+$ cells) was calculated in the ipsi versus contra lymph nodes (4 mice, $p = 0.005$, paired t-test). D) $CD4^+CD69^+$ T cells in the ipsi vs. contralateral draining lymph nodes (4 mice, $p = 0.009$, paired t-test). E) $B220^+CD19^+CD69^+$ B cells in the ipsi vs. contralateral draining lymph nodes (4 mice, $p = 0.001$, paired t-test).

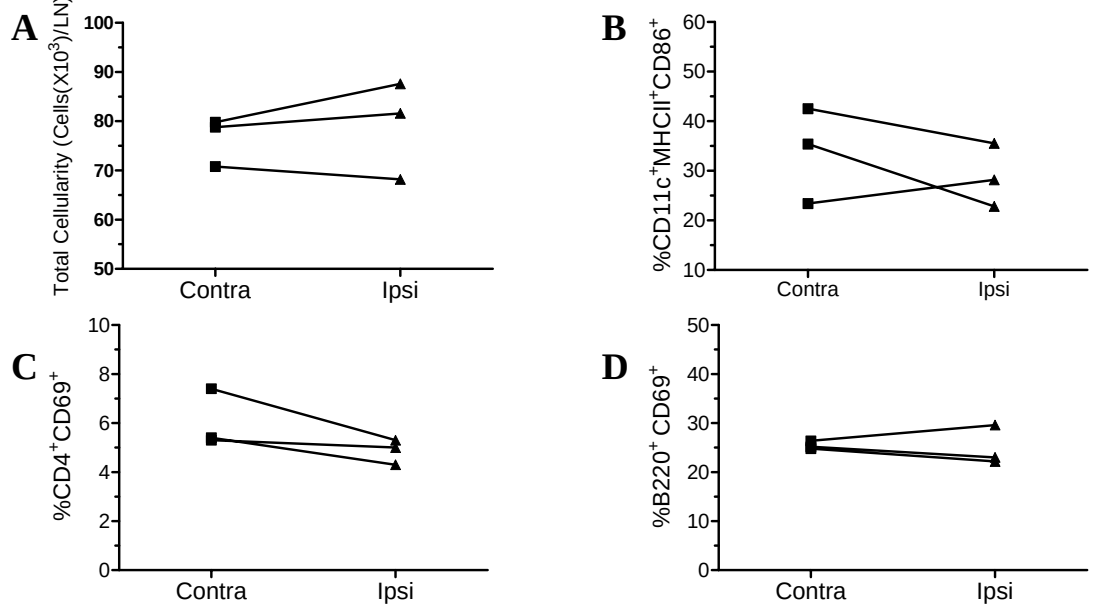

Figure 3-2. RNA is responsible for *in vivo* recruitment and activation of immune cells by U1 RNA. U1 RNA was pretreated with 0.1 μg RNase A per 15 μg U1 RNA for 2 h at 37°C. RNase-treated U1 RNA was then injected into the hind footpads of C57BL/6 mice. Ipsi- and contralateral popliteal lymph nodes were excised and cells were counted with a hemocytometer, stained with fluorescently labeled antibodies, and analyzed by flow cytometry. A) Total cellularity of the ipsilateral versus contralateral LNs (3 mice) was compared ($p = 0.5$, paired t-test). B) Activated DCs were first gated based on forward and side scatter to gate out debris. Dead cells were gated out using Sytox Blue. Cells were then gated on the $CD11c^+MHC\ II^+$ cell population and the percentage of $CD86^{high}MHCII^{high}$ cells in the ipsilateral vs. contralateral draining lymph nodes was determined (3 mice per group, $p = 0.4$, paired t-test). C) $CD4^+CD69^+$ T cells in the ipsi vs. contralateral draining lymph nodes (3 mice, $p = 0.2$, paired t-test). D) $B220^+CD19^+CD69^+$ B cells in the ipsi vs. contralateral draining lymph nodes (3 mice, $p = 0.8$, paired t-test).

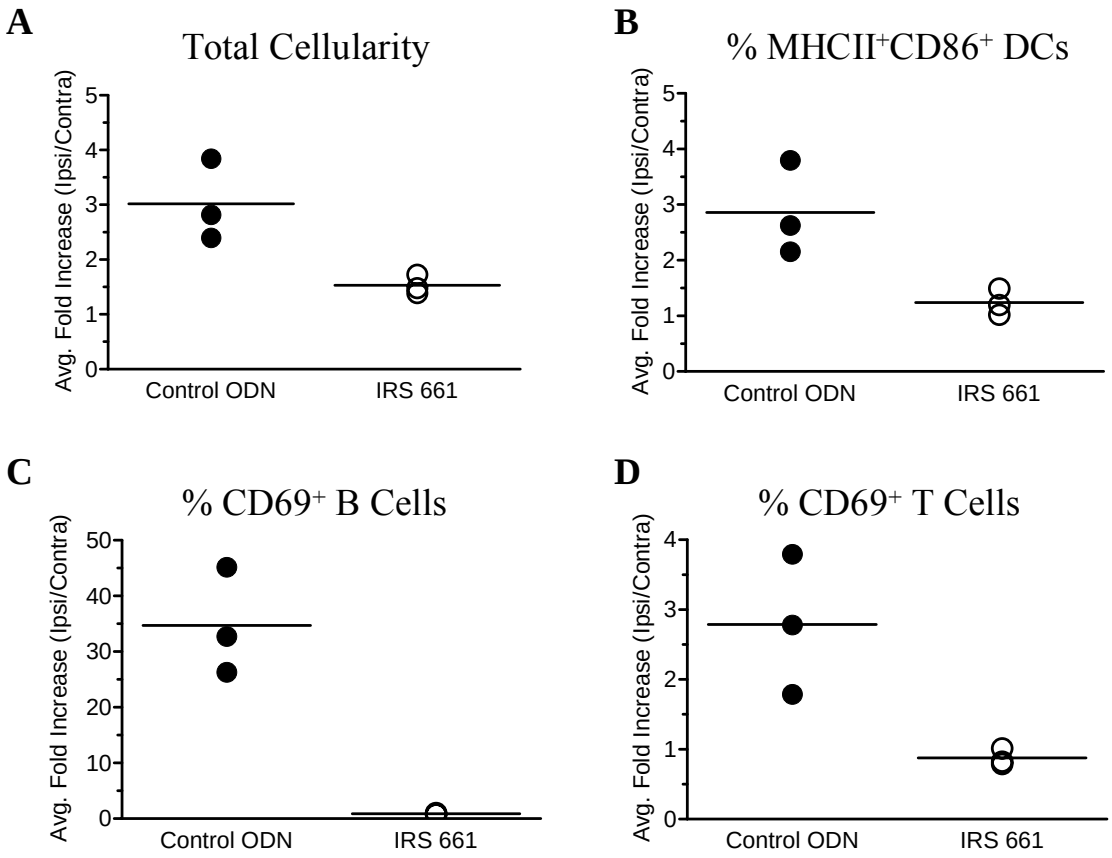

Figure 3-3. U1 RNA is stimulating immune cell recruitment and activation via TLR7. U1 RNA (15 μg) was injected into the hind footpads of C57BL/6 mice with a specific immunoregulatory DNA sequence (IRS 661) that blocks TLR7 signaling or an inactive control ODN, both at a concentration of 5.6 μM. Ipsi- and contralateral popliteal lymph nodes were excised and cells were analyzed by flow cytometry. A) Average fold increase in total cellularity of ipsilateral / contralateral LNs were compared ($p = 0.02$, unpaired t-test). Each point represents one mouse. B) Activated DCs were first gated on forward and side scatter to gate out debris. Dead cells were gated out using Sytox Blue. Cells were then gated on the $CD11c^{+}MHC\ II^{+}$ cell population and the percentage of $CD86^{high}MHCII^{high}$ cells in the ipsi vs. contra draining LNs was determined. The average fold increase of $CD86^{high}MHCII^{high}$ DCs from the ipsi vs. contra LNs is depicted from mice given control or inhibitory ODN ($p = 0.03$, unpaired t-test). C) The average fold increase of the $B220^{+}CD19^{+}CD69^{+}$ B cells in the ipsi vs. contralateral draining lymph nodes (3 mice, $p = 0.004$, unpaired t-test). D) The average fold increase of the $CD4^{+}CD69^{+}$ T cells in the ipsi vs. contralateral draining lymph nodes ($p = 0.03$, unpaired t-test).

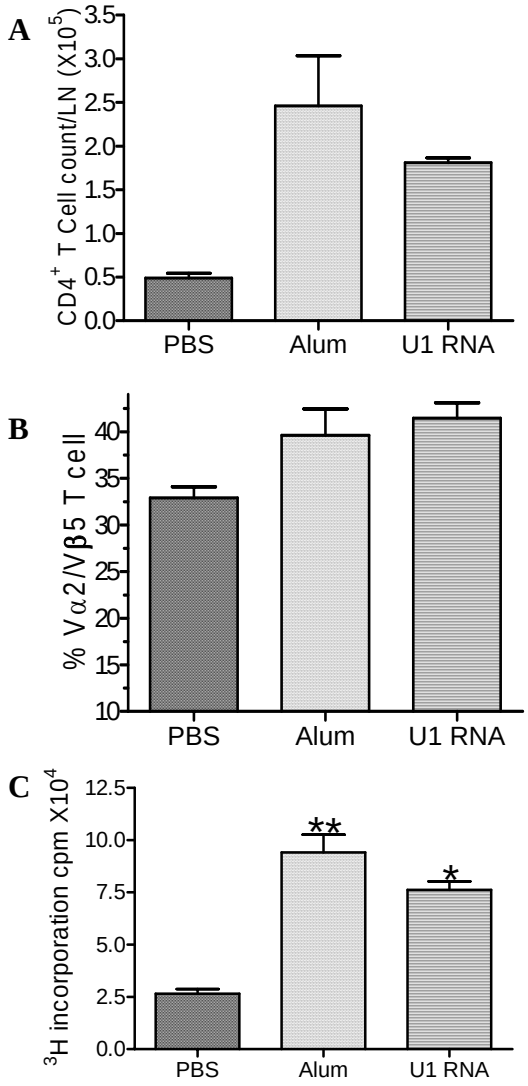

Figure 3-4. U1 RNA augments antigen-specific $CD4^+$ T cell responses. OVA specific MHC Class II transgenic (OT-II) mice (3 per group) were immunized subcutaneously with 100 μg $OVA_{323-339}$ plus alum, U1 RNA, or PBS. Eight days after immunization, draining LNs were harvested and $CD4^+$ T cells were isolated. A) Mean total number of $CD4^+$ T cells in LN, ($p = 0.015$, one way ANOVA). B) Flow cytometry showing the average percentage of Vα2/Vβ5 (OVA specific) T cells in the draining lymph nodes ($p = 0.05$, one way ANOVA) C, T cell proliferation assay, 2.5 X 10^4 $CD4^+$ LN T cells were plated with 2.5 X 10^5 irradiated (3,000 R) CD4-depleted splenocytes in a 96 well round bottom plate in quadruplicate. Cells were either stimulated with 10 μg/mL OVA peptide or left unstimulated. Forty-eight hours later 1μCi of ^3H-thymidine was added to the cells and 18 hours after that the incorporation of ^3H-thymidine was measured using a liquid scintillation counter. The bar graph shows the mean of 3 mice per group. ($p < 0.001$, one-way ANOVA, * $p = 0.002$, PBS vs. U1 RNA and ** $p < 0.001$ PBS vs. alum, Tukey Test).

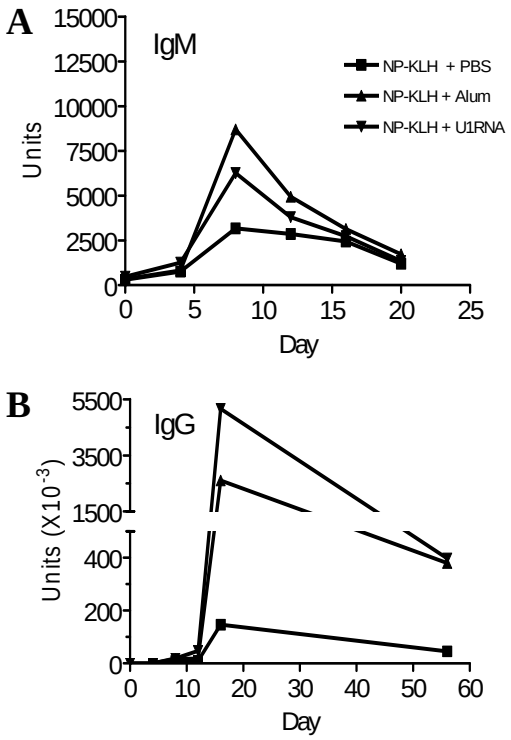

Figure 3-5. Kinetics of anti-NP IgM and IgG antibodies following immunization. C57BL/6 mice (3 per group) were immunized subcutaneously with NP-KLH (100 μg) precipitated in alum or with NP-KLH plus an equal volume of PBS or U1 RNA (50 μg). Mice were bled serially on days 3, 7, 12, 15, 20, and 24. A,B) Anti-NP hapten-specific IgM and IgG levels were quantified using ELISA. Units were determined from a standard curve using positive control serum.

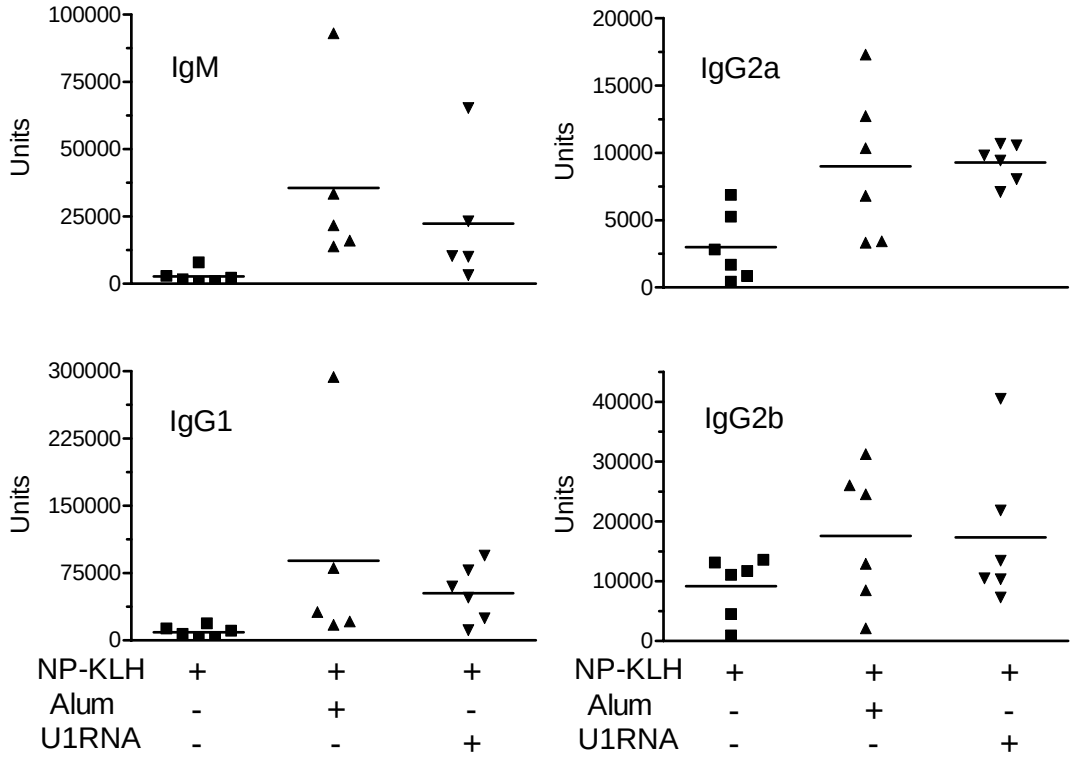

Figure 3-6. U1 RNA increases antigen-specific antibody responses. C57BL/6 mice (6 per group) were immunized subcutaneously, at the base of the tail, with NP-KLH (100 μg) plus PBS, alum, or U1 RNA (50 μg). Mice were bled on day 7 for anti-NP IgM responses ($p = 0.007$, Kruskal-Wallis, $p < 0.05$ Dunn's multiple comparison test vs. PBS control for both alum and U1 RNA) and euthanized on day 16 for anti-NP IgG1 ($p = 0.005$, Kruskal-Wallis, $p < 0.05$ for both alum and U1 RNA Dunn's multiple comparison test), IgG2a ($p = 0.013$, Kruskal-Wallis, Dunn's multiple comparison test $p < 0.05$ for U1 RNA; not significant for alum), and IgG2b ($p = 0.5$, Kruskal-Wallis) responses. Levels of anti-NP antibodies of the various subclasses were determined by ELISA using NP-BSA as the antigen.

CHAPTER 4
ABERRANT ENDOGENOUS SNRNPS BUT NOT INTACT SNRNPS STIMULATE TYPE I INTERFERON *IN VITRO*

Introduction

Systemic lupus erythematosus (SLE) is commonly associated with autoantibodies against endogenous DNA-protein (nucleosomes) and RNA-protein (small ribonucleoprotein) complexes (21). Unmethylated CpG DNA has immunostimulatory (adjuvant) activity, which is mediated by interactions with Toll-like receptor (TLR) 9 (174). Like the unmethylated CpG motifs, U1 and hY1-5 RNA, components of the Sm/RNP and Ro60 ribonucleoproteins, respectively have adjuvant activity *in vivo,* promoting dendritic cell (DC) maturation, B and T cell activation and proliferation, and enhancing antibody responses to exogenous antigens (189). Unlike CpG DNA, this activity is mediated by interactions with TLR7. Purified U1 RNA or synthetic oligoribonucleotides derived from U1 RNA can activate the IFNα/β pathway via the TLR7/8-MyD88 pathway in dendritic cells and splenocytes (172, 179, 180).

U1 RNA, a component of the U1 snRNP, is associated with proteins recognized by anti-Sm (B', B, D, E, F, G) and anti-RNP (70K, A, C) autoantibodies (134, 190). In the cell, U1 RNA is rapidly assembled into complexes with these proteins, a process that occurs within minutes of synthesis (191, 192).

An important and at present largely unexplored question is whether the intact U1 snRNP is immunostimulatory or if U1 RNA is a "latent" adjuvant, which becomes available only after it is released from or exposed within the RNA-protein particle. Immune complexes from patients who are anti-RNP positive can induce plasmacytoid DCs to produce IFNα/β (180) . Although mediated by TLR7, the physical form of the TLR7 ligand in those immune complexes is unclear (180). Anti-RNP positive serum IgG combined with apoptotic cells also enhances IFNα/β production (188), but again the precise nature of the immunostimulatory component present in

the apoptotic cells has not been defined. We have shown that biochemically purified U1 RNA does not need to be incorporated into immune complexes in order to exhibit immunostimulatory effects via TLR7 and that U1 RNA is as potent as a conventional adjuvant, alum, at enhancing immune responses to exogenous antigen. However, it remains unknown whether or not U1 RNA incorporated into snRNP particles is immunostimulatory. Here, we investigated the conditions under which intact snRNPs, partially disrupted snRNPs, and naked U1 RNA become immunostimulatory, an issue that may have implications for the pathogenesis of anti-RNP autoantibodies in human SLE.

Materials and Methods

In Vivo Labeling, Cell Lysis and Immunoprecipitation

[^{35}S]-methionine pulse/chase analyses were carried out as described (191). In brief, pulsed cells were labeled for 3 hours in methionine-deficient RPMI 1640 supplemented with 1 mCi [^{35}S]-methionine/cysteine (Amersham, Piscataway, NJ). Pulsed cells were then resuspended in complete medium (containing methionine) and dialyzed fetal bovine serum at a density of 2 X 10^6 cells/mL and were incubated further at 37ºC in complete RPMI 1640 for 24 hours with or without 30% H_2O_2.

Affinity Chromatography of U snRNPs on H-20 Columns

U snRNPs were isolated from K562 (human erythroleukemia) cells as described (193, 194). Briefly, purified mAb H-20 (Synaptic Systems; Gottingen, Germany), which is specific for the trimethyl G (TMG) cap structure of the U1, U2, U4, and U5 snRNPs, was bound covalently to CNBr-activated Sepharose 4B (GE Healthcare; Piscataway, NJ) according to the manufacturer's protocol. The antibody was immobilized on 1 mL packed beads.

For the isolation of snRNPs, typically 12 mL nuclear extract from 4 X 10^8 K562 cells in lysis buffer (50 mM Tris-HCl, pH 7.5; 500 mM NaCl; 2 mM EDTA; 0.3% NP40 containing 0.5

mM phenylmethylsulfonyl fluoride (PMSF) and 0.3 TIU/ml aprotinin) was incubated twice at 4ºC for 1 hour. The column was washed with 10 mL sterile PBS. The snRNPs were desorbed from the affinity column with 2 mL TMN100 (20 mM Tris/HCl pH 8.0, 10 mM $MgCl_2$, 100 mM NH_4Cl, 0.5 mM PMSF, 0.5 mM dithiothreitol] containing 10 mM m^7G followed by a washing step of 1 mL TMN100. Fractions were pooled and dialyzed for 36 hours against sterile PBS and 12 hours against DMEM + 10% fetal bovine serum (Cellgro, Herdon,VA) supplemented with L-glutamine and penicillin/streptomycin plus 10 mM HEPES (complete DMEM). Columns were regenerated using 2 mL 6M urea in TMN300 (20 mM Tris/HCl pH 8.0, 10 mM $MgCl_2$, 300 mM NH_4Cl, 0.5 mM PMSF, 0.5 mM dithiothreitol) followed by washing with 10 mL TMN300.

The fractions were then collected and split equally into two fractions. One fraction was left as the complete snRNP fraction. The other underwent a phenol-chloroform extraction, ethanol precipitation, and resuspension in sterile PBS. The purified RNA was quantified by spectrophotometry ($A_{260}/A_{280\ ratio}$). The two fractions were considered to contain equal amounts of RNA.

Necrotic/Apoptotic Cell Extracts

K562 cells were made apoptotic or necrotic using 30% H_2O_2 as follows: for apoptotic cells, 2 X 10^6 cells per mL were treated with 20 mM 30% H_2O_2 for 24 hours at 37ºC. For necrotic cells, 2 X 10^6 cells per mL were treated with 80 mM 30% H_2O_2 for 24 hours at 37ºC. Cells were considered apoptotic or necrotic based on Annexin V and propidium iodide (PI) staining as assessed using flow cytometry compared with untreated cells (Table 4-1). The cells were aliquoted at 8 X 10^7 cells per tube.

Sucrose Gradient Analysis

Intact or necrotic K562 cell lysates were fractionated on 5-20% sucrose gradients in lysis buffer with the addition of 0.2 M NaCl (191). Extract from 8 X 10^7 cells was loaded on

precooled gradients (4ºC) and centrifuged in an SW50.1 rotor for 17 hours at 40,000 rpm at 4ºC in a Beckman L5-50B ultracentrifuge. Gradients were fractionated manually (10 X 0.5 mL fractions) and were then immunoprecipitation was carried out using 2.73 (anti-U1-70K) or Y2 (anti-Sm B'B) monoclonal antibodies.

K562 cells (8 X 10^7) were resuspended in 200 uL of lysis buffer and sonicated for 45 seconds at 30% duty cycle and output control 3 then placed on ice for 1 minute, and then sonicated again for 45 seconds (Branson Sonifier 250, Danbury, CT). The cell extract was cleared by centrifugation (30 minutes at 10,000g).

Stimulation of Cells

RAW264.7 (murine macrophage) cells from the American Type Culture Collection (ATCC, Rockville, MD) were maintained in DMEM + 10% fetal bovine serum (Cellgro, Herdon,VA) supplemented with L-glutamine and penicillin/streptomycin plus 10 mM HEPES (complete DMEM) at 37°C in the presence of 5% CO_2. Cells were plated at 5 X 10^5 per well in 12-well plates 24 hours prior to being stimulated for 6 hours with either affinity purified snRNPs or U RNA from an equal amount of snRNPs (from intact, apoptotic, or necrotic cells) .

Real-Time RT-PCR

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and treated with DNase (1 unit/μg, Invitrogen) at 22°C for 15 minutes. The RNA was reverse transcribed using Superscript II First Strand cDNA Synthesis (Invitrogen). Real-time PCR was performed with an Opticon 2 continuous fluorescence detector (MJ Research, Waltham, MA) using SYBR green core reagents and Ampli-Taq gold DNA polymerase from Perkin Elmer-Applied Biosystems (Foster City, CA). Gene expression was normalized to 18S RNA (Ambion, Austin, TX). Primers were designed using the Primer3 Output program. The following primers were utilized: Mx-1,

forward GATCCGACTTCACTTCCAGATGG and reverse CATCTCAGTGGTAGTCAACCC; IL-6 forward CACGGCCTTCCCTACTTCAC and reverse TCAGAATTGCCATTGCACAAC. Optimal reaction concentrations were determined over four logs of linear amplification. Forty-five cycles of PCR were performed in duplicate for each primer. The fold change in gene transcript quantity compared with 18S RNA was measured using the comparative ($2^{-\Delta\Delta Ct}$) method.

Measuring the expression of IFN-I inducible genes such as Mx1 by real-time PCR reliably estimates total IFN-I levels in mice (151) and humans (128).

ELISAs

Microtiter plates (Nunc, Amino F96, Roskilde, Denmark) were coated overnight with 2μg/mL anti-Y2 (Sm B'/B) or 2.73 (U1-70K) monoclonal antibodies in 0.1 M Na_2HPO_4/NaH_2PO_4 pH 9. 100 μL of each sucrose gradient fractions were added to wells and a human polyclonal Sm/RNP positive patient serum (1:2500) was used for a secondary antibody followed by ALP conjugated anti-human IgG antibody before developing with alkaline phosphatase substrate (Sigma-Aldrich). Absorbance at 405 nm was determined using a VERSAmax™ microplate reader and Softmax Pro® version 4.3 (Molecular Devices, Sunnyvale, CA).

Results

Induction of Apoptotic and Necrotic Cells

To test the immunostimulatory effects of snRNPs isolated from dead or dying cells, it was necessary to optimize conditions for generating apoptotic versus necrotic cells. We induced cell death with H_20_2 and by varying the concentration, examined the generation of cells undergoing apoptosis, necrosis, or apoptosis followed by secondary necrosis (Table 4-1). Cells

were considered to be apoptotic if they expressed Annexin V (either PI^+or PI^-). We did not distinguish between early and late apoptosis or secondary necrosis. Necrotic cells are defined as PI^+Annexin V^-.

As shown in Table 4-1, concentrations of H_2O_2 inducing optimal numbers of apoptotic or necrotic cells were 20 mM and 80 mM, respectively. These concentrations were used for the remainder of the studies. At 20 mM H_2O_2 77% of the cells were apoptotic versus 3% necrotic. At 80 mM H_2O_2, 57% of the cells were necrotic and 42% were apoptotic.

Modification of U1 snRNPs During Apoptosis and Necrosis

A time course was performed to investigate the effects of H_2O_2 treatment on U1 snRNP structure. K562 cells were radiolabeled with [^{35}S]-Met/Cys and cells were harvested at various time points (2, 12, 24 hours) after 20 or 80 mM H_2O_2 or no treatment. Immunoprecipitation was performed using a prototype anti-Sm/RNP patient serum. At 2 hours, the normal complement of U snRNP proteins (A-G) was apparent in immunoprecipitates from untreated as well as 20 and 80 mM H_2O_2 treated snRNPs (Figure 4-1). However, the mobilities of several proteins from the apoptotic and necrotic snRNPs were reduced, notably U1-A, Sm B'/B, and U1-C. At 12-24 hours, there were no further mobility shifts, but the snRNPs from necrotic cells could no longer be immunoprecipitated by polyclonal anti-Sm/RNP antibodies, and the intensities of proteins A-G were greatly reduced in the apoptotic extracts. These data suggest that the U snRNPs undergo structural alteration during apoptosis/necrosis. Initially several proteins may undergo post-translational modifications and subsequently the epitopes recognized by anti-Sm/RNP antibodies are destroyed, rendering the snRNPs unable of being immunopreciptiated using human autoimmune serum.

Sucrose Gradient Analysis

To further examine the structural differences of the various isolated snRNPs, sucrose density gradient centrifugation was used to separate snRNP particles or sub-particles of different size. By ELISA (Figure 4-2), necrotic snRNPs had lower mobility and reduced proteins than intact snRNPs in the sucrose gradient. As expected, intact snRNPs migrated as a broad peak, concentrated in fractions 6-7. Figure 4-2A shows the migration of total U snRNPs (captured onto the microtiter plate with an anti-Sm mAb) and Figure 4-2B shows the migration of U1 snRNPs (captured with an anti-U1-70K mAb). U1 snRNPs peaked in fraction 6 and total snRNPs (U1, U2, U4/U6, U5) in fraction 7. In contrast, total snRNPs from necrotic cells peaked in fractions 5-6. U1 snRNPs could not be captured by anti-U1-70K mAb from necrotic cell extracts (Figure 4-2B). These data indicated that the structure U snRNPs is altered in necrotic cells. The U1 snRNPs from necrotic cells are no longer capable of recognition by anti-U1-70K antibodies, whereas snRNPs of altered mobility can be recognized by anti-Sm antibodies in necrotic cell extracts.

Stimulation of RAW 264.7 Cells by snRNPs Versus Purified RNA

We next examined whether snRNPs isolated from intact, apoptotic or necrotic K562 cells or the RNA from these snRNPs had a differential ability to stimulate IFN-I production. As shown in Figure 4-3, naked RNA from control (untreated, A), apoptotic (B), or necrotic (C) snRNPs was immunostimulatory as indicated by an increase in Mx1 and IL-6 mRNA expression. Importantly, snRNPs isolated under gentle conditions from intact or apoptotic K562 cells were unable to stimulate the upregulation of Mx1 or IL-6 mRNA expression (4-3A, B). In contrast, material isolated on the anti-TMG affinity column from necrotic cells (necrotic snRNPs) was stimulatory, enhancing both Mx1 and IL-6 expression (4-3C).

Proteins Associated with snRNPs are Missing in Necrotic snRNPs

In order to demonstrate whether the snRNP proteins actually associated with the RNA in either intact or necrotic snRNPs, we performed an immunoprecipitation using the same antibody used to isolate snRNPs (anti-TMG). K562 cells were radiolabled with [^{35}S]-Met/Cys for 3 hours. These cells were then either treated with 80 mM H_2O_2 or left untreated and cultured for 24 hours. The cells were harvested and washed with PBS and the cell lysate was immunoprecipitated using the anti-TMG antibody (Figure 4-4). In intact K562 cells, all of the U1 snRNP associated proteins (70K, A, B/B', C, D, E/F, G) were present. In addition to the U1 snRNP associated proteins, the 205K doublet that associates with U5 snRNP as well as additional proteins that could be U4/U6 snRNP components also were present. However, only proteins A, B/B', and D, which associate with U1 snRNP and the 205 kD doublet which associates with U5 snRNPs were immunoprecipitated from necrotic cells. Of note, the proteins immunoprecipitated from necrotic cells are greatly reduced compared to those proteins immunoprecipitated from intact K562 cells. This suggests to us that some of the proteins are becoming disassociated from the snRNPs in the necrotic cells. Thus, it is reasonable to conclude that at least some of the RNA from the snRNPs is becoming exposed from necrotic snRNPs, thus rendering them immunostimulatory.

Discussion

Autoantibody production in SLE is focused on a small subset of cellular antigens, consisting mainly of deoxyribonucleoproteins (e.g. chromatin) and ribonucleoproteins such as the Ro60 (SS-A), La (SS-B), and Sm/RNP antigens (146). The U1 snRNP, reactive with anti-Sm and anti-RNP antibodies, consists of proteins U1-70K, A, B'/B, C, D1/2/3, E, F, and G and a single U1 RNA molecule containing four regions of duplex RNA (stem-loops I-IV) interspersed with single-stranded RNA segments (8, 134). There is evidence that U1 RNA and other cellular RNAs can activate immune cells both *in vitro* and *in vivo* (172, 179, 182, 189). Immune

complexes isolated from patients with snRNP autoantibodies also are immunostimulatory (180, 188); however, the nature of the immune complexes has not been fully elucidated. Here we investigated whether intact snRNPs are immunostimulatory.

The present data show that intact snRNPs did not stimulate cells to produce IFN-I; whereas snRNPs from necrotic cells, which migrate aberrantly on sucrose density gradients, stimulated cells to produce IFN-I. One explanation is that intact snRNPs were not immunstimulatory because the immunostimulatory RNA is packaged in proteins that become dissociated, exposing naked RNA during necrotic cell death. Alternatively, modification of either the proteins (see Figure 4-1) or the RNA component might make the intact snRNP more stimulatory. Although, we did not investigate that theory in this report.

In contrast to what has been previously suggested (180, 184), snRNPs isolated from intact cells under gentle conditions were not immunostimulatory (Figure 4-3). The immunostimulation seen with immune complexes could be partly an artifact of the preparation of these immune complexes and the physical state of these immunostimulatory complexes needs to be investigated. Importantly, the immunostimulatory complexes were isolated from SLE patients and therefore may not be intact.

During the transition from apoptosis to necrosis, snRNP proteins undergo post-translational modifications (58), the nature of which was not defined here. The only report of modifications to the U1 snRNP from apoptosis to necrosis is the disappearance of U1-70K, other autoantigens, however, have been found to undergo additional modification during this process (i.e. additional cleavage of proteins) (64). Ultimately, some of these proteins dissociate from the RNA or are degraded, potentially exposing immunostimulatory RNA sequences. It should be kept in mind that the altered structure of U snRNPs in necrosis could influence endosomal

degradation of the snRNPs, potentially influencing the generation of effective TLR7 ligands, which interact with their receptor inside of endosomes (70, 96, 141). It is known that the TMG cap structure is removed from the U RNAs during apoptosis (59). Here we provide evidence that the TMG cap is still present on the U RNAs in necrotic cells, as we can isolate RNA from the cells using the anti-TMG antibody and can see U RNA on an urea-PAGE RNA gel (data not shown).

We show here that during the transition from intact to necrotic cells, it becomes progressively more difficult to immunoprecipitate proteins A-G using polyclonal anti-Sm/RNP antibodies (Figure 4-1). After 12 hours in the presence of 80 mM H_2O_2 (necrosis), proteins A-G could not be visualized by SDS-PAGE of immunoprecipitated K562 radiolabeled cells. Nevertheless, material immunoprecipitated using the anti-TMG antibody, presumably U1, U2, U4/U6, U5 RNAs, stimulated IFN-I production (Mx1 gene expression, Figure 4-3C). Based on these observations, we conclude that naked, TMG-capped, U RNA (or U RNA fragments) may be generated during the process of necrotic cell death. These RNAs appear to have potent immunostimulatory activity for antigen presenting cells, such as RAW 264.7 macrophages used here. We suggest that alterations in the structure of U snRNPs may be required before they become stimulatory.

In SLE patients there is an increase in circulating apoptotic cells and a decrease in phagocytosis of these cells. It seems reasonable to speculate that the apoptotic cells left in circulation may undergo secondary necrosis exposing modified immunostimulatory autoantigens.

Table 4-1. Dose response of H_2O_2 on K562 cells (24 hour time point)

$[H_2O_2]$	Total % dead or dying cells	% Annexin V^+ (apoptotic)	% PI^+Annexin V^- (necrotic)
1 mM	6.2	4.0	1.4
5 mM	16.3	9.0	2.2
10 mM	35.3	30.5	4.8
20 mM	**79.8**	**76.8**	**3.0**
40 mM	84.5	72.3	12.2
50 mM	99	68.6	30.4
80 mM	**99.3**	**42.4**	**57.1**

Apoptotic cells were Annexin V^+ and necrotic cells were PI^+. Doses used in experiments are in bold.

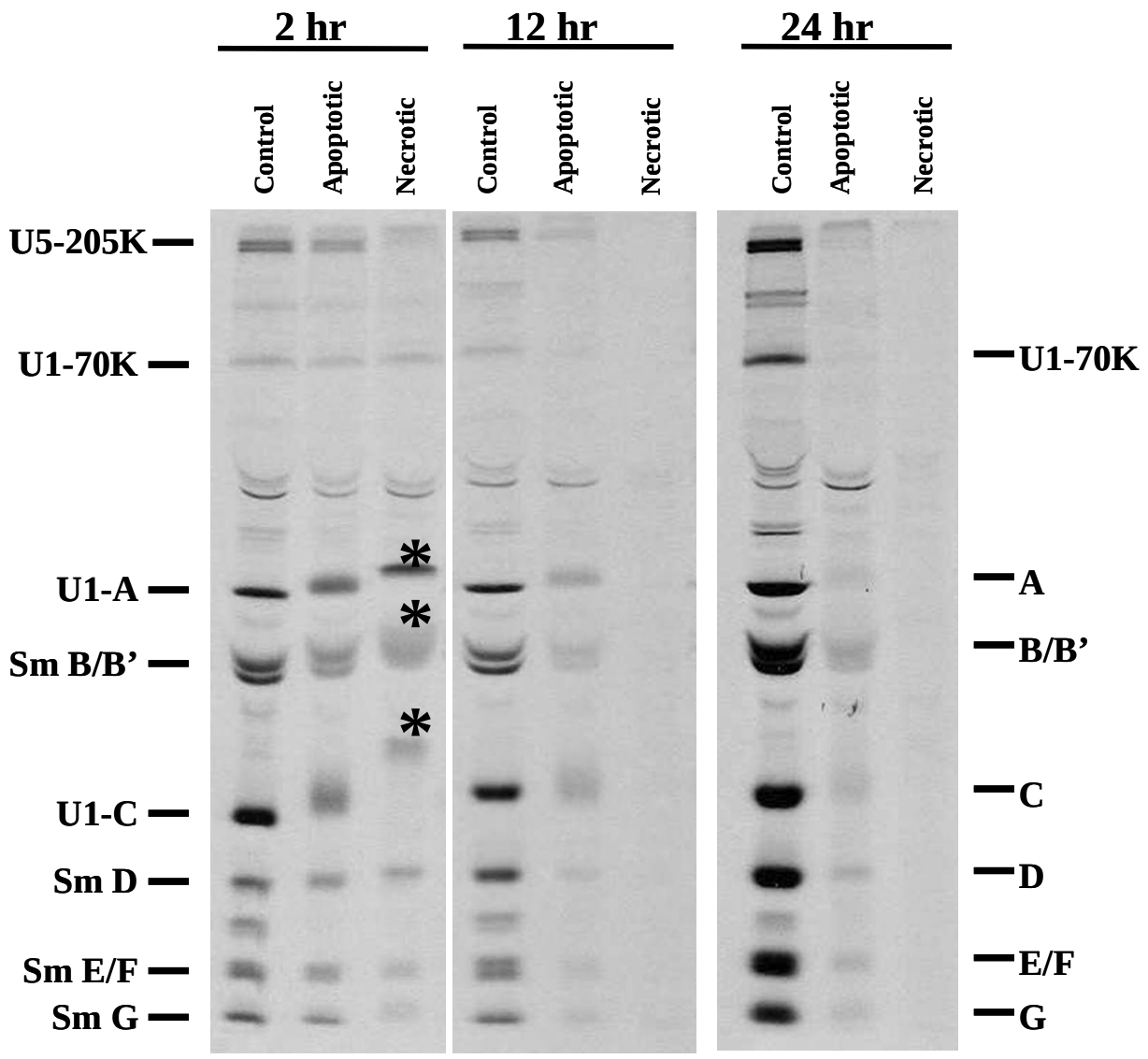

Figure 4-1. Immunoprecipitation of snRNPs isolated from intact, apoptotic or necrotic K562 cells. K562 cells were labeled with [^{35}S] methionine/cysteine for 3 hours and then treated with 20 mM or 80 mM H_2O_2. At 2, 12, and 24 hours cell extracts were analyzed by immunoprecipitation using a human anti-Sm/RNP serum followed by electrophoresis on a 12.5% SDS-polyacrylimide gel followed by fluorography and autoradiography. Positions of the U1 snRNP proteins U1-70K, U1-A, and U1-C, and the Sm B'/B, D, E, F, and G proteins are indicated. Position of the U5-205K doublet also is shown. Note the upward mobility shift of several snRNP proteins, which was particularly evident in necrotic cells (*).

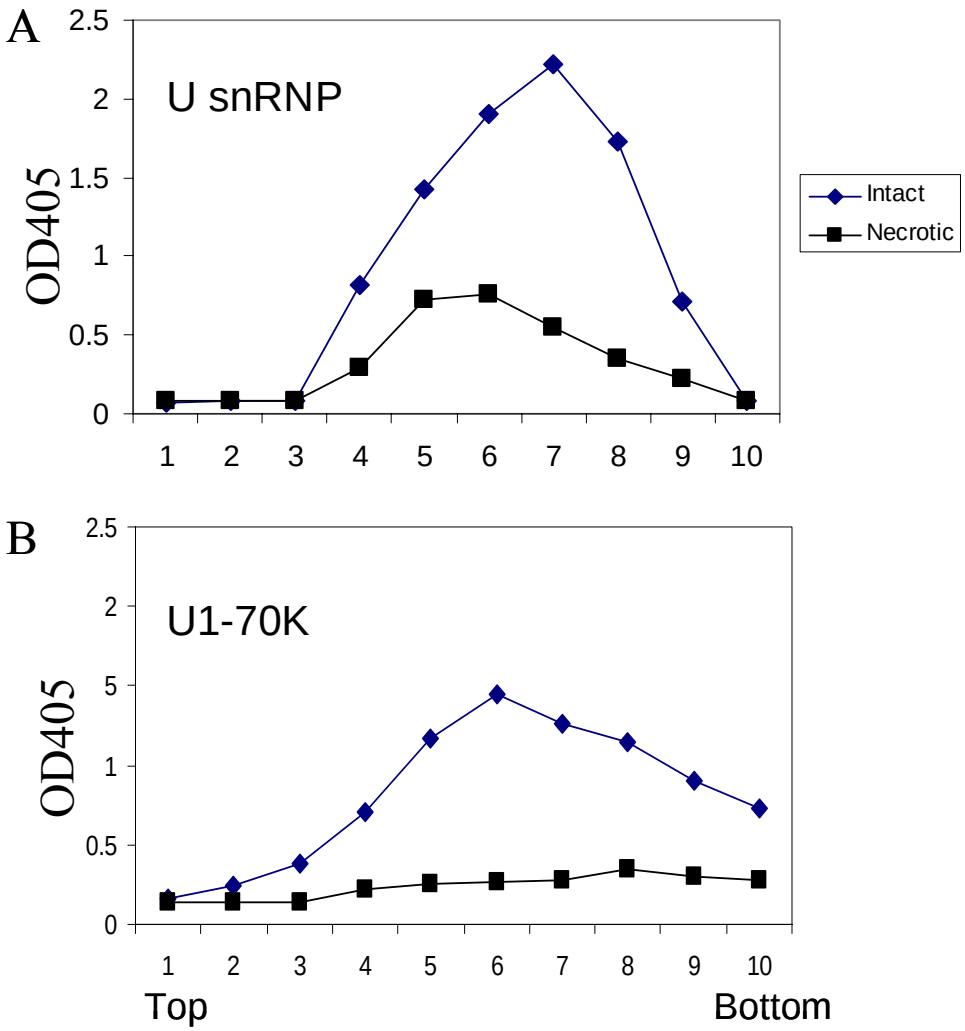

Figure 4-2. Sucrose gradients of snRNPs. Extract from untreated or necrotic (80 mM H_2O_2, 24 hours) snRNPs (8 X 10^7 cell equivalents per gradient) was separated on 5-20% sucrose gradients. Sucrose gradient fractions were tested by ELISA for the presence of U snRNPs and U1-70K. A) Necrotic snRNPs had a lower mobility (peak at Fraction 5) than intact snRNPs (peak at Fraction 7). B) U1-70K peaked in fraction 6 of intact snRNPs and was not present in necrotic snRNPs.

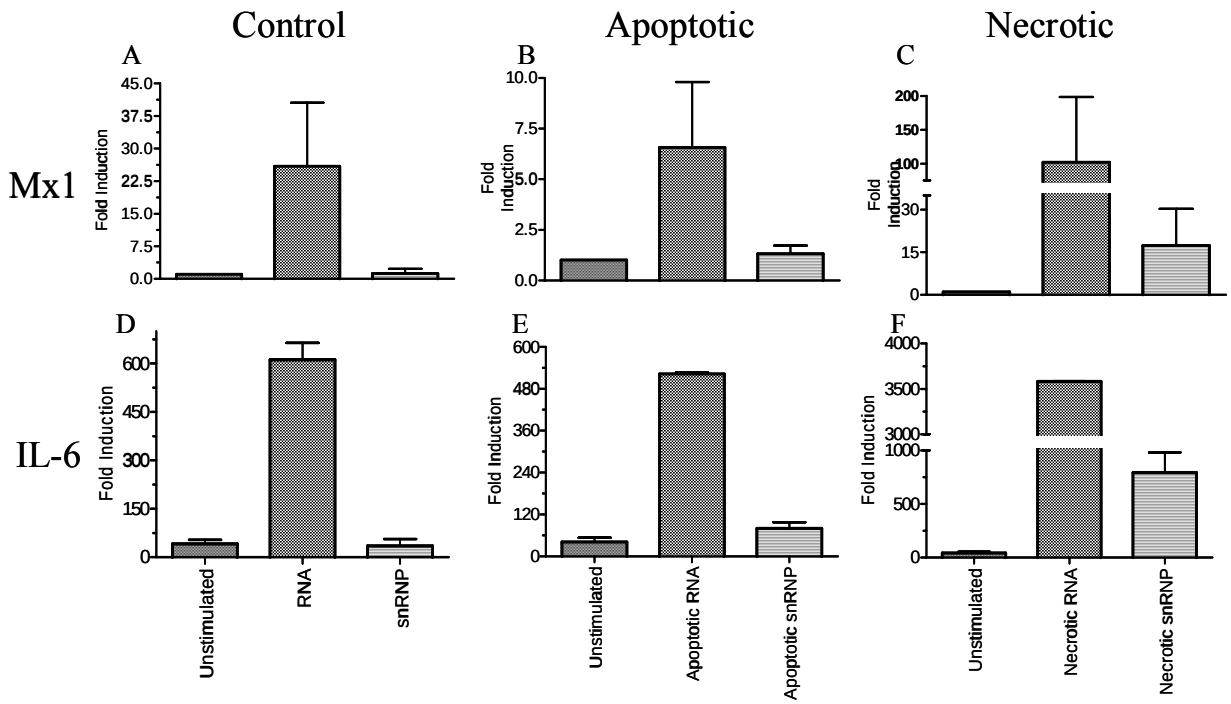

Figure 4-3. Effect of RNA versus intact snRNPs on IFN-I. K562 cells were treated with 20 mM H_2O_2 (apoptotic) or 80 mM H_2O_2 (necrotic) for 24 hours or left untreated (control). U snRNPs were isolated by affinity chromatography. RAW 264.7 cells were stimulated with purified snRNPs or an equivalent amount of purified U RNA. Cells were washed and harvested 6 hours later for RNA isolation (n=3 for each group). Expression of the IFN-I inducible gene, Mx1 and IL-6, was measured by real-time PCR. A-C) Mx1 mRNA expression was induced by naked RNA isolated from control, apoptotic, and necrotic snRNPs. D-F) IL-6 mRNA expression was induced by naked RNA from control, apoptotic and necrotic snRNPs. C,F) Only necrotic snRNPs induced Mx1 and IL-6 expression.

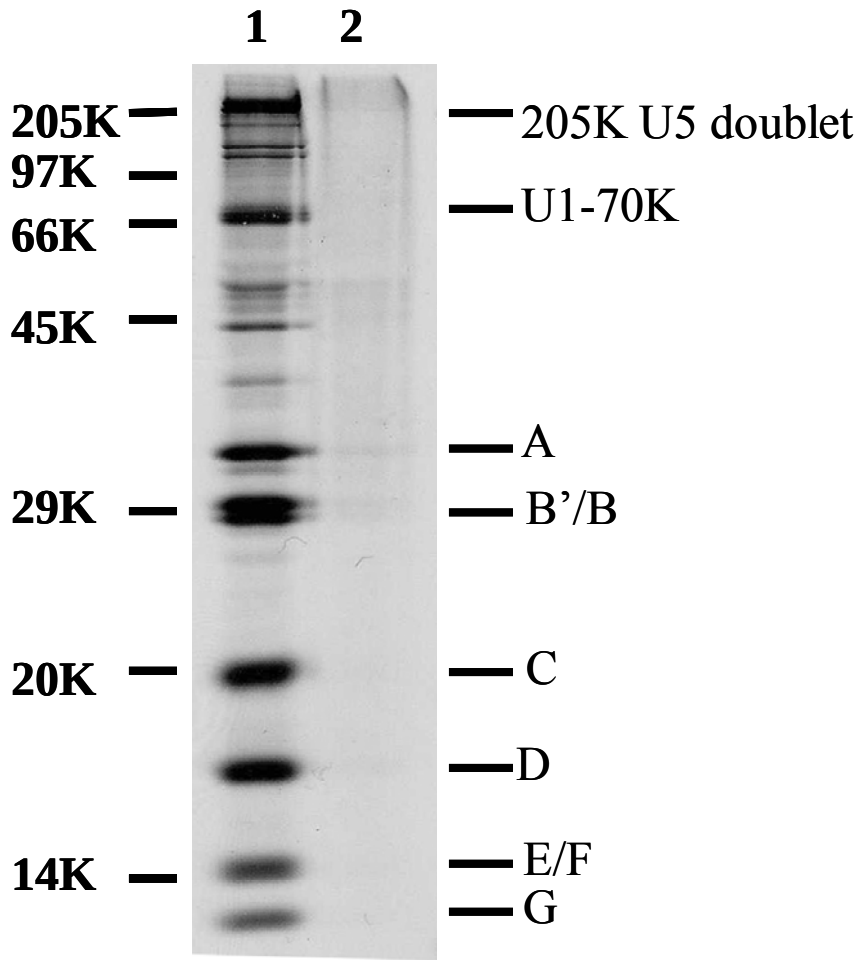

Figure 4-4. Immunoprecipitation using anti-TMG antibody. Lane 1) Radiolabled intact (untreated) Lane 2) and necrotic (80 mM H_2O_2, 24 hours). K562 cell extract were immunoprecipitated with anti-TMG antibodies. Positions of U snRNP proteins U5-205K (indicative of anti-Sm reactivity), U1-A, B'/B, C, D, E/F, and G are indicated on the left. Intact cells immunoprecipitated, U1-70K, A, B/B', C, D, E/F, G as well as the 205K doublet that associates with U5 snRNP. Only proteins A, B/B', D, and the 205kD doublet were immunoprecipitated from necrotic cells.

CHAPTER 5
CONCLUSIONS

It is not known why the most prominent autoantigens in SLE and other autoimmune diseases are nucleic acid-protein complexes. As U1 RNA is an abundant, highly structured cellular RNA associated with a common lupus autoantigen, we investigated the possibility that RNA components of autoantigens might promote autoimmunity by acting as "endogenous adjuvants".

Our data add to the evidence that TLR signaling is involved in autoimmunity. It has been reported that in immune responses to chromatin, the interaction of cellular CpG sequences with TLR9 acts in concert with antigen receptor engagement to promote B cell activation and could play a critical role in generating anti-DNA antibodies (84, 174). In Chapter 2, we proposed a mechanism whereby the activation of dendritic cells by immunostimulatory self-RNAs such as U1 and Y1-5 may drive autoimmune responses to RNP complexes. These complexes may be exposed during endosomal degradation of apoptotic cells (175), in the course of UV irradiation (162) (a known precipitating factor in lupus flares) or because apoptotic/necrotic cells are not disposed of properly in SLE patients. Their interaction with TLR7 stimulates both cytokine production and antigen presenting cell maturation **(**Fig. 2-2B-D**)**, and suggests that the exposed small RNA components of these autoantigens serve as endogenous adjuvants. In view of the importance of self-antigen presentation by immature dendritic cells for immune tolerance (176), the stimulation of dendritic cell maturation by self-RNA molecules in the presence of self-antigen may help explain why ribonucleoproteins are such common targets in SLE and other systemic autoimmune diseases.

Our findings indicate that the U1 RNA component of the Sm/RNP autoantigen stimulates innate immunity via the endosomal TLR, TLR7, and the cytoplasmic dsRNA sensor PKR. U1

RNA induces antigen presenting cell activation and the production of IFN-I and other cytokines including IL-6 and TNF-α. We also found that Y1-5 RNA components of the Ro60 autoantigen can stimulate similar levels of IFN-I, indicating that U1 RNA is not unique in its adjuvant activity, and that the RNA components of other autoantigens are immunostimulatory. In preliminary studies, we have found tRNA is capable of inducing IFN-I (data not shown).

IFN-I has a number of immunomodulatory effects including driving a T_H1 response (93), prolonging the survival of activated B cells (195), facilitating the maturation of B cells into plasmablasts, augmenting antigen specific immunoglobulin production (196), as well as influencing the balance of immunoglobulin isotypes in response to a variety of stimuli (187, 196). SLE patients who produce autoantibodies to Sm/RNP and Ro60/La are found to have higher levels of IFN-I than those SLE patients who do not produce these autoantibodies (113) and increases in IFN-I have been found to be associated with disease activity in SLE (112).

Using the footpad model of local inflammation, we found U1 RNA possesses adjuvant activity *in vivo*. U1 RNA induced IFN-I production, DC maturation (enhanced MHC class II and CD86 expression) and migration into the draining lymph nodes. It also induced the activation of both T and B cells (increased expression of the early activation marker CD69). By effectively blocking the immunostimulatory activity of U1 RNA using a specific inhibitor of TLR7 (IRS 661), we obtained direct evidence that U1 RNA exerts its immunostimulatory effects via TLR7. U1 RNA augmented OVA-specific T cell proliferation and enhanced humoral responses to the NP hapten (following immunization) comparably to the well established adjuvant alum, indicating that this endogenous RNA molecule is as potent as a "standard" vaccine adjuvant. Importantly, U1 RNA was a more potent inducer of IgG2a than IgG1 antibodies **(**Fig. 3-6**),** the major T_H1-type antibody isotype and the isotype of anti-Sm, RNP, and dsDNA autoantibodies

produced in murine lupus (197, 198). The enhancement of T_H1 cytokine production by U1 RNA may help to explain why anti-Sm/RNP responses in murine lupus are predominated by IgG2a autoantibodies. These data suggest *in vivo* that the RNA enhances antigen specific immune responses to standard antigens ovalbuman (OVA) or NP-KLH. By stimulating the innate immune system to produce IFN-I, U1 RNA can drive a more potent humoral response against the snRNP.

Although others have shown that immune complexes from patients who are anti-RNP positive can induce plasmacytoid DCs to produce IFNα/β (180), the physical form of the antigen in those immune complexes has not been characterized. In addition, it has been shown that anti-RNP positive serum IgG combined with apoptotic cells enhances IFNα/β production (188), but again the actual physical state of the U1 snRNP antigen associated with those cells was not defined.

In Chapter 4, we found that intact snRNPs (snRNPs isolated from untreated, live cells), in fact, do not stimulate IFN-I production; whereas necrotic snRNPs (snRNPs isolated from necrotic cells) do stimulate IFN-I production. We show evidence that the necrotic snRNPs have missing and significantly reduced snRNP proteins associated with them, suggesting to us that snRNPs only become stimulatory when proteins are missing and the RNA has become exposed. Further analysis of the stimulatory portion of the snRNP complex is necessary to determine the exact physical state of a stimulatory snRNP complex.

Numerous reports suggest that apoptotic/necrotic cells act as a reservoir for autoantigens. SLE patients have an increase in circulating apoptotic cells that cannot be cleared (49, 51, 52, 199). Our data suggest that apoptotic cells cannot activate an immune response as demonstrated by a lack of an IFN-I response, while necrotic or late apoptotic cells could stimulate IFN-I. This

can be due to the fact that, when left in the circulation, apoptotic cells are likely to undergo secondary necrosis allowing their "adjuvant RNAs" to become exposed, thus giving them the ability to drive antibody responses to their associated proteins. Defects in phagocytosis could be one of the underlying sources of autoimmunity. By fixing this defect, we can eliminate a large reservoir of potential autoantigens, which may help alleviate some symptoms of the disease.

Similar to the idea that chromatin-IgG complexes stimulate via TLR9 and the B cell receptor (BCR) driving an immune response to dsDNA (84), the RNA associated with a variety of autoantigens stimulates via TLR7 inducing IFN-I in DCs (182, 184). We have found that *in vitro*, necrotic snRNPs can drive $CD19^+$ B cells isolated from spleens of TMPD treated mice to produce IgG (unpublished data). These nucleic-acid protein complexes that carry their own adjuvant (RNA or DNA) could be the basis for the selectivity of immune response in SLE and other autoimmune disease.

A difficult question to answer is whether IFN-I drives autoantibody production or whether autoantibody production drives IFN-I production. Our data suggest that RNA associated with common autoantibodies drives IFN-I production. In 2, 6, 10, 14-tetramethylpentadecane (TMPD) – induced lupus, a lupus-like syndrome develops characterized by immune complex-mediated glomerulonephritis, and arthritis and production of autoantibodies against snRNPs as well as dsDNA (197, 200, 201). We have found that IFN-I is required for the formation of these autoantibodies. Similar to human SLE, TMPD-treated mice exhibit an "interferon signature" (151). In mice that lack the IFN-I receptor, injection with TMPD does not cause an increase in the lupus-specific autoantibodies anti-dsDNA or anti-Sm and the severity of the disease is ameliorated (202). Although, the exact mechanism for the increase in IFN-I is still under investigation, it is clear that the IFN-I in this model is dependent on TLR 7 (Lee PY, unpublished

data). The ligand, as of yet, has not been identified, but the RNAs from these snRNP complexes such as U1 RNA stimulating IFN-I via TLR7, remains a likely candidate driving autoimmunity in this model.

The research discussed here aims to improve our understanding of the pathogenesis of autoimmune diseases and help demonstrate why protein-nucleic acid complexes are such prevalent sources of autoantigens. We hope that a better understanding of how these protein-nucleic acid complexes (such as Sm/RNP and Ro 60) become common targets of autoantibodies, it will be possible to develop more specific and better treatments for SLE and other autoimmune diseases.

CHAPTER 6
FUTURE WORK

In chapter 4, we discussed potential mechanisms driving autoimmunity in SLE. We found that if the proteins that are normally associated with snRNPs somehow become disassociated, the complexes are rendered immunostimulatory. In Figure 4-3, we show that snRNPs isolated from necrotic K562 cells and not from intact cells stimulate RAW 264.7 cells to induce Mx1 mRNA expression; yet the RNA extracted from snRNPs isolated from intact, apoptotic, and necrotic cells was immunostimulatory. We found that necrotic snRNPs have fewer and reduced levels of snRNP associated proteins in the gently isolated complex using sucrose gradient analysis and immunoprecipitation, although further analysis of the exact configuration of the snRNP molecule that is immunostimulatory is needed. We performed additional sucrose gradient analyses to determine the exact composition of immunostimulatory snRNP complex.

In Vitro Studies

Stimulation of Cells by snRNPs

We isolated snRNPs from the various sucrose gradient fractions and tested them for their ability to stimulate interferon production *in vitro*. In these experiments, the snRNPs were normalized to the amount of RNA they contained.

All of the wells received snRNPs containing 50 μg of RNA. As expected, all of the fractions isolated from intact snRNPs were not found to be stimulatory. Unexpectedly, only necrotic snRNPs isolated from fraction 9 stimulated RAW 264.7 cells to produce IFN-I as seen by an increase in Mx1 and IFN-β mRNA expression (Fig. 6-1). We had predicted that all of the fractions from necrotic cells would stimulate because the proteins were disassociated from the complex. However, since only fraction 9 from necrotic snRNPs stimulated IFN-I, further investigation is needed to determine what makes fraction 9 stimulatory.

Further Characterization of Sucrose Fractions

Most of the snRNP associated proteins are missing in snRNPs isolated from fractions 6-9 of the sucrose gradients with necrotic cells. However, only fraction 9 was stimulatory. This finding suggests that there is another explanation aside from the lack of proteins for the necrotic snRNPs stimulatory capacity. Currently, we are investigating other possibilities as to why only fraction 9 stimulated IFN-I production. Some theories include the addition of a protein or proteins to the complex, post-translational modifications to proteins in the complex, or modifications to the RNA.

To try to delineate which scenario is correct we performed a number of additional experiments. To investigate the addition of a protein or proteins, we immunoprecipitated U snRNPs, from sucrose gradient fractions, using the anti-TMG antibody. Sucrose gradient fractions from necrotic cells did not contain any proteins (*data not shown*), suggesting that the ability of the necrotic snRNPs to stimulate IFN-I is not due to the addition of proteins to the complex or post-translational modifications to proteins in the complex (as there are no proteins attached to the RNA).

Since we have concluded that the differences in the necrotic snRNPs isolated from fraction 9 may not be solely due to lack of protein association we need to investigate differences at the RNA level. RNA modifications, such as the trimethylguanosine (TMG) cap structure found on the U RNAs or misfolding of the secondary structure could influence the immunostimulatory properties. Interestingly, the Ro60 autoantigen plays a role in the recognition of misfolded RNAs including U snRNPs (28, 162) and mice lacking Ro60 (163) are found to develop a lupus-like syndrome. This suggests that abnormal exposure of potentially immunostimulatory RNAs could promote autoimmunity.

Differences at the RNA Level

Although, what happens to snRNPs during apoptosis has been studied, what happens during necrosis is not known. It is possible that certain modifications render the RNA more stimulatory.

To investigate modifications to the RNA, we analyzed RNA from each fraction by urea PAGE followed by staining with Radiant Red, a fluorescent stain optimized for staining RNA. We did not find any significant differences in the RNA using this method (*data not shown*), a more sensitive and specific method needs to be employed.

Northern blotting is a more sensitive method to investigate differences at the RNA level. Briefly, RNA will be isolated from the various fractions of a sucrose gradient and run on a 2% denaturing formalin agarose gel to separate the small RNAs for analysis. We have designed specific probes for the U RNAs found in our snRNP complexes (including U1 RNA, U2 RNA, U5 RNA and U6 RNA). Table 6-1 lists the probes designed for these studies. The probes will be radiolabeled with [^{32}P]. The RNA gel will be transferred to a nylon membrane and hybridized with the radiolabeled probes. Small differences in the RNA should be evident when RNA isolated from intact or native snRNPs is compared. These experiments will help explain the uniqueness of fraction 9 elucidating the exact physical state/structure or configuration of the immunostimulatory snRNP

Furthermore, determining the physical state of stimulatory snRNPs will also help explain some discordant data in the field. We have clearly shown that intact snRNPs are not immunostimulatory, whereas the naked RNA or necrosis modified snRNPs are. Others have shown that immune complexes from patients who are anti-RNP positive are stimulatory (180) (188). These presumably contain intact snRNPs, but then, the RNAs would not be exposed. By determining the structure of the snRNP molecule within the complexes that are stimulatory we

can identify the configuration of the snRNP needed to drive autoantibody production. The experiments discussed here help explain why RNA containing molecules are such common targets in autoimmunity and how they are stimulatory *in vivo*.

We believe many endogenous molecules may carry their own adjuvant in the form of nucleic acid (either RNA and DNA). If these molecules are not cleared properly during normal cell processes such as apoptosis/necrosis and not cleared by macrophages, they may be taken up by DCs and through TLR signaling, trigger an immune response against those endogenous molecules. Figure 6-2 depicts our model of what is happening in autoimmune diseases such as SLE.

In Vivo Studies

Our studies of the U1 snRNP molecule support the hypothesis that if protein-nucleic acid complexes are disrupted and expose the RNA, an immune response can be elicited against the complex. In other words, the RNA associated with the complex can act as an endogenous adjuvant. All data supporting this hypothesis has been *in vitro*. In order to lend more credence to this hypothesis, *in vivo* experiments are needed.

Immunizations with snRNPs

In Chapter 3, we showed evidence that U1 RNA molecules can act as an adjuvant *in vivo*. In the future, we need to immunize mice with intact versus aberrant (necrotic) snRNPs. If immunizing mice with aberrant snRNPs drives an immune response, measured by antibodies against snRNPs, while immunizations with intact snRNPs leads to no immune response we could definitively say that if the RNA becomes exposed, as is the case in necrotic snRNPs, the molecule is rendered immunostimulatory.

Potential Problems

It is not unreasonable to assume we would encounter problems when these experiments are performed. This section is to address the most obvious issues that may be encountered.

No Differences in the RNA are Seen with the Northern Blots

The possibility remains that the northern blots will show no differences in the RNA extracted from intact versus aberrant (necrotic) snRNPs. This could be for a number of reasons. The first and most obvious reason is that because the RNAs we are looking at are so small (roughly around 200 bases) the differences may be too small to be observed. There are two simple solutions to this problem. The first is to run the gel longer to try to separate the smaller RNAs and the second is to run a denser gel (higher percentage). If this problem is encountered and the solutions mentioned here are tried and we still have no answers other alternatives will need to be looked for. We can sequence the RNA to locate subtle differences between the intact and necrotic snRNPs by using base-specific ribonucleases to generate full-length cDNAs that can be amplified and ligated into a vector. If differences are found in the RNA by sequencing, the exact immunostimulatory portion of the snRNP complex will be known.

Table 6-1. Probes designed for northern blots

RNA of Interest	Probe
U1 RNA	5'GCGCGAACGCAGTCCCCCACTACCAC3'
U2 RNA	5'GTTCCTGGAGGTACTGCAATACTACCAC3'
U4 RNA	5'AAATTTAACCATAAACTCATATACCAC3'
U5 RNA	5'GAACGCTTCACGAATTTGCTACCAC3'

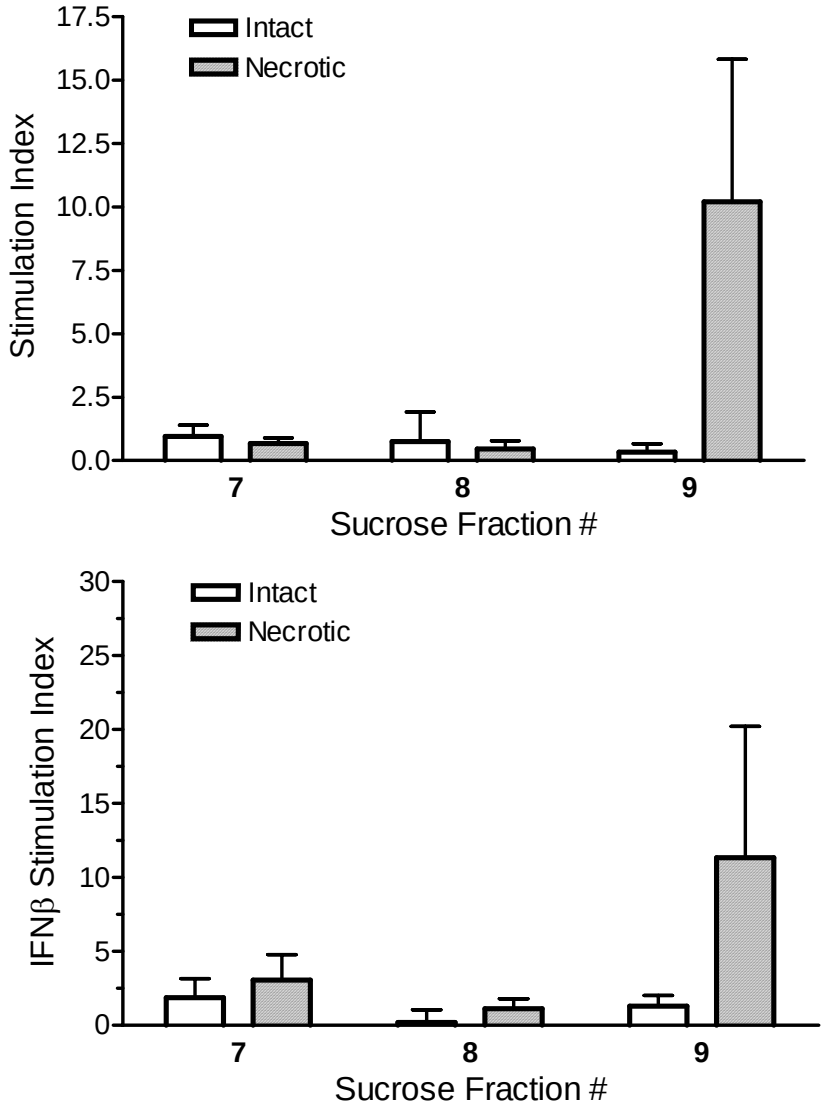

Figure 6-1. Isolated snRNPs from sucrose gradients fractions. Stimulation index [(mRNA expression of gene of interest of unstimulated cells – mRNA expression of gene of interest of stimulated cells)/mRNA expression of gene of interest of unstimulated cells] references to 18S (housekeeping gene) of RAW 264.7cells by snRNPs isolated from sucrose gradient fractions. RAW264.7 cells were stimulated with snRNPs isolated from Fraction 7-9 of 3 pooled sucrose gradients. Only necrotic snRNPs from Fraction 9 stimulated Mx1 and IFN-β mRNA expression.

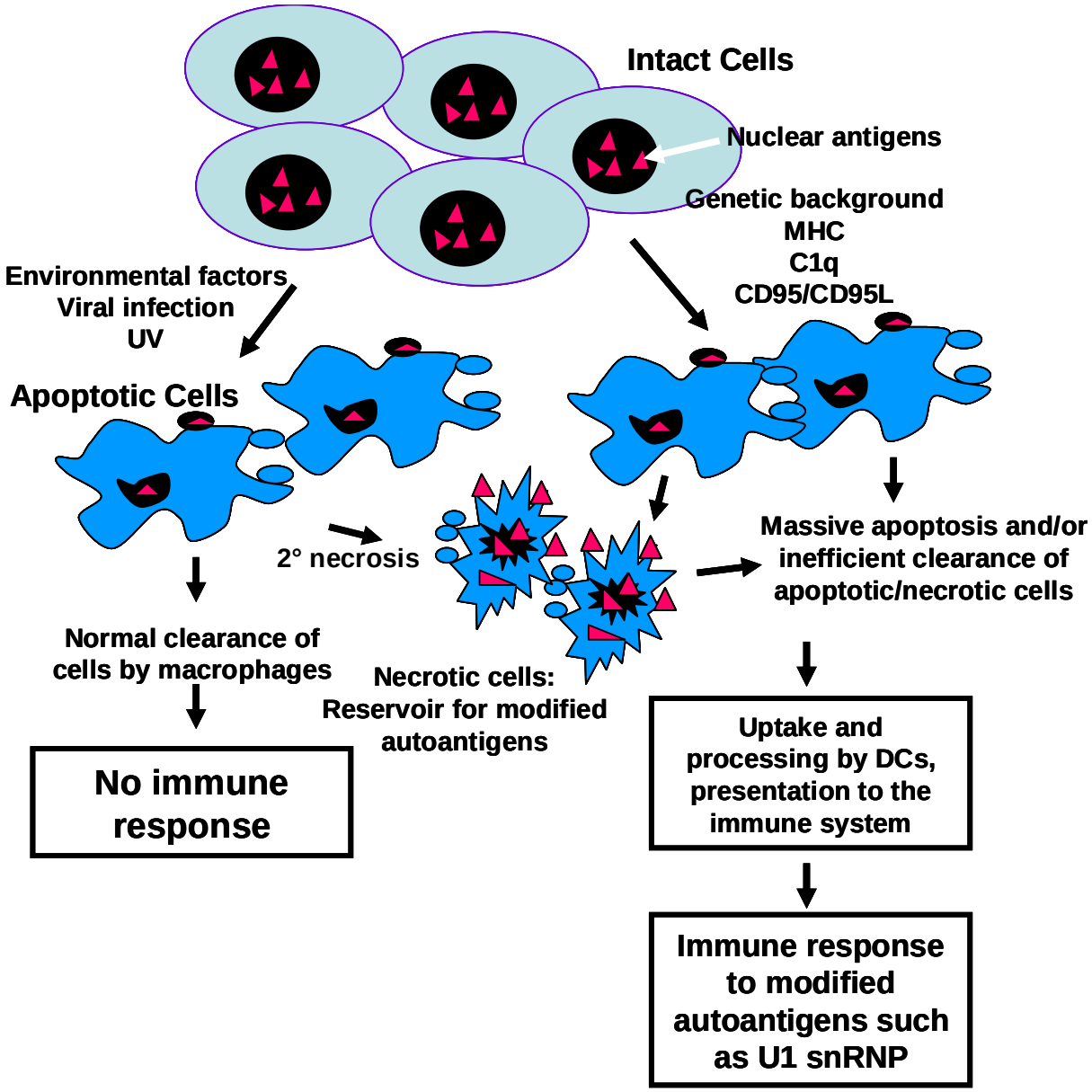

Figure 6-2. Working model of hypothesis. Genetic predisposition or environmental factors lead to increased apoptosis and decreased phagocytosis. Dead and dying cells (apoptotic and necrotic) with modified autoantigens containing nucleic acid are taken up by dendritic cells stimulating TLRs, driving an immune response towards those nucleic acid containing molecules.

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BIOGRAPHICAL SKETCH

Kindra Michele Kelly-Scumpia was born in Palm Beach Gardens, Florida. She attended school in West Palm Beach until the age of 12 when her father, Michael Kelly, received a contracting job in Italy. Kindra was very fortunate to have had the experience of a new culture. Kindra attended a DODD school in Italy for 3 years. Her family then moved back to Florida. Kindra was a sophomore in high school at this point. She graduated from Wellington High School in May 1998. Following high school, Kindra attended the University of Florida where she obtained a Bachelor of Science degree in microbiology and cell sciences. During this period, she worked as a laboratory assistant in the Department of Microbiology under Dr. Samuel Farrah. It was here that she began her research career. Kindra earned several small research scholarships with the Engineering Research Center sponsored by the National Science Foundation. Kindra graduated in December 2001 with honors, and was admitted to the Golden Key National Honor Society and Alpha Lambda Delta during her undergraduate tenure at the University of Florida.

Kindra then started work as a laboratory technician under the supervision of Dr. Bruce Stevens. It was here where is found her place in science. She decided to pursue a career in biomedical sciences and was admitted and entered the Interdisciplinary Program in Biomedical Sciences in the College of Medicine at the University of Florida. Kindra chose to join the immunology concentration and enter the laboratory of Dr. Westley H. Reeves. She began her research on systemic research lupus erythematosus, in particular, the activation of innate immunity by endogenous RNAs, like those associated with common autoantigens. Following her doctorate work, Kindra plans on obtaining a post doctoral position in a different field of immunology where she can widen her field of science. Her ultimate goal is to become an

academic scientist and eventually start her own laboratory. She also hopes to start a family with her husband, Phil with whom she has been married to since May 2006.