Doctoral dissertation title: 5′ terminal nucleotide modulates the immunogenicity of RNA.

Abstract

The innate immune response is the first line of defence in mammalian cells against various pathogens, including RNA viruses. While activation of the innate immune system is crucial to produce antiviral agents such as interferons, overstimulation can lead to autoimmune disorders. One of the key regulators of innate immunity against RNA viruses is the cytoplasmic pattern recognition receptor - retinoic acid-inducible gene I (RIG-I). RIG-I detects double-stranded RNA (dsRNA) transcripts bearing a 5′-triphosphate (5′-ppp) or 5′-diphosphate (5′-pp) moiety, triggering a signaling cascade that includes phosphorylation of IRF3 and induction of type I interferon production.

In addition to viral and certain endogenous intracellular RNA polymerase III transcripts, RIG-I recognizes dsRNA byproducts generated during *in vitro* transcription reaction (IVT), a widely used method to manufacture mRNA therapeutics. Notably, IVT reaction using T7 RNA polymerase exclusively produces transcripts with either 5′-pppA or 5′-pppG as the initiator nucleotide; however, the immunogenic potential depending on the identity of the 5′-terminal nucleotide has not yet been investigated. In the first part of my PhD thesis, I demonstrate that RNA IVT products initiating with 5′-pppA show higher immunogenicity than those starting from 5′-pppG. This immunogenic potential of 5′-pppA initiating RNAs is driven by higher amount of dsRNA byproducts formation during IVT production.

Notably, naturally occurring RIG-I agonists also vary in their 5′-end nucleotide. While many viral RNA genomes begin with 5′-pppA, most polymerase III transcripts in higher eukaryotes initiate with 5′-pppG. The biological significance of this variation remains unexplored. My research shows that dsRNAs with a 5′-pppA end more robustly activate RIG-I/IFN pathway than 5′-pppG dsRNAs in concentrations mimicking early phases of viral infection. Furthermore, I provide potential explanation of this phenomenon showing GTP-binding proteins selectively interact with 5′-pppG dsRNAs, thereby inhibiting RIG-I.

In summary, this study examines both technical and biological aspects of how 5′-terminal nucleotides influence the immunogenic potential of RNA. Technically, it identifies features of IVT that promote immune activation. Biologically, it demonstrates that variation in the 5′-terminal structure of dsRNAs modulates innate immune recognition, establishing a direct link between RNA sequence and immune sensing. The characterisation of IVT mechanisms that trigger immunogenicity provides a foundation for optimising next-generation mRNA therapeutics. The observed reduction in immunogenicity of 5′-pppG dsRNAs further suggests a viral and pol III evasion strategy in which RNAs recruit GTP-binding proteins via 5′-pppG ends. Collectively, these findings reveal a previously underappreciated role for 5′-terminal nucleotides in modulating antiviral immune responses.