CHARACTERIZATION OF STING MEDIATED INNATE IMMUNE SENSING IN THE CONTEXT OF PATHOLOGY

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ABSTRACT

CHARACTERIZATION OF STING MEDIATED INNATE IMMUNE SENSING IN THE CONTEXT OF PATHOLOGY

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M.Sc., Department of Molecular Biology and Genetics
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August 2018, 96 pages

Type I interferonopathies are a group of diseases characterized by excess type I interferon production. Here, we examined the intracellular signal transduction pathways of two interferonopathies, STING-associated vasculopathy with Onset in Infancy (SAVI) and Aicardi-Goutières syndrome (AGS) using in vitro cell line models. SAVI is characterized by constitutive STING activation. M155V and N154S gain-of-function mutations in STING can cause disease in humans. We expressed the M155V mutant STING in STING-knocked out B16 Blue interferon reporter cell line. The effect of STING in tumor progression was investigated in mice using wild type, STING<sup>-/-</sup> or STING<sup>M155V</sup> expressing B16 melanoma cells, with no significant differences between these groups.

Using the STING<sup>M155V</sup> expressing cells, we investigated type I IFN secretion profile to delineate the mechanism behind the cold-induced exacerbation of inflammation observed in SAVI patients. Similarly, TREX1 KO THP cells were used as a model of Aicardi-Goutières syndrome. Exposure to cold upregulated both p-STING and total STING levels in B16 cells, which may account for cold-temperature associated increase in inflammation in patients. Next, we analyzed the type I IFN suppressing
activity of various inhibitors in our cell-line disease models. Results indicated that TBK1/IKKε inhibitors Amlexanox and BX-795 were more effective in controlling chronic type I IFN production than the JAK/STAT inhibitors ruxolitinib and tofacitinib. Finally, we also investigated whether or not cGAS/STING signaling is regulated by circadian rhythms. Preliminary data showed that cGAS/STING-dependent type I IFN production oscillated over time, suggesting that this signaling pathway might indeed be under circadian regulation.

Keywords: interferonopaty, interferon, cGAS, STING, AGS, SAVI, cold-exposure, inhibitor, circadian rhythm
ÖZ

STING ARACILI DOĞAL BAĞIŞLIK UYARIMLARININ PATOLOJİ BAĞLAMINDA KARAKTERİZASYONU

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Tip I interferonopatiler, aşırı tip I interferon üretimi ile karakterize edilen hastalık grubu olarak tanımlanır. Bu çalışmada, in vitro hücre hattı modelleri kullanılarak STING-ilişkili erken başlangıçlı vaskülopati (SAVI) ve Aicardi-Goutières sendromu (AGS) olmak üzere iki interferonopatinin hücre içi sinyal iletim yolaklarını inceledik. SAVI, sürekli STING aktivasyonu ile nitelenir. STING proteininde M155V ve N154S fonksiyon kazanım mutasyonları insanlarda hastalığa neden olmaktadır. STING yoksunu B16 Blue interferon raportör hücre hattında M155V mutant STING proteini ifade edilmiştir. STING yoksunu, mutant STING taşıyan ve sağlıklı STING ifade eden B16 Blue hücre hatlarının tüm gelişimindeki rolü fare deneyleriyle araştırılmış, ancak anlamlı farklar gözlemlenmemiştir.

STING M155V ifade eden hücrelerde, SAVI hastalında soğukla tetiklenen tip I IFN salgı profilinin hücre içi mekanizması araştırılmıştır. Benzer şekilde, TREX1 KO THP hücreleri Aicardi-Goutières sendromunun bir modeli olarak kullanılmıştır. Soğuk stresinin, B16 hücrelerinde hem p-STING hem de total STING seviyelerinde artışa neden olduğu gözlemlenmiş, bu mekanizmanın hastalarda
gözlenen soğukla tetiklenen enfamasyondan sorumlu olabileceği düşünülmüştür. Daha sonra, hücre içi hastalık modellerimizde çeşitli inhibitörlerin tip I IFN baskılayıcı aktivitesi analiz edilmiştir. Sonuçlar, TBK1/IKKe inhibitörleri Amlexanox ve BX-795'in, Jak/Stat inhibitörleri ruxolitinib ve tofacitinibe kıyasla kronik tip I IFN üretimini baskılamada daha etkili olduğu göstermiştir. Son olarak, cGAS/STING sinyal iletiminin sirkadiyen ritim ile düzenlenip düzenlenmediğini de araştırılmıştır. Ön veriler, cGAS/STING'e bağlı tip I IFN üretimini zamana bağlı osilasyon gösterdiğini ve bu sinyal iletim yolunun sirkadiyen regülasyon altında olabileceği düşündürülmüştür.

Anahtar Kelimeler: interferonopati, interferon, cGAS, STING, AGS, SAVI, soğuk stresi, inhibitör, sirkadiyen ritim
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# TABLE OF CONTENTS

ABSTRACT ......................................................................................... v
ÖZ ........................................................................................................ vii
ACKNOWLEDGEMENTS ....................................................................... ix
TABLE OF CONTENTS .......................................................................... xi
LIST OF TABLES ....................................................................................... xv
LIST OF FIGURES ..................................................................................... xvi
LIST OF ABBREVIATIONS ...................................................................... xviii

## CHAPTERS

1. INTRODUCTION .............................................................................. 1
   1.1. Cytosolic Nucleic Acid Sensors .................................................. 1
   1.2. Diseases Associated with Dysregulation of Nucleic Acid Sensing Pathways ................................................................................................................. 6
   1.3. Circadian Rhythm and Control of Immunity .................................... 7
   1.4. Aim of the Study ......................................................................... 9

2. MATERIALS & METHODS ................................................................. 11
   2.1 Materials .................................................................................. 11
      2.1.1. Plasmids ........................................................................ 11
      2.1.2. Bacterial Strain and Culture Media ........................................... 11
      2.1.3. Cell Lines ........................................................................ 12
      2.1.4. Cell Culture Media, Buffers and Solutions ............................... 13
      2.1.5. Stimulants and Inhibitors ...................................................... 13
   2.2. Methods ................................................................................ 16
2.2.1. Bacterial Growth ................................................................. 16
2.2.2. Preparation of Chemically Competent Cells ................................. 17
2.2.3. Plasmid Transformation of Competent Cells ................................. 17
2.2.4. Plasmid Isolation ................................................................. 17
2.2.5. Plasmid Integrity and Contamination Test by Gel Electrophoresis ........ 18
2.2.6. Establishment of Antibiotic Kill Curve in B16-Blue cells .................. 18
2.2.7. Transfection with plasmid DNA and Construction of Stable Transfected Cells ................................................................. 18
2.2.8. Counting of Cells .................................................................. 19
2.2.9. Detection of Type I IFN Production using the Quanti-BLUE Reagent ................................................................................. 19
2.2.10. Flow Cytometric Analysis of Samples ......................................... 20
2.2.10.1. Cell Fixation ................................................................... 20
2.2.10.2. Intracellular STING Staining ............................................... 20
2.2.11. Cold Induction Experiments ..................................................... 21
2.2.12. Determination of Protein Concentration ..................................... 21
2.2.13. Western Blot ......................................................................... 21
2.2.14. in Vivo Experiments ............................................................... 23
2.2.14.1 Animal Handling ................................................................. 23
2.2.14.2. Tumor Injection, Tumor Size Measurements and Preparation of Tumor-infiltrating Single Cells ......................................................... 23
2.2.14.3. Treg and Tumor Associated Macrophage panel staining ...... 24
2.2.15. Circadian Rhythm Experiments ................................................ 26
2.2.16. Quanti Luc luciferase IFN expression reporter assay for Dual Cell Lines ................................................................................. 26
2.2.17. Quanti Blue SEAP NF-κB activation reporter assay for Dual Cell lines ................................................................................. 26
2.2.18. Statistical Analysis ................................................................. 27

3. RESULTS & DISCUSSION .................................................................. 29
3.1. Generation of Antibiotic Selection Kill curve .................................. 30
3.2. Optimization of Plasmid DNA Transfection ........................................31
3.3. Generation of human STING\textsuperscript{M155V} expressing B16 Blue cell line...........33
3.4. Functional Assessment of STING\textsuperscript{M155V} B16 Cell Line ......................35
   3.4.1. Spontaneous Type I IFN secretion profile of STING\textsuperscript{M155V} B16 Cells .................................................................35
   3.4.2. Determination of Responsiveness of STING\textsuperscript{M155V} B16 Cells to Stimulation with Intracellular Nucleic Acid Ligands ...............36
   3.4.3. Testing of potential inhibitors to suppress spontaneous Type I IFN secretion in STING\textsuperscript{M155V} B16 Cells ........................................38
   3.4.4. Cold Induced Type I IFN exacerbation in STING\textsuperscript{M155V} B16 Cell Line .................................................................39
3.5. The Effect of STING mediated Type I IFN secretion on Tumor Progression .................................................................44
3.6. Assessment of TREX1\textsuperscript{−/−} THP1 Cells as an \textit{in vitro} Model of Aicardi Goutieres Syndrome (AGS) .................................................................48
   3.6.1. Spontaneous Type I IFN secretion by TREX1\textsuperscript{−/−} THP1 Cell Line ...49
   3.6.2. Effect of Cold Exposure on Type I IFN secretion from TREX1\textsuperscript{−/−} THP1 Cells .................................................................50
   3.6.3. Inhibition Strategies for Elevated Spontaneous Type I IFN secretion in TREX1\textsuperscript{−/−} THP1 Cell Line .................................................................55
3.7. Investigation of Changes in Cytosolic Nucleic Acid Sensing Mechanisms in Relation to Circadian Clock .................................................................58
4. CONCLUSIONS .........................................................................................67
REFERENCES ........................................................................................................71
APPENDICES
A. Vector Map of pUNO1-hSTING-M155 ........................................................................79
B. Culture Media, Buffers & Solutions Used in Bacterial Growth, Transformation & Competent Cell Preparation .................................................................81
C. Culture Media, Buffers & Solutions Used In Mammalian Cell Culture .........83
D. Western Blot Buffers & Solutions .................................................................85
E. Graphs & Regarding Parameters Used For IC_{50} Value Calculation ..........89
F. Flow Cytometry Gating Strategies ...............................................................91
G. Basal Level Oscillation Patterns .................................................................93
H. Permission to Copyrighted Material ............................................................95
LIST OF TABLES

Table 2.1. Stimulants and inhibitors used in experiments........................................14
Table 2.2. Immunosuppressive and control ODNs used in experiments..............14
Table 2.3. Stimulants and their working concentrations used in Dual THP1, TREX
KO THP1, cGAS KO THP1, STING KO THP1, and STING KI N154S cell line
experiments..............................................................................................................15
Table 2.4. Inhibitors and corresponding working concentrations used in Dual
THP1, TREX KO THP1, cGAS KO THP1, STING KO THP1, and STING KI
N154S cell line experiments...................................................................................16
Table 2.5. List of antibodies used in western blot.........................................................23
Table 2.6. List of antibodies and their fluorophores used in Treg.......................25
Table 2.7. List of antibodies and their fluorophores used in TAM......................25
LIST OF FIGURES

Figure 1.1. STING mediated nucleic acid sensing mechanism; adapted from (Galluzzi, Vanpouille-Box, Bakhoum, & Demaria, 2018) .................................. 5
Figure 1.2. Circadian rhythm characteristics .......................................................... 9
Figure 2.1. Representative image of the Area used for counting of cells in a haemocytometer ........................................................................................................... 19
Figure 3.1. Viability of STING\(^{-/-}\) B16 cell line incubated with various concentrations of blasticidin ........................................................................................................ 31
Figure 3.2. Optimization of plasmid DNA transfection using EGFP expression plasmid and Lipo2000 transfection reagent in STING\(^{-/-}\) B16 cells .................................. 32
Figure 3.3. Evaluation of STING expression following transfection of STINGM\(^{155V}\) expression plasmid into STING\(^{-/-}\) B16 Cell Line .................................................. 34
Figure 3.4. Assessment of SEAP reporter activity-based spontaneous IFN production in STING\(^{wt}\), STING\(^{-/-}\), STING\(^{M155V}\) B16 cell lines ........................................ 36
Figure 3.5. Type I IFN response of STING\(^{wt}\), STING\(^{-/-}\), STING\(^{M155V}\) B16 cells to stimulation with nucleic acid receptor agonists ........................................... 37
Figure 3.6. Effect of A151 on spontaneous Type I IFN secreted from STING\(^{M155V}\) B16 cell line ................................................................. 39
Figure 3.7. Experimental Setup of Cold Induction ..................................................... 40
Figure 3.8. Effect of Amlexanox on cold-induced spontaneous type I IFN expression in B16 STING variant cell lines ............................................................... 41
Figure 3.9. Experimental setup to investigate the mechanism of cold-induced STING activation ........................................................................................................ 43
Figure 3.10. Western Blot images of p-STING, total STING and their corresponding semi-quantitative protein expression levels ............................................. 44
Figure 3.11. Tumor volumes and Kaplan-Meier percent survival curves of STING\(^{wt}\), STING\(^{-/-}\), STING\(^{M155V}\) B16 cell lines ......................................................... 46
Figure 3.12. Tumor infiltrating macrophage and Treg percentages in tumors derived from STING knockout or STING variant expressing B16 melanoma cells. ..........47

Figure 3.13. IFN expression and NF-κB activity of WT, STING−/−, cGAS−/−, TREX1−/− THP Dual Cell Lines .................................................................................................................. 49

Figure 3.14. Effect of Cold-exposure on IFN expression and NF-κB activity of WT, STING−/−, cGAS−/−, TREX1−/− THP Dual Cell Line .......................................................................................... 51

Figure 3.15. Effect of Application time of Cold-exposure on IFN expression and NF-κB activity of WT, STING−/−, cGAS−/−, and TREX1−/− THP Dual Cells ......53

Figure 3.16. IFN secretion and NF-κB activation profile of WT, STING−/−, cGAS−/− and TREX1−/− THP Dual Cell Lines stimulated with recombinant IFN-β and cold exposure. ........................................................................................................... 54

Figure 3.17. IFN secretion and NF-κB activation profile of TREX1−/− THP Dual Cell Line in response to increasing doses of various inhibitors ......................... 56

Figure 3.18. Determination of type I IFN secretion from dexamethasone synchronized TREX−/− and STINGN154S THP Dual Cell Lines as a function of time. ................................................................................................................................. 58

Figure 3.19. Determination of type I IFN secretion from dexamethasone synchronized cytosolic nucleic acid or IFN-β stimulated WT, ......................... 62

Figure 3.20. Determination of NF-κB activation in dexamethasone synchronized cytosolic nucleic acid or IFN-β stimulated WT, STING−/− and cGAS−/− THP Dual Cell Lines as a function of time. ................................................................. 64

Figure A.1. The vector map of hSTING-M155(Invivogen, USA) ........................................ 79

Figure A.2. IC_{50} Graphs of Amlexanox ...................................................................... 89

Figure A.3. M1-M2 Flow cytometry gating strategies and individual plots of mice. ................................................................................................................................. 91

Figure A.4. Treg Flow cytometry gating strategies and individual plots of mice. 92

Figure A.5. During 48-hour period of circadian time, basal levels of type I IFN oscillations. .................................................................................................................. 93
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGS</td>
<td>Aicardi-Goutières syndrome</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>BB</td>
<td>Blocking buffer</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cGAMP</td>
<td>Cyclic GMP-AMP</td>
</tr>
<tr>
<td>cGAS</td>
<td>Cyclic GMP-AMP synthase</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>DF</td>
<td>Dilution factor</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>GMP</td>
<td>Guanosine monophosphate</td>
</tr>
<tr>
<td>GOF</td>
<td>Gain of function</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>hSTING</td>
<td>human STING variant</td>
</tr>
<tr>
<td>IFNs</td>
<td>Interferons</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factors</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon-stimulated gene</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon-stimulated response element</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>KI</td>
<td>Knocked in</td>
</tr>
<tr>
<td>KO</td>
<td>Knocked out</td>
</tr>
<tr>
<td>MAVS</td>
<td>Mitochondrial antiviral-signaling protein</td>
</tr>
<tr>
<td>MDA-5</td>
<td>Melanoma Differentiation-Associated protein 5</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MO</td>
<td>Monocytes</td>
</tr>
<tr>
<td>Mφ</td>
<td>Macrophage</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa B</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene I</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SAVI</td>
<td>STING-associated Vasculopathy with Onset in Infancy</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEAP</td>
<td>Secreted embryonic alkaline phosphatase</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>STING</td>
<td>Stimulator of interferon genes</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor associated macrophages</td>
</tr>
<tr>
<td>TANK</td>
<td>TRAF family member-associated NF-kappa-B activator</td>
</tr>
<tr>
<td>TBK-1</td>
<td>TANK-binding kinase 1</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cells</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumor necrosis factor receptor-associated factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>TREX1</td>
<td>three prime repair exonuclease 1</td>
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CHAPTER 1

INTRODUCTION

1.1. Cytosolic Nucleic Acid Sensors

The Immune system consists of two distinct but communicating arms: innate and adaptive immunity. Innate immunity generates the first line of host defense to evolutionary conserved molecular structures. In cooperation with innate immunity, adaptive immunity takes the lead in cases where the innate immunity fails to combat the infection. In contrast to innate immune system, adaptive immunity mounts a highly specific response through clonal selection of T and B cells. However, this response is directed by innate immunity. In this well-established cooperation, certain lymphoid or myeloid origin immune subsets such as B-cells, dendritic cells and macrophages, process and present antigens to T-cells. Furthermore, these professional antigen presenting cells express specialized receptors to recognize molecular structures that are associated with pathogen and damage, termed as pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). These molecular structures are perceived by germ-line encoded receptors collectively termed as pattern recognition receptors (PRRs). The distinction of self/non-self is made through recognition of PAMPS, whereas self/damaged-self discrimination is made through recognition of DAMPs. In this context, both foreign and self-nucleic acids can be recognized by immune cells as PAMP or DAMPs (Medzhitov, 2009).

The idea that nucleic acids can stimulate an immune response dates back to early 60ties wherein long double stranded synthetic RNA was reported to induce interferon production (Isaacs, Cox, & Rotem, 1963). However, subsequent to this
finding, the mechanisms that operate in nucleic acid sensing was discovered 3 decades later. First, bacterial DNA was reported to promote type I interferon production (Yamamoto, Kuramoto, Shimada, & Tokunaga, 1988). Next ground-breaking discovery was the first receptor-ligand affiliation. Toll like receptor 9 (TLR 9) was identified as the receptor for unmethylated CpG dinucleotide containing DNA (Hemmi et al., 2001). During 2008-2009, the role of Stimulator of interferon genes (STING) as the essential adaptor protein operating in cytosolic DNA/cyclic dinucleotide sensing was revealed by 4 independent research groups including Glen N. Barber, Hong-Bing Shu, Zhengfan Jiang, John Cambier’s group (Tao, Zhou, & Jiang, 2016). Following this, AIM2 was shown to detect cytosolic DNA (Hornung & Latz, 2010) and this recognition was linked to inflammasome activation that led to IL-1β production but not type I IFN synthesis. Although STING protein was known to function in cytosolic DNA sensing and type I IFN production, which protein functioned upstream of STING was not fully understood. In 2012 cyclic GMP-AMP synthase (cGAS) was identified as the dominant cytosolic DNA sensor that synthesized a new second messenger, 2’3’-cyclic guanosine–adenosine monophosphate (2’3’-cGAMP) which in turn acted as ligand of downstream STING (Lijun, Wu, Fenghe, Xiang, & Chen, 2009). Later, scientific inquiry of this pathway revealed various positive and negative regulators and mutations residing in STING by multiple research groups. As the main focus of this research, cGAS-STING-mediated cytosolic nucleic acid recognition pathway will be explained in more detail in the following pages.

Cytosolic nucleic acid sensing is an evolutionary conserved protection mechanism against pathogens including viruses, some bacteria and protozoa. However, recent evidence suggests that nucleic acid sensing pathways and DNA damage response also overlap in the host. As mentioned previously, TLR9 was the first identified receptor for DNA sensing. However, TLR9 expression is restricted to plasmacytoid dendritic cells (pDC) and B cells, whereas other cell populations such as fibroblasts lack TLR9, yet remain responsive to stimulation with cytosolic DNA and produce
Cyclic GMP–AMP (cGAMP) synthase (cGAS), a nucleotidyl transferase binds dsDNA in a length-dependent but sequence-independent manner and undergoes modification aiding cGAS dimerization. Once activated, cGAS catalyzes the synthesis of two phosphodiester bonds between a GMP and an AMP, thus resulting in the generation of 2′-3′ cGAMP (Tao et al., 2016; Zevini, Olagnier, & Hiscott, 2017). dsDNA, stem-loop forming ssDNA, Y-form DNA, and DNA:RNA hybrids were reported to be sensed by cGAS (Tao et al., 2016). Presence of [G(2′,5′)-pA(3′,5′)] phosphodiester bond found in 2′-3′ cGAMP defined as metazoan cGAMP, has the highest affinity to STING among other agonists such as the cyclic dinucleotides (CDN) of bacterial origin. Molecular mimicry between the mammalian second messenger 2′-3′ cGAMP and the bacterial cyclic-di-AMP, cyclic-di-GMP, 3′-3′ cGAMP, enables STING-mediated recognition of bacterial CDNs, albeit with a lower affinity (Cai, Chiu, & Chen, 2014). Under unstimulated conditions, STING resides as an inducible homodimer on the endoplasmic reticulum (ER) membrane. Upon stimulation with STING agonist, STING undergoes conformational change, leading to its dimerization and post-translational modification such as K27-linked polyubiquitination. Altered conformation of STING allows for the recruitment of TANK-binding kinase 1 (TBK1). This provisional complex migrates from the ER to Golgi perinuclear endosomes. Activation induced homing in the trans-Golgi network results in palmitolaytion at Cys88 and Cys91 residues of STING. This event leads to the phosphorylation of STING by TBK1 at Ser366 (Zevini et al., 2017). Concomitantly, STING-TBK1 complex activates interferon regulatory factor 3 (IRF3) via its phosphorylation at Ser396 and induce IRF3 dimerization. IRF3 dimers translocate to the nucleus to initiate expression of type I IFN expression (Dobbs et al., 2015; Hu et al., 2016). Following release of type I IFNs and type-I IFN-receptor-mediated signaling,
multiple interferon stimulated genes (ISGs) are expressed. ISG expression reinforces anti-viral response. Although this pathway is usually triggered by viral infections conferring anti-viral cytokine production, initiation of signal transduction can also be stimulated by pathogen-independent leakage of DNA fragments into the cytosol, particularly when there is extensive unrepaired DNA damage. The promoter region of the IFN-β gene contains at least four regulatory cis-elements: the positive regulatory domains (PRDs) I, II, III and IV and promoter region of the IFN-α accommodates PRD-like elements (PRD-LE). Despite absence of multiple confirmations, transcription of IFN-β is directed through homodimer IRF3, homodimer IRF7, heterodimer IRF3/IRF7 complexes that engage p300/CBP complex. These transcription factor complexes act on PRD1-PRDIII sequences and dictate type I IFN expression. IFR3 dimer activation also triggers IP-10 expression (Honda, Yanai, Takaoka, & Taniguchi, 2005). Once type I IFN pathway is initiated, interferon-stimulated gene (ISG) products are generated by feed forward circuit of type I IFNs. ISG products have diverse functions: I) Some of these products bolster anti-viral immune response, collectively termed as positive regulators. STAT1, STAT2, RIG like receptors (RLR), cGAS, IRF3, IRF7, IRF9 are among these positive regulators. II) Some ISG products maintain IFN memory and play important roles in resolution of the “IFN-primed state”. These are called negative regulators. Pronounced examples of these include ISG15, USP18 and SOCS. III) Another group of ISGs interfere with evading pathogens and are called as effector products such as viperin. These products are cell intrinsic mechanistic inhibitors of pathogens (Schneider, Chevillotte, & Rice, 2014). Schematic summary of cytosolic innate immune nucleic acid sensing mechanism is depicted in Figure1.1.
Apart from the cGAS-STING axis mediated DNA recognition and subsequent type I IFN production, cytosolic RNA receptors specifically RIG-I and MDA-5 also contribute to type I IFN secretion and pro-inflammatory cytokine production by binding to different groups of dsRNA. Similar to the adaptor protein function of STING in cGAS mediated DNA sensing, RIG-I and MDA-5 transduce their RNA recognition signal through mitochondrial antiviral-signaling protein (MAVS). Conveying a signal to TBK1 and IκB kinase (IKK), MAVS activation results in phosphorylation of IRF3 and IRF7 (Kato et al., 2006; Seth, Sun, Ea, & Chen, 2005). As previously explained, IRF3 and IRF7 transcription factors generate type I IFN response.

In summary, nucleic acid recognition-dependent type I interferon production constitutes a major anti-viral response. However, dysregulation in this signaling pathway causes catastrophic outcomes for the organism.
1.2. Diseases Associated with Dysregulation of Nucleic Acid Sensing Pathways

Under normal circumstances, nucleic acid sensing mechanism operate in response to pathogens and the elevated type I IFN response is terminated following clearance of the pathogen. More recently, a group of diseases, characterized by sterile inflammation stemming from elevated levels of type I IFNs have been defined and named as “type I interferonopathies”. In this thesis, we investigated two of these diseases, STING-associated Vasculopathy with Onset in Infancy (SAVI) and Aicardi-Goutières syndrome (AGS) among other interferonopathies. Germline mutations M155V, N154S, V147L, G166E in the dimerization domain or mutations in C terminal domain C206Y, R281Q,R,284M, R284G, R284S of STING causes SAVI pathology (Y. Liu et al., 2014; Saldanha et al., 2018; Tao et al., 2016). These gain-of-function mutations in STING culminates in spontaneous type I IFN production, originating from constitutively active signal transduction of STING. Systemic inflammation, cutaneous rash, pulmonary manifestations such as interstitial lung disease, and severe small vessel vasculopathy are hallmarks of SAVI pathology (Stoffels & Kastner, 2016; Volpi, Picco, Caorsi, Candotti, & Gattorno, 2016). Gain of mutations of STING can be inherited by autosomal dominant Mendelian pattern, displaying symptoms of SAVI starting with infancy. However, de novo gain of function mutations of STING during early development may also lead to somatic mosaicism and could be a potential causative candidate for contributing pathology of late-onset rheumatological diseases (Stoffels & Kastner, 2016).

AGS is caused by genetic mutations in multiple genes including, Adenosine to Inosine RNA editing receptor Adenosine deaminase acting on RNA (ADAR1), dNTP level controlling SAMHD1, DNA damage response regulating nucleases like TREX1, RNase H2 subunits hydrolyzing RNA in RNA: DNA hybrids, all of which results in AGS pathology (Hartmann, 2017a). While mutations in TREX1 and RNase H subunits contribute to type I IFN production through cGAS-STING-IRF3
axis, mutations in ADAR1, RIG-I or MDA-5 promotes type I IFN production via RIG-I/MDA-5-MAVS-IRF3 axis (Rodero & Crow, 2016). In this study, we investigated TREX1 deficient cell line model of AGS. TREX1 is an 3’ to 5’ repair exonuclease 1 and responsible for degradation of cytosolic ds- and ss-DNA species. TREX1 is found localized to the endoplasmic reticulum membrane (Stetson, Ko, Heidmann, & Medzhitov, 2008). TREX1 deficient mice suffer from inflammatory myocarditis and are fully rescued if cGAS is knocked out, indicating the importance of cGAS/STING pathway in this model. TREX1 deficiency results in accumulation of genome-derived DNA fragments in the cytosol stemm from impaired DNA damage response (Ablasser et al., 2014). This evidence implies that TREX1 deficiency-dependent DNA accumulation provokes cGAS-STING mediated spontaneous type I IFN production in AGS patients with TREX deficiency.

1.3. Circadian Rhythm and Control of Immunity

Recent research in the emerging field of chrono-immunology revealed that several aspects of immunity are under circadian control. Circadian rhythm is a type of biological clock consisting of repetitive 24-hour cycles stemming from rotation of earth. Host-pathogen interactions, trafficking of immune cells and activation of innate and adaptive immunity are thought to be regulated by specific clock genes. Subsequent to photonic impulse, mammals demonstrate circadian rhythm dependent gene regulation through suprachiasmatic nucleus (SCN) located in the anterior hypothalamus that converts light input into electrochemical signals for synchronization. This pacemaker system generates central circadian oscillations. SCN neurons persistently sustain their autonomous rhythmicity even in ex vivo conditions, unlike peripheral tissues. Several set of intertwined rhythmic transcription-translation regulation circuits maintain mammalian circadian core clockwork. Circadian locomoter output cycles protein kaput (CLOCK), brain and muscle arNT-like 1 (BMaL1) and neuronal Pas domain-containing protein 2 (neuronal Pas2) are among cardinal circadian regulatory transcriptional activators. By forming heterodimeric complexes, they act on e-box elements for controlling
target genes, including period circadian protein homologue 1 (PER1) and PER2, cryptochrome 1 (CRY1) and CRY2. Per-CrY complexes function as transcriptional repressors on e-box promoter of CLOCK and BMaL1. This leads to the decreased expression of CLOCK and BMaL1. Decay of Per-CrY which coincides with dark phase, leads to enhanced expression of CLOCK and BMaL1 (Scheiermann, Gibbs, Ince, & Loudon, 2018).

In this study, we used dexamethasone for in vitro circadian synchronization. Dexamethasone is an agonist of glucocorticoid receptor that maintains circadian rhythmicity through PER1, PER2 and CLOCK (So, Bernal, Pillsbury, Yamamoto, & Feldman, 2009). As a synthetic alternative of glucocorticoid hormone, dexamethasone treatment upregulates the PER1-2 genes. Upregulation of PER genes correspond to light phase in vivo and decay coincides with the dark phase (Labrecque & Cermakian, 2015; So et al., 2009).

For evaluation of circadian regulated responses, 3 fundamental circadian clock parameters are assessed: acrophase, period length and signal amplitude (Figure 1.2.). Acrophase is the time point where signal peak is reached. Period is the time interval between two signal peaks. Amplitude is the scaler measure of signal peak. Furthermore, another important chronobiological terminology is the Zeitgeber, which essentially represents a synchronizing agent which sets the time after which the circadian oscillations take place.
1.4. Aim of the Study

This study aimed to investigate the consequences of dysregulated intracellular signaling responsible for excess type I IFN and inflammation in SAVI and AGS. To pursue this aim, we generated stably transfected STING<sup>M155V</sup> expressing B16 melanoma cells to duplicate the chronic type I IFN secretion profile observed in SAVI. Similarly, TREX deficient THP monocytes were used to mimic AGS. These in vitro cell line models were used to analyze the mechanism underlying cold-induced exacerbation of type I IFN production in terms of intracellular signaling pathways. Since type I IFN production is stimulated by both type I IFN receptor-mediated autocrine positive feedback loop as well as cell intrinsic STING-dependent pathway, we also intended to determine the predominant signaling pathway responsible for constitutive type I IFN production through the use of specific inhibitors. For these studies, Jak/Stat inhibitors ruxolitinib and tofacitinib, and TBK1/IKKe inhibitors amlexanox and BX795 were employed. Since STING expression was suggested to impact tumor progression in certain cancers, we also examined whether wild type, STING<sup>−/−</sup> or STING<sup>M155V</sup> expressing B16 melanoma tumors differed in terms of progression and tumor immune cell infiltration. Finally,
we also investigated whether or not cGAS/STING signaling is regulated by circadian rhythms.
CHAPTER 2

MATERIALS & METHODS

2.1 Materials

2.1.1. Plasmids
In order to inspect mutant STING behavior, plasmid encoding for hSTING-M155 was purchased from Invivogen (USA). pUNO1 vector backbone carrying hSTING-M155 encodes human STING variant with gain-of-function mutation on residue 155 from valine (VAL) to methionine (MET). It encompasses the following sequences and their genetic employments: EF-1α / HTLV hybrid promoter consisting of Elongation Factor-1α (EF-1 α) and 5’UTR of the Human T Cell Leukaemia Virus (HTLV), providing enhanced expression in all cell cycles by ensuring steady-state transcription and hence increased translation efficiency. SV40 pAn is Simian Virus late polyadenylation signal which supports mutant STING mRNA stabilization. pMB1 ori site serves as the origin of replication in E. coli for plasmid amplification. By localizing upstream of blasticidin resistance gene, CMV promoter and enhancer controls blasticidin expression in mammalian cells. Similarly, EM7 promoter controls bacterial blasticidin expression. Bsr gene administers resistance to blasticidin. Human beta-Globin polyA is responsible for bsr gene stabilization without interfering with SV40 polyadenylation signal. hSTING-M155 plasmid map is shown in Appendix A. For optimization of transfection, EGFP plasmid was used due to its green reporter signal.

2.1.2. Bacterial Strain and Culture Media
To expand the hSTING-M155 gain of function variant encoding plasmid, E. coli GT116 strain [F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZM15 ΔlacX74 recA1 rspL]
(StrA) endA1 ΔsbcC-sbcD genotype] was obtained from Invivogen (USA). Fast Media Blas TB pouches consisting of terrific broth supplemented with blasticidin and Fast Media Blas Agar pouches was acquired from Invivogen (USA) and were prepared according to manufacturer’s instructions. Non-transformant E. coli were grown in Luria Broth or Luria Broth agar. Media preparation is described in Appendix B.

2.1.3. Cell Lines

Murine, C57BL/6, B16 melanoma cell line derived B16-Blue IFN-α/β Cells and STING knocked out B16-Blue ISG-KO-STING Cells were obtained from Invivogen (USA). These two cell lines were selected considering their IFN reporter activity. B16-Blue IFN-α/β is an engineered cell line through stable transfection with a SEAP reporter gene under the control of the IFN-α/β-inducible ISG54 promoter enhanced by a multimeric ISRE. B16-Blue™ IFN-α/β cells are non-responsive to IFN-γ, due to the inactivation of IFN-γ receptor. B16-Blue ISG-KO-STING cells were generated from the B16-Blue ISG cell line, by stable knockout of the STING gene. Secreted embryonic alkaline phosphatase (SEAP) is expressed under the control of the I-ISG54 promoter enhanced by a multimeric ISRE. In short, SEAP reporter activity enables monitoring of type I IFN signaling/production from the culture supernatant. Both cell lines were maintained under zeocin (100 µg/ml) selective pressure. Type I IFN levels were measured via QUANTI-Blue (Invivogen, USA)-dependent detection of secreted SEAP. B16-Blue ISG-KO-STING cell line was used to derive stably transfected hSTING-M155 expressing B16 cell line. In a different set of experiments, another variant of THP1 cell line was used: THP1-Dual (thpd-nfis), cGAS KO THP1-Dual (thpd-kocgas), STING KO THP1-Dual (thpd-kostg), were all obtained from Invivogen (USA). For SAVI and AGS in vitro model experiments, TREX KO THP1-Dual (thpd-kotrex) and STING KI N154S (thpd-s154) were used. These cell lines were obtained from Invivogen (USA). All THP1-Dual cell lines express inducible luciferase and soluble alkaline phosphatase as a result of IRF3/IRF9 activation or NF-κB activation, respectively.
2.1.4. Cell Culture Media, Buffers and Solutions
RPMI 1640 cell culture media, heat inactivated FBS, Calcium and Magnesium free Dulbecco's Phosphate Buffered Saline (DPBS), 1 M HEPES, MEM Non-Essential Amino Acids Solution (100X), Sodium Pyruvate Solution (100 mM), Penicillin/Streptomycin, Cell Culture grade water, molecular biology grade water were purchased from Biological Industries (Israel). Opti-MEM Reduced Serum Media was obtained from Life Technologies, USA. Normocin, Blasticidin QUANTI-Blue SEAP detection reagent and QUANTI-Luc luciferase detection reagent were supplied from Invivogen(USA). For B16 Blue reporter cell line group, 100 µg/ml Zeocin (Invivogen, USA) selection antibiotic was used. For stably transfected hSTING$^{M155V}$ B16 cell line, 10 µg/ml Blasticidin was added to culture medium for maintenance of hSTING$^{M155V}$ gene expression. 100 µg/ml Zeocin and 10 µg/ml Blasticidin were used for THP1 Dual cell lines. Details of cell culture media, buffers and solutions used in experiments are denoted in Appendix C.

2.1.5. Stimulants and Inhibitors
The following information indicates materials that were used in this study.

All concentrations are given for 96-well plate assay using a final volume of 200 µl. All stimulants were transfected with Lipofectamine 2000 transfection reagent (ThermoFisher Scientific, USA) through complexation with 1.5µL transfection reagent per ml media ratio.
Table 2.1. Stimulants, inhibitors and corresponding working concentrations used in B16 and B16 variant cell line experiments.

<table>
<thead>
<tr>
<th>Inhibitor/ligand</th>
<th>Description</th>
<th>Working Concentration</th>
<th>Company</th>
<th>Cat. #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amlexanox</td>
<td>TBK1/IKKe inhibitor</td>
<td>1 - 256 µg/ml</td>
<td>Invivogen (USA)</td>
<td>inh-amx</td>
</tr>
<tr>
<td>pl: C (HMW)</td>
<td>a synthetic analogue of double-stranded RNA</td>
<td>low: 0.1 µg/well high: 1 µg/well</td>
<td>Invivogen (USA)</td>
<td>tlrl-pic</td>
</tr>
<tr>
<td>pdA: dT</td>
<td>a synthetic analogue of B-DNA.</td>
<td>low: 0.1 µg/well high: 1 µg/well</td>
<td>Invivogen (USA)</td>
<td>tlrl-patn</td>
</tr>
<tr>
<td>2’3’- cGAMP</td>
<td>ligand of STING</td>
<td>10 µg/ml low: 1.5 µg/well high: 15 µg/well</td>
<td>Invivogen (USA)</td>
<td>tlrl-nacga23</td>
</tr>
<tr>
<td>HSV-60 naked</td>
<td>60 bp oligonucleotide containing viral DNA motifs</td>
<td>5 µg/ml low: 0.5 µg/well high: 5 µg/well</td>
<td>Invivogen (USA)</td>
<td>tlrl-hsv60n</td>
</tr>
</tbody>
</table>

Table 2.2. Immunosuppressive and control ODNs used in all experiments.

<table>
<thead>
<tr>
<th>ODN</th>
<th>Working Concentration</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>A151 24-mer, 5’-(TTAGGG) 4-3’)</td>
<td>3µM</td>
<td>Alpha DNA</td>
</tr>
<tr>
<td>K3-flip 20-mer, 5’-ATGCACTCTGCAGGCTTCTC-3’</td>
<td>3µM</td>
<td>Alpha DNA</td>
</tr>
</tbody>
</table>
Table 2.3. Stimulants and their working concentrations used in Dual THP1, TREX KO THP1, cGAS KO THP1, STING KO THP1, and STING KI N154S cell line experiments.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Working Concentration</th>
<th>Brand/ Source</th>
<th>catalogue code</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-60 low</td>
<td>2.5µg/ml</td>
<td>Invivogen,USA</td>
<td>tlr-l-hsv60n</td>
</tr>
<tr>
<td>HSV-60 medium</td>
<td>5 µg/ml</td>
<td>Invivogen,USA</td>
<td>tlr-l-hsv60n</td>
</tr>
<tr>
<td>HSV-60 high</td>
<td>25 µg/ml</td>
<td>Invivogen,USA</td>
<td>tlr-l-hsv60n</td>
</tr>
<tr>
<td>cGAMP low</td>
<td>5 µg/ml</td>
<td>Invivogen,USA</td>
<td>tlr-nacga23</td>
</tr>
<tr>
<td>cGAMP medium</td>
<td>15 µg/ml</td>
<td>Invivogen,USA</td>
<td>tlr-nacga23</td>
</tr>
<tr>
<td>cGAMP high</td>
<td>50 µg/ml</td>
<td>Invivogen,USA</td>
<td>tlr-nacga23</td>
</tr>
<tr>
<td>Poly dA:dT low</td>
<td>0.5 µg/ml</td>
<td>Invivogen,USA</td>
<td>tlr-patn</td>
</tr>
<tr>
<td>Poly dA:dT high</td>
<td>5 µg/ml</td>
<td>Invivogen,USA</td>
<td>tlr-patn</td>
</tr>
<tr>
<td>Poly I:C low</td>
<td>0.5 µg/ml</td>
<td>Invivogen,USA</td>
<td>tlr-pic</td>
</tr>
<tr>
<td>Poly I:C high</td>
<td>5 µg/ml</td>
<td>Invivogen,USA</td>
<td>tlr-pic</td>
</tr>
<tr>
<td>IFN-β low</td>
<td>5 ng/ml</td>
<td>REBIF44</td>
<td>clinical drug</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>not applicable</td>
</tr>
<tr>
<td>IFN-β medium</td>
<td>50 ng/ml</td>
<td>REBIF44</td>
<td>clinical drug</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>not applicable</td>
</tr>
<tr>
<td>IFN- β high</td>
<td>500 ng/ml</td>
<td>REBIF44</td>
<td>clinical drug</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>not applicable</td>
</tr>
</tbody>
</table>
HSV, cGAMP, polydA:dT, polyI:C were transfected with Lipofectamine 2000 transfection reagent (ThermoFisher Scientific, USA) through complexation with 1.5µL transfection reagent per ml media ratio. IFN-β stimulation was performed as such in the absence of any transfection.

Table 2.4. Inhibitors and corresponding working concentrations used in Dual THP1, TREX KO THP1, cGAS KO THP1, STING KO THP1, and STING KI N154S cell line experiments.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Descriptions</th>
<th>Used Working Concentrations</th>
<th>Brand/ Source</th>
<th>catalogue code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tofacitinib (CP-690550)</td>
<td>JAK3 Inhibitor</td>
<td>1 nM, 10 nM, 100 nM, 1000 nM</td>
<td>Invivogen, USA</td>
<td>tlr1-cp69</td>
</tr>
<tr>
<td>Ruxolitinib</td>
<td>JAK1 and JAK2 Inhibitor</td>
<td>10 nM, 100 nM, 1000 nM, 10 000 nM</td>
<td>Invivogen, USA</td>
<td>tlr1-rux</td>
</tr>
<tr>
<td>BX-795 hydrochloride</td>
<td>TBK1/IKKe inhibitor</td>
<td>10 nM, 100 nM, 1000 nM, 10 000 nM</td>
<td>Merck, Germany</td>
<td>SML0694</td>
</tr>
<tr>
<td>Amlexanox</td>
<td>TBK1/IKKe inhibitor</td>
<td>1 µg/ml, 4 µg/ml, 16 µg/ml, 64 µg/ml</td>
<td>Invivogen, USA</td>
<td>inh-amx</td>
</tr>
</tbody>
</table>

2.2. Methods

2.2.1. Bacterial Growth

*E. coli* were grown overnight at 37°C on agar plates. Single colonies were inoculated in 10 ml Luria Broth. Similarly, liquid cultures were grown overnight at 37°C with 200 round per minute shaking. A demand for larger volumes of liquid culture was met according to 1/100 dilution in volumes if otherwise stated.
2.2.2. Preparation of Chemically Competent Cells

Chemically competent cells were prepared from *E. coli* GT116 strain. Grown overnight culture of cells was 1/10 diluted to 200 ml Luria broth media in order to obtain a fresh culture. Once optical density reached 0.4 (0.2-0.5 range is optimal), cells were transferred to 50 ml falcons, kept on 4°C for 10 minutes and centrifuged at 3000 rpm for 10 minutes. The supernatant was removed and cell pellets were dissolved in 20% of the initial culture volume of pre-cooled TSS buffer and distributed to 4°C pre-cooled 1.5 ml tubes as 1 ml per tube. Then, each tube was submerged into liquid nitrogen for snap freezing. As such prepared chemically competent cells were stored at -80°C.

2.2.3. Plasmid Transformation of Competent Cells

Competent cells were thawed over ice and were dispersed to pre-cooled 1.5 ml tubes (100 µl/tube). 1 µg of the desired plasmid was added to tubes, gently mixed, left on ice for 30 minutes and then heated to 42°C for 30 seconds. Following heat pulse, tubes were incubated on ice for 2 minutes. Subsequently, 900 µl of SOC medium was added to each tube, incubated at 37°C with 200 round per minute shaking for 1 hour to ensure the presence of plasmid inheritor bacteria. Following these steps, Streak plate on to selective agar plate containing blasticidin was performed to attain single colonies.

2.2.4. Plasmid Isolation

Plasmid isolation was achieved by means of Macherey-Nagel (MN, Germany) plasmid purification kit. Mini-prep plasmid purification was conducted according to manufacturer’s protocol. Notwithstanding, midi-prep plasmid purification was optimized in order to enhance plasmid yield. At the 6th hour of growth (mid-log phase) of the plasmid containing *E. coli* overnight culture, 100 µg/ml Chloramphenicol (Preparation is explained in Appendix B.) was added to the culture medium. By allowing plasmid replication independent of bacterial chromosomal replication inhibition, chloramphenicol provided accumulation of
plasmid DNA in bacterial cytosol. Cultures were maintained for an additional 12 hours, followed by midi-prep plasmid purification protocol according to manufacturer’s instructions.

2.2.5. Plasmid Integrity and Contamination Test by Gel Electrophoresis
One percent agarose gel was prepared in 1X TAE and Ethidium Bromide (EtBr) was added to the gel solution shortly before decanting the gel. Plasmid DNA samples were mixed with 6X loading dye. Each lane was loaded with 20 ng plasmid DNA as such or cut with NdeI restriction enzyme (NEB, UK) that has only one cut site in the hSTING-M155 plasmid. GeneRuler DNA ladder mix (ThermoFisher Scientific, USA) was used for determining the exact size of plasmids. Agarose gel was run using the runVIEW gel system (Cleaver Scientific, UK) at 120 V and images were taken.

2.2.6. Establishment of Antibiotic Kill Curve in B16-Blue cells
B16-Blue ISG-KO-STING Cells were plated at a density of $3 \times 10^5$/well in a 6-well-plate in 1.5 ml culture medium with no antibiotics. The following day, culture media was replaced with fresh media containing assigned experimental concentrations of blasticidin. Cell culture media were renewed every 2 days without trypsin detachment of cells. Cell death after 2 weeks was detected using JuLI cell imaging microscope (Baker and Baker Ruskinn, UK).

2.2.7. Transfection with plasmid DNA and Construction of Stable Transfected Cells
Transfection of hSTING-M155 plasmid was achieved using Lipofectamine 2000 transfection reagent (ThermoFisher Scientific, USA). For transient transfections, 1.5 µl/ml lipofectamine to culture medium ratio was used. For stable transfection, optimal plasmid DNA/transfection reagent ratio was determined qualitatively based on JuLI images showing EGFP expression levels. Upon transfection of plasmid DNA, cells were incubated for 48 h without antibiotics. After 48 h, cells were
exposed to selective antibiotic pressure for 2 weeks. Polyclonal colonies were subjected to expansion under selective pressure.

2.2.8. Counting of Cells
For cell counting, both flow cytometry and manual counting through a haemocytometer were used. In the flow cytometric method, live cell populations were gated according to their FSC/SSC characteristics and the number of cells in a specific volume was determined. For the haemocytometer-based method, re-suspended cells were diluted with 1X PBS accordingly and dilution factor was noted. Then, the diluted sample was mixed with Trypan Blue (Cat.# 17-942E, Lonza, Switzerland) at 1:1 ratio. This is required for exclusion of dead cells from the count. 10 µL of mixed cell suspension was loaded onto a haemocytometer. Cells were counted from the area depicted in black in Figure 2.1 under 40X magnification and total cell number was calculated as shown below:

Total cell count= (counted cells in 4 of red squares /4) X10^4 X (Dilution factor) X 2 X (1 ml)

![Figure 2.1. Representative image of the Area used for counting of cells in a haemocytometer.](image)

2.2.9. Detection of Type I IFN Production using the Quanti-BLUE Reagent
20 µl of cell culture supernatants from unstimulated or stimulated wild type, knock-out or mutant cells were combined with 180 µl of QUANTI-Blue in 96-well-plates.
After 4 hour incubation at 37°C in the dark, colorimetric measurements were taken at 645 nm with a Multiskan GO Microplate Spectrophotometer (ThermoFisher Scientific, USA).

2.2.10. Flow Cytometric Analysis of Samples

Protocols regarding flow cytometric analysis include cell fixation and intracellular STING staining and are explained separately below. Results were analyzed using a BD (USA) Accuri C6 or Acea Biosciences (USA) Novocyte flow cytometer.

2.2.10.1. Cell Fixation

Ensuring the end of indicated incubation periods, cells were centrifuged at 300xg for 10 minutes to obtain cell pellets. Cell fixation was performed by dropwise addition of 100 µL Fixation Medium, (Medium A, ThermoFisher Scientific, USA) while vortexing at the same time. Next, cells were incubated for 15 minutes at room temperature. Once incubation period ended, cells were washed with 1 ml FACS buffer, followed by centrifugation as before. Supernatants were discarded through aspiration and pellets were re-suspended in FACS Buffer for later use (max. 1 week at 4°C) or pellets were stained with antibody as denoted in the following section.

2.2.10.2. Intracellular STING Staining

After fixation, sample pellets were disrupted by vortexing. For intracellular STING staining, STING (D2P2F) rabbit monoclonal antibody (Cat. # 13647) was purchased from Cell Signalling Technologies, USA. 5 µg/ml STING antibody was diluted in 100 µL Permeabilization Medium, (Medium B, ThermoFisher Scientific, USA) and added onto the pellet. Following vortexing, samples were incubated at room temperature for 30 minutes in the dark and washed with FACS buffer. Secondary antibody, anti-rabbit AlexaFluor 488 conjugated antibody (Becton Dickinson, USA) was diluted to 1 µg/ml in 100 µL Medium B and transferred to
sample tubes. Following incubation for 30 minutes at room temperature in the dark, cells were washed with FACS buffer and re-suspended in PBS.

2.2.11. Cold Induction Experiments
All cold-induced samples were incubated at +4°C. Cold exposure duration was designated as 30 minutes or 1 hour or 2 hours depending on experimental setup.

2.2.12. Determination of Protein Concentration
Prior to Western Blot, the protein concentration of cell lysates was determined by Micro BCA protein assay kit (ThermoFisher Scientific, USA) According to manufacturer’s instructions. BSA standard dilutions were adjusted to range between 40 µg/ml to 0.5 µg/ml as follows: 40, 20, 10, 5, 2.5, 2, 1, 0.5, 0. All standards and experimental samples were prepared as duplicates. To obtain more precise measurements, 1:60 and 1:30 diluted samples were used instead of a single dilution. All samples were transferred to a 96-well-plate and mixed with working reagent at a 1:1 volumetric ratio. Samples were incubated at 37°C for 2 hours, allowed to cool down to room temperature for 10 minutes and the absorbance values of standards and lysates were measured at 562 nm on a Multiskan GO Microplate Spectrophotometer (ThermoFisher Scientific, USA). The concentrations of lysates were estimated using the BSA standard curve.

2.2.13. Western Blot
Immunoblotting was performed to study the expression levels of the protein of interest. To observe monomeric versus activation-induced dimeric forms of proteins, non-reducing and reducing SDS-PAGE was used, respectively. Appendix D describes the preparation of buffers and solutions used in this section. Control or treated cells were washed with PBS and lysates were collected by scraping in cold lysis buffer. Following 30 seconds of sonication, extracts were centrifuged for 10 min at 14000xg in a cold centrifuge. Supernatants were removed and total protein concentrations were determined by Micro BCA protein assay kit (ThermoFisher Scientific, USA) as previously described. Extracts were kept at -80°C until further
Before loading of samples on the gel, samples were heated to 95°C for 5 minutes in Laemmli buffer and then cooled on ice. Equal amount and volume of each sample was loaded into wells (10 µg or 20 µg total protein in 10-20 µl volume). For gel casting, TGX Stain-Free FastCast Acrylamide Kit, 10% (Cat. #161-0183, Bio-Rad, USA) was assembled as stated in manufacturer’s protocol. PageRuler Prestained 10-180 kDa (Cat. # 26616, ThermoFisher Scientific, USA) was used as marker for size separation. After loading of wells with samples, the gel was run at 200 V on a BioRad Miniprotean Tetra Vertical Electrophoresis Cell (USA). Subsequently, proteins on the gel were semi-dry transferred to a nitrocellulose membrane at +4°C, applying 30 V for 1 hour 15 min. Membranes were blocked in blocking buffer for 1 hour at room temperature and washed 4 times with TBST, 1,1,2 and 3 minutes respectively. Following incubation in primary antibody for 1 hour at room temperature or overnight at +4°C, membranes were washed 4 times with TBST again. Membranes were then incubated in HRP-linked secondary antibody for 1 hour at room temperature or overnight at +4°C followed by washing in TBST 4 times as before. For development, SantaCruz Western Blotting Luminol Reagent (sc-2048, USA) was mixed at equal volumes as instructed. Membrane images were taken and analysed using a BioRad (USA) ChemiDoc imaging system.
Table 2.5. List of antibodies used in western blot.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Origin and Isotype</th>
<th>Dilution in BB</th>
<th>Brand</th>
<th>Cat. #</th>
</tr>
</thead>
<tbody>
<tr>
<td>STING (D2P2F)</td>
<td>Rabbit IgG</td>
<td>1:1000</td>
<td>CST, Cell Signaling Technologies(USA)</td>
<td>13647</td>
</tr>
<tr>
<td>Phospho-IRF-3 (Ser396)</td>
<td>Rabbit IgG</td>
<td>1:1000</td>
<td>CST, Cell Signaling Technologies(USA)</td>
<td>29047</td>
</tr>
<tr>
<td>IRF-3 (D83B9)</td>
<td>Rabbit IgG</td>
<td>1:1000</td>
<td>CST, Cell Signaling Technologies(USA)</td>
<td>4302</td>
</tr>
<tr>
<td>β-actin (8H10D10)</td>
<td>Mouse IgG2b</td>
<td>1:2000</td>
<td>CST, Cell Signaling Technologies(USA)</td>
<td>3700</td>
</tr>
<tr>
<td>Anti-rabbit HRP conjugate 2nd Ab</td>
<td>Goat</td>
<td>1:2000</td>
<td>Dako, Agilent Technologies (USA)</td>
<td>P0448</td>
</tr>
<tr>
<td>Anti-mouse HRP conjugate 2nd Ab</td>
<td>Horse</td>
<td>1:2000</td>
<td>CST, Cell Signaling Technologies(USA)</td>
<td>7076</td>
</tr>
</tbody>
</table>

2.2.14. *in Vivo* Experiments

2.2.14.1 Animal Handling

12 weeks old female C57BL/6 mice were maintained in the Bilkent University Animal Housing Facility of the Department of Molecular Biology and Genetics for *in vivo* experiments. Housing conditions for all mice were 22°C with 12-hour light and 12-hour dark cycles. Mice had had unlimited access to water and food. Experimental procedures were approved by the animal ethical committee of Bilkent University.

2.2.14.2. Tumor Injection, Tumor Size Measurements and Preparation of Tumor-infiltrating Single Cells

To investigate how STING-expression impacted tumor development, 3 experimental groups (n=5) were formed: B16<sup>wt</sup>, STING<sup>-/-</sup> B16 and human
STING<sup>M155V</sup> expressing B16 cells (5x10<sup>5</sup> in 100 µl PBS) were injected subcutaneously into shaved backs of mice. Tumor development was monitored on a regular basis using a caliper and tumor volumes (length* height* width) were recorded in mm<sup>3</sup>. On day 18, mice were euthanized by cervical dislocation. To determine the distribution of immune cell subsets in the tumor, tumors were first minced with scalpels into small fragments and transferred into 50 ml falcons. 1 ml Liberase (Cat. # 5401160001, Roche, Switzerland) was added to tumor tissue suspension and incubated for 30 minutes at 37 °C. Samples were filtered using a 40 µm strainer, washed with 1X PBS, and cell pellets were suspended with 2 ml ACK lysis buffer (Cat. #10-54BE, Lonza, Switzerland) for 1 minute to deplete red blood cells. Samples were washed in 18 ml wash medium two times. Tumor-infiltrating cell phenotypes were determined using specific markers of Treg and/or TAMs (Tumor Associated Macrophage).

**2.2.14.3. Treg and Tumor Associated Macrophage panel staining**

Tumor-infiltrating cells were fixed, permeabilized and incubated with Zombie Aqua Fixable Viability Kit (Live/Dead), (Cat #423102, Biolegend, USA) to gate live cell population and TruStain fcX (anti-mouse CD16/32) (Cat. # 101319, Biolegend, USA) at 1:100 dilution for blocking non-specific binding of immunoglobulin to the Fc receptors. After washing samples, antibody cocktails (in 100 µL FACS buffer/sample) were prepared as described in Tables 2.4 and 2.5 for Treg and TAM-specific markers, respectively. Single-stained samples were also prepared for compensation adjustment of flow cytometer. Samples were incubated with the antibody mixtures for 30 minutes in dark and washed. Then, samples were analysed on a Novocyte flow cytometer equipped with Violet, Blue and Red lasers. All antibodies, regarding brands, catalogue numbers, clone, working dilution factors are depicted in table 2.6. and table 2.7. for Treg and TAM panels, respectively.
Table 2.6. List of antibodies and their fluorophores used in Treg Panel.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Expressed Population</th>
<th>Brand</th>
<th>Cat. #</th>
<th>Clone</th>
<th>DF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3-PE</td>
<td>T cells (killer, helper, regulatory)</td>
<td>Biolegend (USA)</td>
<td>100206</td>
<td>17A2</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD4-APC</td>
<td>Th, DC</td>
<td>Biolegend (USA)</td>
<td>100516</td>
<td>RM4-5</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD25-PeCy5</td>
<td>Th, MO, Mφ</td>
<td>Biolegend (USA)</td>
<td>102010</td>
<td>PC61</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD45-RF710</td>
<td>leukocytes</td>
<td>Tonbo (USA)</td>
<td>80-0451-U025</td>
<td>30-F11</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table 2.7. List of antibodies and their fluorophores used in TAM Panel.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Expressed Population</th>
<th>Brand</th>
<th>Cat. #</th>
<th>Clone</th>
<th>DF</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4/80-FITC</td>
<td>Mφ</td>
<td>Biolegend (USA)</td>
<td>123108</td>
<td>BM8</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD11b-APC</td>
<td>MO, Mφ, NK, neutrophils, granulocytes</td>
<td>Biolegend (USA)</td>
<td>101211</td>
<td>M1/70</td>
<td>1:1000</td>
</tr>
<tr>
<td>Gr1-PE (Ly-6G/Ly-6C)</td>
<td>a subset of MO, Mφ, neutrophils, eosinophils</td>
<td>Biolegend (USA)</td>
<td>108408</td>
<td>RB6-8C5</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD3-PE</td>
<td>T cells</td>
<td>Biolegend (USA)</td>
<td>100206</td>
<td>17A2</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD19-PE</td>
<td>B cells</td>
<td>Biolegend (USA)</td>
<td>115508</td>
<td>6D5</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD206-PerCPCy5.5</td>
<td>Mφ</td>
<td>Biolegend (USA)</td>
<td>141716</td>
<td>C068C2</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD45-RF700</td>
<td>leukocytes</td>
<td>Tonbo (USA)</td>
<td>80-0451-U025</td>
<td>30-F11</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
2.2.15. Circadian Rhythm Experiments

For circadian rhythm experiments, cells were synchronized in culture medium containing 0.1 μM dexamethasone (Invivogen, USA, cat code: tlrl-dex) for 2 hours. Following fresh medium replacement devoid of dexamethasone, cells were distributed to 96-well plates at a density of $10^6$ cell/ml. Cells were then transfected with intracellular ligands using 0.3 μL/well Lipofectamine 2000 (ThermoFisher Scientific, USA) or stimulated with IFN-β (REBIFF44) without transfection as explained in Table 2.3. Synchronized cells were stimulated at 4 hour intervals for the duration of 48 hours and culture supernatants were collected 12h after each stimulation time point. Supernatants were assayed for Quanti Luc luciferase IFN reporter assay and Quanti Blue SEAP NF-κB reporter assay as described in 2.2.16 and 2.2.17.

2.2.16. Quanti Luc luciferase IFN expression reporter assay for Dual Cell Lines

After collection of cell supernatants, 10 μL of each sample was transferred to white 96-well plates (Costar, USA, cat #3912). Luciferase readings were conducted by Turner BioSystems Modulus micro-plate reader. Luminometer parameters were set as 50 μL per injection, 4-second delay and 0.1-second integration time. Quanti-Luc solution was prepared according to manufacturers’ instructions and was transferred to the Turner BioSystems Modulus micro-plate reader. Injection per well was executed by reader automatically.

2.2.17. Quanti Blue SEAP NF-κB activation reporter assay for Dual Cell lines

After collection of cell supernatants, 20 μL of each sample was transferred to flat bottomed 96-well plates. 180 μL of Quanti Blue solution was introduced to each well. Following 4-hours of incubation at 37°C, OD readings were acquired using a Multiskan GO Microplate Spectrophotometer (ThermoFisher Scientific, USA) at 645 nm.
2.2.18. Statistical Analysis

Graphical representations and statistical analysis were conducted using the GraphPad Prism 5 software (GraphPad Software Inc., USA). Two-tailed Mann Whitney U test was used to define significantly higher IFN producing group compared to wild type. Mantel-Cox test was applied to test any significant difference in Kaplan-Meier Survival Curve. Kruskal Wallis was used to detect any significance between Treg percent distribution and M1-M2 macrophage polarization distributions in tumor lysates. For all comparisons, P values of <0.05 were considered significant. For indication of the significance, P values of <0.05 denoted with *, P values of <0.01 denoted with **, P values of <0.001 denoted by ***, P values of <0.0001 denoted by ****.
CHAPTER 3

RESULTS & DISCUSSION

Type I interferonopathies represent a group of diseases characterized by elevated type I interferon signature. Interferon secretion is an evolutionary conserved response to viral invasion in healthy individuals predominantly induced by presence of cytosolic DNAs and RNAs of microbial origin. In the case of type I interferonopathies, persistent type I IFN production stems from aberrant accumulation of self-nucleic acids in cytoplasm and/or mutations causing chronic activation of nucleic acid sensing pathways. In this thesis, we aimed to generate in vitro models of two type I interferonopathies to study pathogenic disease mechanisms and to test the activity of various inhibitors that might be used in the clinic. One disease we concentrated on was stimulator of IFN genes (STING)-associated vasculopathy with onset in infancy (SAVI). In healthy individuals, the adaptor protein STING that is encoded by TMEM173 gene, binds the second messenger cGAMP and is activated to trigger downstream signaling pathways, culminating in type I IFN production. However, in SAVI patients, gain of function mutations in STING protein results in adoption of the active dimeric form regardless of cGAMP presence, leading to constitutive signal transduction and hence spontaneous type I IFN production. Defined mutations in TMEM173 include M155V, N154S, V147L (Y. Liu et al., 2014). To delineate spontaneous type I IFN producing intracellular signal transduction mechanism of SAVI pathology, disease-associated human gain-of-function variant of STING M155V (STING<sup>M155V</sup>) encoding plasmid was stably transfected into STING KO B16 Blue cell line.
(STING\textsuperscript{−/−} B16). Our attempts to generate a similar mutant cell line in human THP-1 cells was unsuccessful (data not shown), owing to the difficulties of transfecting this non-adherent cell line. Therefore, due to its adherent characteristics, easy to transfect feature, availability of a STING knockout variant and given the presence of IFN monitoring reporter system, we decided to use the STING KO B16 Blue cell line (STING\textsuperscript{−/−} B16) to express the STING\textsuperscript{M155V} as an \textit{in vitro} SAVI model. Selection, maintenance and transfection optimization for stable cell line generation is described in the following Sections.

3.1. Generation of Antibiotic Selection Kill curve

In order to determine optimal antibiotic concentration for selection and maintenance of stably transfected cell line, prior to plasmid transfection, a kill curve was constructed. For this, differential antibiotic concentrations ranging from 1 to 32 µg/ml with a 2-fold increase was tested. Cell viability against blasticidin concentration was assessed according to bright field microscopy images as shown in the Figure 3.1.

One week after blasticidin addition, 16µg/ml and 32 µg/ml blasticidin added groups showed no viability (Images are not shown.) After two weeks, 8, 16, and 32 µg/ml blasticidin added groups showed no cell viability. Thus, optimal blasticidin concentration for selection and maintenance was set as 10 µg/ml.
Figure 3.1. Viability of STING−/− B16 cell line incubated with various concentrations of blasticidin.

STING−/− B16 cells were seeded at a concentration of 6x10^4/well into 48-well-plates. Prior to antibiotic addition, cells were allowed to reach 70-80% confluency (24h incubation). Next day, culture medium was replaced with medium containing the corresponding blasticidin concentration. Culture media was then renewed every other day. Bright field images were taken after 14th day.

3.2. Optimization of Plasmid DNA Transfection

To establish optimal transfection efficiency, different plasmid DNA and transfection reagent ratios were tested (Figure 3.2.). For these experiments, to evaluate transfection efficiency, an enhanced green fluorescent protein (EGFP) expression plasmid was used. Expression levels were determined qualitatively using fluorescence microscopy. Optimal plasmid and transfection reagent concentration was chosen based on maximum transfection efficiency and minimal expenditure of plasmid DNA and transfection reagent. Based on data presented in
Figure 3.2, optimum ratio was determined to be 2µg plasmid/ 4µL Lipofectamine 2000 transfection reagent (Lipo2000).

**Figure 3.2.** Optimization of plasmid DNA transfection using EGFP expression plasmid and Lipo2000 transfection reagent in STING<sup>−/−</sup> B16 cells.

*STING<sup>−/−</sup>B16 cells were plated at a density of 10<sup>5</sup> cells/well in 12-well-plates. Prior to transfection, cells were allowed to reach 70-80% confluency (36 h incubation). Binary combinations of 2, 4 or 8 µg EGFP expression plasmid and 1 2 or 4 µL*
Lipofectamine 2000 transfection reagent were used in transfection experiments. All images were taken 24h post-transfection.

3.3. Generation of human STING$^{M155V}$ expressing B16 Blue cell line.

Once transfection efficiency and transfected cell line maintenance parameters were optimized, pUNO1-hSTING-M155 plasmid was transfected to STING$^{-/}$ B16 cell line. To assess gain of function mutant STING protein expression, transfected cells were intracellularly stained with human STING-specific antibody and analyzed by flow cytometry. As expected, pUNO1-hSTING-M155 plasmid transfected STING$^{-/}$ B16 cell line stably expressed human STING$^{M155V}$ protein. As shown in Figure 3.3.a., a 3-fold higher FITC mean fluorescent intensity was observed in human STING-M155V transfected STING$^{-/}$ B16 when compared to STING$^{-/}$ B16 cell line. As indicated in Figure 3.3.b., the transfection efficiency was 76.43%.
Figure 3.3. Evaluation of STING expression following transfection of STINGM$^{155V}$ expression plasmid into STING$^{-/-}$ B16 Cell Line.

STING$^{-/-}$ B16 cell line was transfected with the pUNO1-STINGM155V plasmid according to the optimized protocol. Following two weeks of blasticidin selective pressure, polyclonally expanded transfected cells vs non-transfected cells were
compared for STING expression. a. shows MFI of STING-specific staining. b. percent of STING expressing cells following flow cytometric analysis.

3.4. Functional Assessment of STING\textsuperscript{M155V} B16 Cell Line

Having shown that stably transfected cell line constitutively expressed gain of function variant STING\textsuperscript{M155V} protein, we next assessed whether the mutant protein expressing cells constitutively produced type I interferons and therefore can be utilized as a model of SAVI (Junt & Barchet, 2015; Liu et al., 2014; Gul et al., 2017).

3.4.1. Spontaneous Type I IFN secretion profile of STING\textsuperscript{M155V} B16 Cells

Previous research showed that SAVI patients’ cells spontaneously produced high levels of type I IFNs (Gul et al., 2017; Y. Liu et al., 2014). To test whether STING\textsuperscript{M155V} B16 cells replicated these findings and could be considered as an \textit{in vitro} SAVI model, basal levels of type I IFN production of wild type STING (STING\textsuperscript{wt}), STING knock out (STING\textsuperscript{−/−}) and mutant STING (STING\textsuperscript{M155V}) expressing B16 Blue IFN reporter cells were determined at different cell densities. As shown in Figure 3.4.a., at a density of 4\times10^5 cells/ml, STING\textsuperscript{M155V} cells showed 1.5-fold more type I IFN production when compared to STING\textsuperscript{wt} or STING\textsuperscript{−/−} cells, although the difference was not statistically significant. In contrast, at higher cell densities, STING\textsuperscript{M155V} cells secreted significantly higher concentrations of type I IFN (Figure 3.4.b. and Figure 3.4.c; P<0.01 when compared to STING\textsuperscript{wt} or STING\textsuperscript{−/−}). These findings suggest that STING\textsuperscript{M155V} cell line has sufficient functional characteristics to be considered as an \textit{in vitro} model of SAVI, allowing testing of inhibitory agents for mitigating spontaneous type I interferon production.
Figure 3.4. Assessment of SEAP reporter activity-based spontaneous IFN production in STING\textsuperscript{wt}, STING\textsuperscript{-/-}, STING\textsuperscript{M155V} B16 cell lines. STING\textsuperscript{wt}, STING\textsuperscript{-/-}, STING\textsuperscript{M155V} cells were seeded at a. 4x10\textsuperscript{5}, b. 8x10\textsuperscript{5}, c. 1.6x10\textsuperscript{6} cells/ml densities. Samples were incubated for 24h at 37\textdegree C. Supernatants were collected for Quanti Blue IFN reporter assay. Data are presented as individual data points of three independent experiment and group means with SD. Statistical comparisons between groups were based on Two-tailed Mann Whitney U test. ** indicates p<0.01.

3.4.2. Determination of Responsiveness of STING\textsuperscript{M155V} B16 Cells to Stimulation with Intracellular Nucleic Acid Ligands

Having established that STING\textsuperscript{M155V} cell line had favorable functional properties to be considered as a SAVI in vitro model, we next assessed the cytosolic nucleic acid sensing response of STING\textsuperscript{M155V} B16 cells in comparison to the wild type and knock out cell lines. For this purpose, HSV60, cGAMP, polydA:dT and polyI:C stimulation-dependent type I IFN secretion was screened and compared among STING\textsuperscript{wt}, STING\textsuperscript{-/-}, STING\textsuperscript{M155V} B16 cell lines. HSV60 is an oligonucleotide containing Herpes Simplex Virus 1 DNA motifs. It induces type I IFN production in a TLR-, DAI- and RNA Pol III-independent, but cGAS-STING-dependent manner (Unterholzner et al., 2010). 2’3’-cGAMP is a STING agonist, inducing STING dimerization and subsequent type I IFN production (Bhat & Fitzgerald, 2014). polydA:dT is another dsDNA oligonucleotide, containing repetitive synthetic of polydA:dT sequence and is used as a synthetic analogue of B-DNA, inducing type I IFN production in an RNA pol III-dependent manner. RNA pol III
synthesises dsRNA with a 5’-triphosphate moiety (5’ppp-dsRNA) using polydA:dT as a template. 5’ppp-dsRNA is recognized by the cytosolic RNA sensor RIG-I, which initiates type I IFN production through TBK1-IRF3 axis (Ablasser et al., 2009). PolyI:C is a synthetic analogue of double-stranded RNA (dsRNA). It induces NF-κB through TLR3 (Alexopoulos, Czopik Holt, Medzhitov, & Flavell, 2001) and IRF3 activation through RIG-I/MDA5 axis, resulting in type I IFN production (Kato et al., 2006).

Figure 3.5. Type I IFN response of STING<sup>wt</sup>, STING<sup>-/-</sup>, STING<sup>M155V</sup> B16 cells to stimulation with nucleic acid receptor agonists.

**STING<sup>wt</sup> B16 (a), STING<sup>-/-</sup> B16 (b), STING<sup>M155V</sup> B16 (c)** cells were seeded at a density of 8x10<sup>3</sup> in 96-well plates. Samples were stimulated with low and high concentrations of HSV60 (low: 0.5 µg/well; high: 5 µg/well), cGAMP (low: 1.5 µg/well; high: 15 µg/well), polydA:dT (low: 0.1 µg/well; high: 1 µg/well), polyI:C (low: 0.1 µg/well; high: 1 µg/well). Samples were incubated for 24h at 37°C. Supernatants were collected for Quanti Blue IFN reporter assay. Basal levels of
type I IFN production in unstimulated cell lines are shown in (d). Bar graphs show mean and SD of two biological replicates of three independent experiments.

Analysis of response of STING\textsuperscript{M155V} cells to stimulation with cytosolic nucleic acid ligands revealed that these cells had more robust type I IFN production to low dose HSV and high dose of cGAMP, whereas stimulation with RNA-sensor-dependent ligands polydA:dT and polyI:C yielded similar IFN production levels when compared to STING\textsuperscript{−/−} and STING\textsuperscript{wt} B16 cells (Figures 3.5 a-c). As expected, STING\textsuperscript{−/−} B16 cells were unresponsive to HSV60 and cGAMP stimulation, since these two ligands activate STING dependent signal transduction. Type I IFN responses of STING\textsuperscript{−/−} B16 cells to intracellular RNA-sensor ligands polydA:dT and polyI:C was uncompromised (Figure 3.5.b.). The trend in type I IFN response of STING\textsuperscript{M155V} cell line against HSV60 or cGAMP stimulation (Figure 3.5.c.) is different from the response of STING\textsuperscript{wt} B16 cells (Figure 3.5.a.). While STING\textsuperscript{wt} shows elevated type I IFN response to increasing concentration of HSV60 stimulation, minimal HSV60 stimulation is sufficient for avid type I IFN levels in STING\textsuperscript{M155V} B16 cells. Of note, increasing cGAMP concentration resulted in decreased type I IFN levels in STING\textsuperscript{wt} B16, whereas, STING\textsuperscript{M155V} B16 cells continued to produce high levels of type I IFNs in the presence of excess cGAMP (Figure 3.5.c.). Whether this difference stems from a dysregulated negative regulation mechanisms in the STING-TBK1-IRF3 axis in the SAVI model, remains to be elucidated.

3.4.3. Testing of potential inhibitors to suppress spontaneous Type I IFN secretion in STING\textsuperscript{M155V} B16 Cells

Herein, the aim was to inspect the immunosuppressive potential of candidate molecules to mitigate spontaneous type I IFN production in STING\textsuperscript{M155V} B16 Cells. Our first candidate inhibitor was A151. A151 is a suppressive oligonucleotide (ODN) containing mammalian telomeric repetitive TTAGGG motifs, that showed promising pre-clinical effects against many inflammatory diseases (Gursel et al.,
Therefore, experiments were designed to test the ODN A151’s suppressive effect in the in vitro SAVI model (Figure 3.6).

Contrary to our expectation, 3 µM dose of A151 did not show significant inhibitory effect on elevated type I IFN signature of STING\(^{M155V}\) B16 cells (Figure 3.6.).

3.4.4. Cold Induced Type I IFN exacerbation in STING\(^{M155V}\) B16 Cell Line

Next, we wanted to analyze the effect of cold exposure on spontaneous type I IFN production by STING\(^{M155V}\) B16 in comparison to STING\(^{wt}\), STING\(^{-/-}\). Previous research has shown that SAVI patients have cold induced flares (de Jesus, Canna, Liu, & Goldbach-Mansky, 2015). We wanted to test that whether these cold induced flares are associated with escalation of spontaneous type I IFN production. Provided that cold exposure would enhance type I IFN secretion, in these
experiments we also wanted to test the utility of a potent TBK1/IKKε inhibitor: Amlexanox. This drug was previously proven to inhibit metabolic inflammation in animal models, (Reilly et al., 2013). Since this inhibitor was never tested in the context of SAVI, we hypothesized and therefore wanted to evaluate Amlexanox as a good drug candidate for alleviation of symptoms of SAVI.

**Figure 3.7. Experimental Setup of Cold Induction**

As indicated in Figure 3.7., STING\textsuperscript{wt} B16, STING\textsuperscript{-/-} B16, STING\textsuperscript{M155V} B16 cell lines were divided into two groups: cold induction and always 37°C groups. Following plating of cells and allowing them to attach to the bottom of the wells (1 to 2 hours), cells were either untreated or were treated with various concentrations of Amlexanox (ranging from 1 to 256 µg/ml by 4-fold increments) at 37°C for 1 hour. Then, each cell line was further subdivided into two groups. One group was left untreated and the other one was transfected with 10 µg/ml cGAMP. Next, “cold induction groups” were incubated at 4°C for 1 hour followed by 22-hours incubation at 37°C. In contrast, “always 37°C groups” were just incubated for 23 hours at 37°C. At the end of the incubation period, cell supernatants were collected and subjected to Quanti Blue assay. Results revealed that cold-induced exacerbation in type I IFN production compared to STING\textsuperscript{wt} B16 and STING\textsuperscript{-/-} B16 in response to cGAMP stimulation (Figure 3.8.a.). Cold exposure had no effect on type I IFN levels in STING\textsuperscript{wt} and STING\textsuperscript{-/-} B16 cell lines (Figure 3.8.a.). For STING\textsuperscript{M155V} B16 cells, the inhibitory efficacy of Amlexanox was less pronounced.
in cold exposed cells when compared to the “always 37°C” group. Calculated IC$_{50}$ values were 20.63 (R$^2$=0.81) and 32.85 (R$^2$=0.94) for “always 37°C group” and “cold induced group”, respectively (IC$_{50}$ values were calculated according to the equation of the polynomial best line and R$^2$ as described in APPENDIX E). These results suggest that following cold exposure, higher doses of Amlexanox might be required to dampen spontaneous type I IFN production. A similar strategy of increasing the dose of the clinically suitable inhibitor can be utilized to prevent the cold-induced flares of patients. In summary, we hypothesize that higher doses of Amlexanox might alleviate the cold exacerbated symptoms of SAVI.

Since the amount of spontaneously produced type I in IFN STING$^{M155V}$ B16 cells in the absence of cGAMP stimulation was only slightly higher than background levels of wild type cells, detecting the effect of cold-exposure was not possible due to the sensitivity limitations of the assay (Figure 3.8.b.).

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**Figure 3.8.** Effect of Amlexanox on cold-induced spontaneous type I IFN expression in B16 STING variant cell lines.
**STING**

**wt**, **STING**−/−, **STING**M155V cells were seeded at a density of 8x10^5 cells/ml. All cell lines were treated with 1, 4, 16, 64, or 256 µg/ml of Amlexanox concentrations and incubated at 37°C for 1 hour. Next, each cell line was subdivided into two groups. One group was transfected with 10 µg/ml cGAMP (a.) and the other one was left untreated (b.). Then, while “cold induction groups” were incubated at 4°C for 1 hour followed by 22-hour 37°C incubation, “always 37°C groups” were incubated for 23 hours at 37°C. Supernatants were then collected for Quanti Blue Assay. Mean of two duplicates and SD values taken from one of three independent experiments with similar results was shown.

Since we saw a small difference in **STING**M155V B16 cell line in response to cold induction, we wanted to delineate the mechanism that might be responsible for the enhanced type I IFN production under cold conditions. Considering type I IFN production is affected by STING activation and positive autocrine mechanism through IFNAR (Interferon α/β receptor)-JAK/STAT pathway, we wanted to investigate the contribution of these pathway to the cold-induced augmentation of IFN production.

For this purpose, we designed an experimental set up as shown in the Figure 3.9. Experimental groups were designated as “always 37°C”, “stimulation-cold stress”, “cold stress-stimulation” to indicate post- or pre-cold exposure conditions with respect to the cGAMP stimulation time point, respectively.

Next, we wanted to investigate whether activation and expression of STING was affected by cold stress. For this, amounts of total STING and phosphorylated active form of STING (pSTING) were analyzed by immunoblotting in samples treated as described in Figure 3.9. The anti-human STING antibody had a lower specificity towards mouse STING in wild type B16 cells (Figure 3.10.a.). Therefore, we were not able to discern STING activation in this cell line. As expected, **STING**−/− cells did not express the adaptor protein.
Figure 3.9. Experimental setup to investigate the mechanism of cold-induced STING activation.

In contrast, consistent with previous results, STING^{M155V} B16 cells had high levels of STING and p-STING expression (Figure 3.10.a.). Comparison of total and phospho-STING expression levels between “always 37°C”, “stimulation-cold stress” and “cold stress-stimulation” groups revealed that exposure to cold prior to cGAMP stimulation upregulated both p-STING and total STING levels. Furthermore, “stimulation-cold stress” group had also higher p-STING and STING levels than “always 37°C” group, although this effect was less pronounced than the “cold stress-stimulation” group (Figure 3.10. a. and b.). Taken together, these results imply that cold induction increases STING expression and activation in STING^{M155V} B16 cells. Hence, cold-induced escalation of type I IFN upregulated may stem from increased STING expression and therefore enhanced activation in the case of gain-of-function mutations. How cold induction upregulates STING expression remains to be elucidated. One possible mechanism could be related to the expression of CIRP (Cold inducible RNA binding protein). CIRP is a cold-shock protein which can be induced after exposure to a mild cold-shock. Having role in regulation of mRNA stability through its target sites and ER stress (Zhong & Huang, 2017), a-CIRP-related mechanism could be responsible for upregulation of cold-induced STING expression.
Figure 3.10. Western Blot images of p-STING, total STING and their corresponding semi-quantitative protein expression levels.

STING\textsuperscript{wt} B16, STING\textsuperscript{-/-} B16, STING\textsuperscript{M155V} B16 cells were divided into three groups: always 37°C group, “stimulation-cold stress” group, and “cold stress-stimulation” group. After cell seeding, “always 37°C group” and “stimulation-cold stress” groups were transfected with 10 µg/ml cGAMP. While “always 37°C group” was incubated only at 37°C for 1 hour 30 minutes, “stimulation-cold stress” cells were kept at 37°C for 30 minutes and were then exposed to 4°C cold stress for 30 minutes followed by another 30 minutes of incubation at 37°C. “Cold stress-stimulation” group was first exposed to cold stress for 30 minutes, followed by transfection with 10 µg/ml cGAMP stimulation and another 1 hour 30 minutes incubation at 37°C. At the end of the incubation periods, whole cell lysates were prepared with RIPA buffer. \textbf{a.} Immunoblots with long and short exposure times for STING, phosphorylated-STING and β-actin band intensities are displayed. \textbf{b.} Graphical presentation of band pixel densities were normalized to β-actin. Band pixel densities were measured using the BioRad Image Lab software.

3.5. The Effect of STING mediated Type I IFN secretion on Tumor Progression

Apart from its crucial role in nucleic acid sensing, STING has a critical role in tumor development. Since type I IFNs contribute to anti-tumor immunity driven through the recruitment of CD8\textsuperscript{+} cytotoxic T cells, STING activation was
previously shown to exert an anti-tumor effect (Pépin & Gantier, 2017). However, evidence also suggests that STING activation can trigger tumor development (He et al., 2017), possibly through angiogenesis and proliferation inducing effects of STING-stimulated cytokine, chemokine and growth factor(s) (Barber, 2015). Conversely, STING agonist mediated type I IFN signal can initiate an anti-tumor effect in a variety of tumor models (Downey, Aghaei, Schwendener, & Jirik, 2014). Taken together, STING activation can have opposing effects in tumor development and the role of STING may be tumor type-dependent (Barber, 2011, 2015; Brencicova & Diebold, 2013; Deng et al., 2014; He et al., 2017; Xia, Konno, Ahn, & Barber, 2016).

To clarify the role of STING activation in tumor progression, mice were challenged with wild type, knockout or gain-of-function STING expressing B16 melanoma cells. For this, STING\textsuperscript{wt} B16, STING\textsuperscript{−/−} B16 and STING\textsuperscript{M155V} B16 cell lines (5x10\textsuperscript{5} cells in 100 µl PBS) were injected subcutaneously into dorsal flanks of 12 weeks old female C57BL/6 mice (n=5 per group). All mice were kept at 22°C with 12-hour light and 12-hour dark cycles and had unlimited access to water and food. Tumor development was monitored on a regular basis. On day 18, mice were euthanized by cervical dislocation, tumors were excised and analyzed for tumor-infiltrating cell types.
Figure 3.11. Tumor volumes and Kaplan-Meier percent survival curves of STING\(^{wt}\), STING\(^{-/-}\), STING\(^{M155V}\) B16 cell lines.

STING\(^{wt}\) B16, STING\(^{-/-}\) B16 and STING\(^{M155V}\) B16 cells were injected into dorsal flanks of C57BL/6 mice (\(n=5;\ 5\times10^5\) cells in 100 µl PBS) \(a\). Tumor volumes were recorded through the use of a caliper on a daily basis. Data represents mean tumor volumes and SEM \(b\). Kaplan-Meier percent survival curves of tumor-challenged mice.

As can be seen from Figure 3.11.a., tumor volume progression and survival percentages (Figure 3.11.b.) did not show striking differences between groups. Although STING\(^{M155V}\) challenged mice had smaller tumors that progressed more slowly, the difference was not statistically significant and a repeat experiment with a higher sample size might be required to reveal possible differences between groups. Furthermore, since the B16 melanoma model is extremely aggressive, tumor volumes reached the maximal ethically allowable limits within 18 days, which may have overpowered the cumulative effect of type I IFNs on tumor development. Despite these negative results, we still wanted to evaluate whether there were any differences between groups in terms of immune cell infiltration of tumors.
Figure 3.12. Tumor infiltrating macrophage and Treg percentages in tumors derived from STING knockout or STING variant expressing B16 melanoma cells.

Single cell tumor suspensions were prepared with liberase treatment as described in Section 2.2.14.2. 2 x 10⁶ tumor cells were stained for TAM and Treg staining. a. and b. show M₁ and M₂ macrophage percent present in STING⁺/⁻ B16, STING⁻/⁻ B16 and STINGM155V B16 tumors, respectively c. shows M₁/M₂ ratios within STING⁺/⁻ B16, STING⁻/⁻ B16 and STINGM155V B16 tumors. d. shows regulatory T cell percentages within STING⁺/⁻ B16, STING⁻/⁻ B16 and STINGM155V B16 tumors. No significant differences between groups were detected by Kruskal-Wallis test. Data represents individual measurements, mean and SEM.

Since M2-macrophage and regulatory T cell (Treg) infiltration was reportedly associated with unfavorable tumor progression (Sica, Schioppa, Mantovani, & Allavena, 2006), the percentages of these cell phenotypes in tumor tissues were determined in tumor tissues. Tumors were dissociated into single cell suspensions through the use of liberase treatment as described in Section 2.2.14.2. 2 x 10⁶ tumor
cells were subjected to staining for markers of tumor associated macrophage subtypes (TAM) and T<sub>reg</sub>. Staining panels were described in detail in Table 2.4 and Table 2.5 for T<sub>reg</sub> and TAM specific markers, respectively. Single-stained controls were also prepared for compensation adjustment of flow cytometer. CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>CD206<sup>-</sup> macrophages were considered as M<sub>1</sub> phenotype and CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>-</sup>CD206<sup>+</sup> cells were considered as M<sub>2</sub> macrophages. Gating strategy and bi-axial density plots of each sample is presented in APPENDIX F. To distinguish the T<sub>reg</sub> population, CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> triple positive population was used. T<sub>reg</sub> gating strategy and bi-axial density plots of each sample is presented in APPENDIX G. Figure 3.12. a, b and c shows that there were no statistically notable differences in the percent of M1 or M2 macrophage infiltration between groups. Similarly, tumor infiltrating T<sub>reg</sub> percentile distribution was also non-significant (Figure 3.12. d). In summary, we were not able to discern the contribution of STING activation to tumor progression possibly due to the aggressive nature of the B16 melanoma model. A less aggressive tumor model and a larger sample size might allow for evaluation of STING-mediated changes in tumor microenvironment.

3.6. Assessment of TREX1<sup>−/−</sup> THP1 Cells as an in vitro Model of Aicardi Goutieres Syndrome (AGS)

In previous sections, we presented data on a cell line expressing a gain of function STING mutant in as an in vitro model of the type I interferonopathy, SAVI. In this section, we focused on TREX1 deficient THP1 cells as an in vitro model model of another type I interferonopathy, the AGS (Aicardi-Goutières syndrome). Unlike SAVI, in this disease chronic type I IFN production stems from increased cytosolic burden of self-nucleic acids (Volpi et al., 2016). TREX1 (the major cytosolic 3′-5′ DNA exonuclease, DNase III) degrades both double- and single-stranded cytosolic DNA species which may arise as a result of DNA damage-induced release of cytosolic genomic DNA. TREX1 deficiency contributes to accumulation of cytosolic nucleic acids through impaired clearance of self-DNA. Accumulation of DNA in the cytosol results in signal transduction through the cGAS-STING axis.
cGAS recognizes DNA and converts it into cGAMP. cGAMP binds to STING and induces its activation (Cai et al., 2014; Hartmann, 2017b), culminating in TBK1/IRF3 activation and elevated levels of type I IFN production.

### 3.6.1. Spontaneous Type I IFN secretion by TREX1<sup>-/-</sup> THP1 Cell Line

As explained above, AGS manifests itself with high type I IFN signature. To test whether TREX1<sup>-/-</sup> THP1 cells replicated a similar type I IFN signature and could be used as an <i>in vitro</i> model of AGS, we investigated basal levels of type I IFN production together with NF-κB activation in TREX1<sup>-/-</sup> THP1 cell line. Consistent with our expectations, TREX1<sup>-/-</sup> THP1 cells produced more type I IFNs compared to wild type THP1 cells. Depending on cell densities of 5x10<sup>5</sup> cells/ml, 10<sup>6</sup> cells/ml, or 2x10<sup>6</sup> cells/ml, TREX deficient cells produced 8-15- or 20-fold more type I IFN with respect to WT cells (Figure 13.3.a). NF-κB activation of TREX1<sup>-/-</sup> THP1 cell line was also 2- to 3-fold increased compared to wild type cells. These results suggest that TREX1<sup>-/-</sup> THP1 cells constitute a suitable <i>in vitro</i> model to study AGS.

![Figure 3.13. IFN expression and NF-κB activity of WT, STING<sup>-/-</sup>, cGAS<sup>-/-</sup>, TREX1<sup>-/-</sup> THP Dual Cell Lines](image)

Cell lines were incubated at 37°C for 24h without stimulation and supernatants were collected at the end of the incubation. IFN and NF-κB activation was assessed
by Quanti Luc and Quanti Blue assays, respectively. **a.** shows IFN expression based on IFN signaling-dependent luciferase expression (relative luciferase units). **b.** shows NF-κB activity based on NF-κB activation-dependent SEAP production. 3 biological replicas were used in each experiment, and all data points from 2 independent experiments are shown. Relative luciferase units (RLU) are presented as ratio of individual values with respect to average of WT recordings taken using a cell density of 5x10^5 cells/ml. Two-tailed Mann Whitney U test was used for statistical comparison of population means. Box-whiskers show mean and SD. For all comparisons, P values of <0.05 were considered significant. For indication of the significance, P values of <0.01 were denoted with **.

3.6.2. Effect of Cold Exposure on Type I IFN secretion from TREX1^{-/-} THP1 Cells
Similar to SAVI, 40% of AGS patients are reported to have chilblains exacerbated by cold exposure (Abdel-Salam et al., 2010). To address whether cold induction exacerbates type I IFN production in AGS, we tested spontaneous type I IFN production from TREX^{-/-} THP1 cells exposed to 2-hour cold (+4°C) treatment in comparison to “always 37°C” incubated samples. In AGS patients, high levels of IFN-α contribute to leukocyte accumulation and hence, increase in immune cell density in tissues (Kolivras et al., 2008). To mimic this in vivo aspect of AGS, we also tested different cell densities.

Data presented in Figure 3.14.a. shows that, as expected, type I IFN production correlated with increase in cell density. When effect of cold exposure was analyzed, at a density of 5x10^5 cells/ml, cold induced versus 37°C incubated cells produced similar levels of type I IFN production. In contrast, at the highest cell density (2x10^6/ml), TREX deficient cells secreted higher levels of type I IFNs compared to 37°C incubated cells. These results suggest that cold-exposure might exert its pathological effect when the type I IFN concentrations reached a threshold value. This hypothesis might explain the absence of cold-induced pathology in healthy
individuals versus exacerbation of symptoms in case of chronically elevated levels of IFNs. Results presented in Figure 3.14.b. also shows that, except for STING\textsuperscript{+/−} cells, cold-exposure also contributed to a modest increase NF-κB activation in all cell lines. Since STING-mediated signaling pathway also leads to NF-κB activation and therefore pro-inflammatory cytokine secretion such as IL-1β (Broz & Dixit, 2016; Mayer-Barber & Yan, 2017; Surucu & Gursel, 2017), our results indicate IRF and NF-κB-dependent pathways can simultaneously contribute to cold-induced exacerbation of symptoms in AGS patients. Whether the observed cold-induced effect stems from increased STING expression as previously shown in the SAVI in vitro model, remains to be elucidated.

Figure 3.14. Effect of Cold-exposure on IFN expression and NF-κB activity of WT, STING\textsuperscript{−/−}, cGAS\textsuperscript{−/−}, TREX1\textsuperscript{−/−} THP Dual Cell

WT, STING\textsuperscript{−/−}, cGAS\textsuperscript{−/−} and TREX1\textsuperscript{−/−} Dual THP cells, were incubated at the indicated densities without any stimulation. Following 24h 37°C incubation or 2h at 4°C followed by 22h 37°C incubation, cell supernatants were collected. IFN production (a.) and NF-κB activation (b.) were assessed as described before. Relative luciferase units (RLU) are presented as ratio of individual values with respect to, all data points divided by average of WT, recordings taken using a cell density of
5x10^5 cells/ml group. Pairwise 37°C and cold induced samples were compared statistically according to Mann Whiney U test. No significance was detected. Individual scatter points, means and SD are shown. Data is representative of one of three independent experiments, each containing three biological replicates.

Since cold-exposure seemed to exert an effect at the highest cell density (corresponding to higher levels of type I IFNs), we hypothesized that accumulation of type I IFNs in the cell culture might be a pre-requisite in cold-induced exacerbation of interferonopathies. To test this hypothesis, we designed an experiment in which plated cells were exposed to cold stress at different time points. For this, 3 different concentrations of cells were either kept at 37°C or were exposed to a 1 hour cold at 1, 12, 18 or 21th hours of a total of 24 h incubation period. Type I IFN production and NF-κB activation were then assessed as described before. Although the results were not statistically significant, at the highest cell densities, a modest increase in both type I IFN production (Figure 3.15.a.) and NF-κB activation (Figure 3.15.b.), following cold-induction at the latest time point (21st hour) group, suggested that cold-triggered exacerbation of AGS symptoms might be dependent on type I IFN accumulation. Therefore, for further experiments, the 21st hour was chosen as the optimal time point to introduce cold-stress.
Previously, accumulated type I IFN in TREX deficient cells correlated with a modest enhancement of cold-induced IFN and NF-κB activity. To test whether cold-induction targets cGAS-STING-IRF pathway or type I IFN-receptor dependent autocrine IFN effect, TREX1⁻/⁻ THP cells were stimulated with 10-fold increasing doses of recombinant IFN-β and were either kept at 37°C or were
exposed to cold at 21st hour of the 24 h incubation period. IFN-dependent luciferase expression and NF-κB activation were monitored as before.

Figure 3.16. IFN secretion and NF-κB activation profile of WT, STING−/−, cGAS−/− and TREX1−/− THP Dual Cell Lines stimulated with recombinant IFN-β and cold exposure.

WT, STING−/−, cGAS−/− and TREX1−/− Dual THP cells were seeded at 10⁶ cells/ml. Cells were either left untreated or were stimulated with 5 ng/ml, 50 ng/ml or 500 ng/ml of recombinant IFN-β after seeding. One group was incubated at always 37°C for 24 hour. Other groups were exposed to 1-hour cold treatment at 4°C starting from 21st hour of a total of 24 h incubation period. Cell supernatants were then collected and evaluated for IFN production a. and NF-κB activity b. by Quanti Luc and Quanti Blue assays, respectively. Relative luciferase units (RLU) are presented as ratio of individual values with respect to, all data points divided by average of WT, recordings taken using a cell density of 5x10⁵ cells/ml group. Two biological replicates were used and plots as representative three independent experiments, with similar results.

Using this experimental setup, cold-stress was found to moderately increase NF-κB activation but not IFN signaling in 50 ng/ml rec. IFN-β stimulated WT and TREX−/− cells (Figure3.16.). Although our results failed to demonstrate a clear effect of
cold exposure on STING-mediated immune activation in AGS model, we nevertheless hypothesized that type I IFN autocrine signaling and NF-κB activation pathways that depend on TBK1/IKKe kinase activation might simultaneously contribute to the pathology of AGS, suggesting that inhibitors targeting both TBK1 and IKKe might be of value in the clinic (Hervas-Stubbs et al., 2011).

3.6.3. Inhibition Strategies for Elevated Spontaneous Type I IFN secretion in TREX1−/− THP1 Cell Line

Although we tried to investigate the contribution of signaling pathways in AGS using immunoblotting, our results were inconclusive (data not shown). Next, to investigate the contribution of JAK/STAT versus TBK1/IKKe-dependent immune activation in our AGS model, we decided to use specific inhibitors targeting either the JAK/STAT pathway (tofacitinib and ruxolinitib) or the TBK1/IKKe pathway (BX-795 and amlexanox) (Hervas-Stubbs et al., 2011; Mascarenhas & Hoffman, 2012; Reilly et al., 2013; Rosengren, Corr, Firestein, & Boyle, 2012). We also included the suppressive ODN A151 as another candidate inhibitor. In these experiments, TREX deficient THP dual cells were incubated without or with different inhibitor concentrations and spontaneous type I IFN production or NF-κB activation was monitored as described before.
Figure 3.17. IFN secretion and NF-κB activation profile of TREX1−/− THP Dual Cell Line in response to increasing doses of various inhibitors.
TREX−/− THP Dual cells were seeded at a density of 10^6 cells/ml. Cells were either left untreated or treated with DMSO as vehicle control or indicated doses of Tofacitinib, Ruxolitinib, BX-795, Amlexanox, A151 or control ODN K-flip. Cells were incubated at 37°C for 24 hour. Type I IFN production was assessed by Quanti Luc reporter assay (a). Relative luciferase units (RLU) were reported as fold induction over the untreated TREX−/− THP Dual group. NF-κB activity was determined using the Quanti Blue assay (b). Cell viability 24 h after inhibitor treatment was determined using the FCS-SSC characteristics of TREX−/− THP Dual cells and flow cytometry (c). To. Two biological replicates, were used in each experiment. Data is representative of three independent experiments with similar results.

Figure 3.18.a. shows that type I IFN production from TREX deficient cells was not affected by JAK/STAT inhibitors tofacitinib and ruxolitinib. Treatment with 3 μM A151 was also ineffective. In contrast, BX-795 and Amlexanox exerted a potent inhibitory effect on spontaneous type I production. 10 μM BX-795 inhibited type I IFN production by 50% and 64 μg/ml Amlexanox suppressed 75% of spontaneous type I IFN production. The observed suppressive effects of inhibitors were not the result of drug-induced cytotoxicity as cell viabilities remained relatively high following inhibitor use (Figure 3.17.c.). Even at the highest dose of (64 μg/ml) Amlexanox, 75% of cells remained viable, whereas with 10 μM BX-795 the viability was 80%. Taken together, these results had two implications: 1) In AGS, STING/TBK1/IKKε-dependent type I IFN production is more dominant than autocrine type I IFN-mediated JAK/STAT-dependent immune activation and 2) Amlexanox is the most effective inhibitor of spontaneous type I IFN production in TREX deficient THP cells. Since currently, there is no established treatment for AGS, our data suggest that Amlexanox might be an effective inhibitor to combat the interferon-dependent pathology in AGS or other type I interferonopathies.
3.7. Investigation of Changes in Cytosolic Nucleic Acid Sensing Mechanisms in Relation to Circadian Clock

The immune system, like many other physiological systems, is under circadian control (Scheiermann et al., 2018). Evidence suggests that innate and adaptive immune cell trafficking, susceptibility to bacterial, viral and parasitic infections, expression of certain pattern recognition receptors and their downstream signaling pathways, phagocytosis, cytokine and chemokine secretion show circadian oscillations (Kiessling et al., 2017; Muldoon et al., 2016; Ohdo, Koyanagi, Suyama, Higuchi, & Aramaki, 2001; Silver, Arjona, Walker, & Fikrig, 2012). Furthermore, pathological manifestations of certain autoimmune diseases (eg., rheumatoid arthritis) also vary with circadian rhythmicity (Nakao, 2014). Considering the aforementioned information and absence of data on circadian rhythm associated changes in cytosolic DNA recognition pathways, we aimed to assess possible fluctuations in cGAS/STING-mediated immune activation pathway over a period of 48 hours. We also hypothesized that provided that there were circadian oscillations in cGAS/STING related sensing, we would also see similar fluctuations in in vitro cell line models of SAVI and AGS. Such a finding would be of considerable clinical importance since this would mean that the timing of inhibitor administration would impact the therapeutic outcome.

For these experiments, we also included the recently available THP1 N154S STING variant knocked in cell line. The N154S is another gain-of-function STING mutation that was originally described in some SAVI patients (Y. Liu et al., 2014; Melki et al., 2017).

As an initial setup, we first wanted to analyze spontaneous type I IFN production and NF-κB activation in WT, STING−/−, cGAS−/−, TREX−/− and STINGN154S Dual THP cells subsequent to circadian rhythm synchronization. To pursue this aim, all cells were incubated in culture medium containing 0.1 µM dexamethasone for a total period of 2 hours of synchronization. Following this, cells were washed and
distributed into 96-well plates at a density of $10^6$ cell/ml. Subsequent to a 12-hour incubation period allowing for synchronization and type I IFN accumulation, cell culture supernatants were collected at 4-hour intervals for a period of 48 hours. Since this experimental setup necessitated a 12-hour delay for IFN detection, designation of synchronized light-dark phases was adjusted for this delay. Glucocorticoid hormone dexamethasone upregulates the PER1-2 genes. Upregulation of PER genes corresponds to light phase in vivo. Because of this 12-hour delay, the response curves were shown to start with the dark cycle (Labrecque & Cermakian, 2015; So et al., 2009).

WT, STING−/− or cGAS−/− THP1 cell lines did not produce substantial levels of type I IFNs and there were no meaningful oscillations (shown in Appendix H). Conversely, TREX−/− and STINGN154S Dual THP cells displayed substantial fluctuations of Type I IFN production over time (Figure 3.18a). Both AGS and SAVI models showed 4 major activity peaks during the 48-hour zeitgeber time measurement. However, light to dark and dark to light phase transitions seemed to display different patterns (Figure 3.18a.), suggesting that accumulation of certain soluble factors and/or expression of interferon stimulated genes might have modulatory effects, impacting transition times. Taken together, our data showed a 4-peak pattern of type I IFN production over a period of 48 hours, indicative of an ultradian secretion pattern. Data further suggests that early-morning and early night application of inhibitors might work more efficiently for AGS and SAVI patients. For NF-κB activation, we could not discover tangible periodic changes for AGS or SAVI (Figure 3.18b.).
Figure 3.18. Determination of type I IFN secretion from dexamethasone synchronized TREX\textsuperscript{--/} and STING\textsuperscript{N154S} THP Dual Cell Lines as a function of time.

All cells were treated with 0.1 \( \mu \)M dexamethasone for 2 hours for circadian clock synchronization and were washed and plated at a density of \( 10^{6} \) cells/ml in dexamethasone-free medium. Cell supernatants were collected 12 hours later at 4 hour intervals. Type I IFN production was evaluated using the Quanti Luc luciferase reporter assay. (a) Fold change in mean relative luciferase units (RLU) and (b) NF-\( \kappa \)B activation are presented with respect to time point matched WT cell line’s corresponding values. Results are the average of two biological replicates.

In Section 3.6.3., we showed that in AGS, STING/TBK1/IKK\( \varepsilon \)-dependent type I IFN production was more dominant than autocrine type I IFN-mediated JAK/STAT-dependent immune activation. Next, we aimed to examine whether
cGAS/STING-mediated or Type I IFN-dependent immune activations were regulated by circadian rhythm. Rhythmic interknitted core clock protein fluctuations activate or repress genes through the e-box sequence (Scheiermann et al., 2018). To assess whether innate nucleic acid sensing pathways and type I IFN signaling could be regulated through clock genes, we first searched the “Eukaryotic Promoter Database” for the presence of e-box sequence in proximal promoter regions of cytosolic nucleic acid sensing pathway elements in human. We found that STING, MAVS, TBK-1, IRF3 and STAT1 had e-box sequence(s) in their proximal promoter sites. To substantiate these findings, we then examined HSV60, cGAMP, polydA:dT, and low dose recombinant IFN-β stimulation-time dependent response of WT, STING−/− and cGAS−/− THP Dual Cell lines in terms of type I IFN production and NF-κB activation. Similar to the previous experimental setup, all cells were synchronized with 0.1 µM dexamethasone for 2 hours. Following washing, cells were distributed to 96-well plates at a density of 10^6 cell/ml in dexamethasone negative fresh medium. Cells were either left untreated or stimulated with nucleic acid sensor agonists every 4-hours for a period of 48 hours. 12-hours after each stimulation, cell supernatants were collected and assessed for type I IFN production or NF-κB activation.

These preliminary experiments revealed that, type I IFN production from WT cells oscillated with time over a period of 48 hours (Figure 3.19). There were 4 maxima points, 2 corresponding to the dark cycles and 2 corresponding to the light cycles. It is probable that the peaks observed during the dark cycle may result from direct stimulation of nucleic acid sensors whereas the peaks that follow may be results of autocrine Type I IFN signaling-dependent secondary cytokine production. Periodic fluctuation pattern in STING−/− cell line seemed to be disrupted. Considering the fact that STING is one of the probable circadian regulated adaptor proteins, this disruption may have been caused by STING deficiency. The partial wave pattern in polydA:dT and rec. IFN-β stimulated STING−/− cells may be due to other circadian regulation adaptors. Specifically, in the case of polydA:dT stimulation;
polydA:dT can be sensed and converted to RNA through RNA Pol III and consecutively induce signaling through RIG-I/MDA5 and MAVS axis. Of note, MAVS was found to have e-box promoter sequence in its proximal promoter region. Hence, MAVS dependent circadian regulation might be partially recapitulating STING dependent circadian regulation in response to polydA:dT stimulation in STING\textsuperscript{−/−} cell line. In case of recombinant IFN-β stimulation, Type I IFN receptor-dependent STAT1 activation might be responsible for the observed partial wave pattern in STING\textsuperscript{−/−} cell line due to its e-box sequence.

**Figure 3.19.** Determination of type I IFN secretion from dexamethasone synchronized cytosolic nucleic acid or IFN-β stimulated WT, STING\textsuperscript{−/−} and cGAS\textsuperscript{−/−} THP Dual Cell Lines as a function of time.
All cells were treated with 0.1 µM dexamethasone for 2 hours for circadian clock synchronization. Following washing, WT, STING\textsuperscript{−/−}, cGAS\textsuperscript{−/−} Dual THP cells were plated at 10\textsuperscript{6} cells/ml to 96-well plates in dexamethasone negative fresh medium. Cells were then left untreated or stimulated with 5 µg/ml HSV60, 15µg/ml cGAMP, 5 µg/ml polydA:dT or 5 ng/ml recombinant IFN-β every 4-hours and culture supernatants were collect 12 hours later. Type I IFN production was evaluated using the Quanti Luc luciferase reporter assay. Fold change in mean relative luciferase units (RLU) is presented with respect to time point matched WT cell line’s corresponding values. Results are the average of two biological replicates.

The cGAS\textsuperscript{−/−} cell line showed a more similar fluctuation pattern to WT than STING\textsuperscript{−/−} cell line, yet the periodic pattern still seemed to be disrupted. As expected, stimulation with the cGAS ligand HSV60 did not elicit any response in this cell line. In contrast, following stimulation with cGAMP, cGAS\textsuperscript{−/−} cell line also had a fluctuating pattern of type I IFN production. However, compared to the WT cells, the type I IFN peaks were delayed. Whether the absence of cGAS affects the major circadian regulation mechanisms, remains to be determined. In case of polydA:dT stimulation, response of the cGAS\textsuperscript{−/−} cell line was too modest to determine any fluctuations. Since transfected polydA:dT represents another cytosolic dsDNA ligand and therefore an agonist of cGAS, this result is not unexpected. Finally, consistent with WT cells, stimulation with recombinant IFN-β caused a similar pattern of oscillating response in cGAS\textsuperscript{−/−} cells. Taken together, our data suggests that STING might be one of the adaptor proteins regulated by circadian rhythm. Further studies regarding circadian clock regulation of cGAS/STING/TBK1-mediated type I IFN production would be required to clarify the validity of these preliminary findings.
Figure 3.20. Determination of NF-κB activation in dexamethasone synchronized cytosolic nucleic acid or IFN-β stimulated WT, STING−/− and cGAS−/− THP Dual Cell Lines as a function of time.

All cells were treated with 0.1 µM dexamethasone for 2 hours for circadian clock synchronization. Following washing, WT, STING−/−, cGAS−/− Dual THP cells were plated at 10⁶ cells/ml to 96-well plates in dexamethasone negative fresh medium. Cells were then left untreated or stimulated with 5 µg/ml HSV60, 15µg/ml cGAMP, 5 µg/ml polydA:dT or 5 ng/ml recombinant IFN-β every 4-hours and culture supernatants were collect 12 hours later. NF-κB activation was evaluated using the Quanti Blue reporter assay. Fold change in NF-κB activity is presented with respect to time point matched WT cell line’s corresponding values. Results are the average of two biological replicates.
For the circadian rhythm controlled NF-κB activation of WT, STING^{−/−} and cGAS^{−/−} THP Dual Cell Lines, stimulated fold-inductions were relatively lower and no clear fluctuation patterns could be discerned.

In summary, our preliminary experiments suggest that cytosolic nucleic acid stimulated-type I IFN production might be regulated by circadian clock and STING might play a role in this regulation. Our results also indicate that for AGS and SAVI, type I IFN secretion period and amplitude might be affected by light to dark and dark to light transitions. Based on the timing of oscillations, optimal inhibitor application time might impact the therapeutic utility and therefore management of disease symptoms.
CHAPTER 4

CONCLUSIONS

Dysregulation in the nucleic acid sensing pathways has detrimental consequences. Constitutively elevated type I IFN production in SAVI and AGS shortens patients’ life span and the chronic inflammation associated manifestations, impact their quality of life. Pathology causing congenital mutations in SAVI patients were previously identified to include M155V, N154S, V147L, G166E (dimerization domain) C206Y, R281Q,R,284M, R284G, R284S (C terminal domain) mutations (Y. Liu et al., 2014; Saldanha et al., 2018; Tao et al., 2016). These gain-of-function mutations in STING protein instigate spontaneous type I IFN production. In another interferonopathy, mutations in ADAR1, SAMHD1, TREX1 or RNase H2 were shown to cause AGS (Hartmann, 2017a). While mutations in TREX1 and RNase H subunits contributes to type I IFN production through the cGAS-STING-IRF3 axis, mutations in ADAR, RIG-I or MDA-5 promotes type I IFN production through the RIG-I/MDA-5-MAVS-IRF3 axis (Rodero & Crow, 2016). Herein, we investigated TREX1 deficiency and consequent cGAS-STING mediated spontaneous type I IFN production as an in vitro AGS model.

First, we generated a stably transfected cell line as a model for SAVI. The M155V STING variant was stably expressed as evidenced by flow cytometric analysis (Figure 3.3). Next, we assessed whether or not our model replicated the chronic type I IFN secretion pattern observed in SAVI. Results confirmed that STING(M155V) B16 Blue cell line constitutively produced detectable levels of type I IFNs (Figure 3.4 and Figure 3.5). Next, we investigated the phenomenon of cold- induced type I IFN exacerbation in our SAVI and AGS in vitro models using multiple experimental approaches. Our data showed that cold-induced type I IFN
exacerbation in SAVI *in vitro* model stems from STING protein upregulation and consecutive STING phosphorylation (Figure 3.10.).

Although we tried to investigate the contribution of cold-exposure to signaling pathways in AGS using immunoblotting, our results were inconclusive (Data not shown). However, through the use of inhibitors we showed that chronic type I IFN production depended more on the STING/TBK1/IKKe-dependent pathway rather than the autocrine/paracrine type I IFN-mediated JAK/STAT pathway. (Figure 3.14. - Figure 3.15. - Figure 3.16.). Next, we demonstrated that Amlexanox was the most effective inhibitor capable of reducing type I IFN signature down to 20% without exerting significant cytotoxicity in TREX deficient THP cells (Figure 3.17.).

In a separate study, we aimed to inspect the impact of constitutive STING activation to tumor progression. However, the aggressive nature of the employed tumor B16 melanoma tumor model did not allow us to identify possible contribution of STING signaling to tumor progression (Figure 3.11- Figure 3.12). Our tumor challenge experiment data suggest that a less aggressive tumor model and a larger sample size might permit future studies to shed light on STING-tumor progression association.

Recent evidence suggests that innate and adaptive immune activation and regulation show circadian oscillations (Kiessling et al., 2017; J. Liu et al., 2006; Muldoon et al., 2016; Nakao, 2014). In this thesis, we wanted to explore whether the cGAS-STING-dependent nucleic acid sensing was controlled by the circadian rhythm. Taking into consideration that circadian regulation works through activation or repression of genes through the e-box sequence (Scheiermann et al., 2018), we searched the “Eukaryotic Promoter Database” and noted that STING, MAVS, TBK-1, IRF3 and STAT1 had e-box sequence(s) in their proximal promoter sites. In support of this, *in vitro* evidence indicated that STING deficient cells lost their periodicity (Figure 3.19). Circadian regulation in AGS and SAVI were analyzed through time-dependent type I IFN secretion suggesting the presence of light to dark and dark to light transitory differences (Figure 3.18.). These results
implicate that optimal inhibitor application time might impact the therapeutic utility and therefore management of disease symptoms. Our data propose that late night or early morning application of inhibitor might be more efficacious. Further studies regarding circadian clock regulation of cGAS/STING/TBK1-mediated type I IFN production would be required to clarify the validity of these preliminary findings.
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APPENDIX A

A. VECTOR MAP OF pUNO1-hSTING-M155

Figure A.1. The vector map of hSTING-M155 (Invivogen, USA).
APPENDIX B

B. CULTURE MEDIA, BUFFERS & SOLUTIONS USED IN BACTERIAL GROWTH, TRANSFORMATION & COMPETENT CELL PREPARATION

Luria Broth (LB):
5 g NaCL
5 g Tryptone
2.5 g Yeast extract
500 ml dH₂O
Autoclaved.

LB Agar:
5 g NaCL
5 g Tryptone
7.5 g Agar
500 ml dH₂O
Autoclaved.

TSS:
To make 50 ml:
5g PEG 8000
1.5 ml 1M MgCl₂ (or 0.30g MgCl₂*6H₂O)
2.5 ml DMSO
Add LB to 50 ml
Filter sterilize (0.22 µm filter) pH 6.5
**SOB:**

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<td>Bacto Tryptone</td>
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<tr>
<td>Yeast extract</td>
<td>0.5%</td>
<td>5 g</td>
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<tr>
<td>NaCl</td>
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<td>0.584 g</td>
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<td>KCl</td>
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</tr>
<tr>
<td>MgSO₄</td>
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<td>2.465 g</td>
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Add deionized water to final volume (1L)

**SOC:**

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<th>Ingredient</th>
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<tr>
<td>Glucose</td>
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Add deionized water to final volume (1L).

**Chloramphenicol (Applichem, Germany)**

For 1000x stock solution

0.34 g chloramphenicol in 50 ml absolute ethanol

Stored at -20 °C.
APPENDIX C

C. CULTURE MEDIA, BUFFERS & SOLUTIONS USED IN MAMMALIAN CELL CULTURE

10 % FBS containing RPMI 1640 (Referred as culture medium):
50 ml FBS (inactivated at 55°C)
5 ml Penicillin/ Streptomycin
5 ml Sodium Pyruvate
5 ml HEPES
5 ml NEAA (non-essential amino acid)
Final volume was reached by addition of 500 ml RPMI-1640 (w/L-Glutamine, w/Phenol Red) from BI, Israel. 100 µg/ml Normocin is used to prevent mycoplasma and fungal contamination.

2 % FBS containing RPMI 1640 (Referred as wash medium):
10 ml FBS (inactivated at 55°C)
5 ml Penicillin/ Streptomycin
5 ml Sodium Pyruvate
5 ml HEPES
5 ml NEAA (non-essential amino acid)
Final volume was reached by addition of 500 ml RPMI-1640 (w/L-Glutamine, w/Phenol Red) from BI, Israel.
**PBS (Phosphae Buffered Saline, 10X):**

80 g NaCl  
2 g KCl  
8.01 g Na$_2$HPO$_4$. 2H$_2$O  
2 g KH$_2$PO$_4$  

pH is adjusted to 6.8. Completed to 1L with dH2O.  
Diluted 1X PBS (pH≈7.2) autoclaved prior to use.

**FACS Buffer (1L):**

1L 1X PBS  
10 g BSA (1%)  
250 mg Na-Azide (0.25 %)

**QUANTI-Blue:**

Content of pouch is poured and mixed with 100 ml of endotoxin-free water according to manufacturer’s instructions. Stored at 4°C.
APPENDIX D

D. WESTERN BLOT BUFFERS & SOLUTIONS

Lysis Buffer (180 µL):
18 µl from 10xRipa buffer
26 µl from 10x stock solution of Roche complete mini EDTA free tablet solution
18 µl from 10x stock solution of Roche PhosSTOP
118 µl from MBG H2O

Running Buffer (10X)
30 g Tris base
144 g Glycine
10 g SDS
in 1L of filtered dH2O
stored at room temperature, diluted with filtered dH2O to 1X and pre-cooled before use.

6X Laemmli Sample Loading Buffer (for non-reducing and reducing):
1.2gr SDS (sodium dodecyl sulfate)
6mg bromophenol blue
4.7ml glycerol
1.2ml Tris 0.5M pH6.8
2.1ml dH2O
Completely dissolved.
0.93gr DTT was added only for reducing SDS-PAGE. Aliquots were kept frozen at-20°C.
**Blotting Buffer(10x):**

30 g Tris  
144 g Glycine  
pH is adjusted to 8.3 in 1 L dH₂O. Stored at room temperature.

**Transfer Buffer(1x):**

700 ml filtered +4°C cold dH₂O  
200 ml absolute Methanol  
100 ml 10X Blotting Buffer  

Shown addition order is important to prevent formation of precipitation. Pre-cooled at +4°C before use.

**TBS (10x, 100 ml):**

3g Tris  
8g NaCl  
0.2g KCl  
the volume completed with 100 ml dH₂O.

**TBST (1x, 1 L):**

100 ml of TBST(10x)  
pH is adjusted to 7.4, then complete the volume with dH₂O 1 L and add 0.1% Tween 20  

**Blocking Buffer (1x, 50 ml)**

2.5 g Skimmed Milk (5% w/v) with 50 ml TBST

**Wash Buffer**

1x TBST
**Antibody Dilution Buffer**
v:v dilution with 10 ml blocking buffer (1x TBST with 5% BSA)

**Stripping Buffer (1L)**
Glycine 15 g
In 1L of filtered dH₂O

**Mild Stripping Buffer (1L)**
Glycine 15 g
SDS 1g
Tween20 10 ml
pH was adjusted to 2.2, then completed with dH₂O to 1L.
APPENDIX E

E. Graphs & Regarding Parameters Used For IC\textsubscript{50} Value Calculation

Always 37\textdegree C Group

\[ y = 1 \times 10^{-0.05}x^2 - 0.0311x + 1.607 \]
\[ R^2 = 0.81498 \]

\[ 0 \quad 50 \quad 100 \quad 150 \quad 200 \quad 250 \quad 300 \]

4\textdegree C Cold Induced Group

\[ y = 9 \times 10^{-0.05}x^2 - 0.0289x + 1.8316 \]
\[ R^2 = 0.94469 \]

\[ 0 \quad 50 \quad 100 \quad 150 \quad 200 \quad 250 \quad 300 \]

Figure A.2. IC\textsubscript{50} Graphs of Amlexanox
APPENDIX F

F. Flow Cytometry Gating Strategies

Figure A.3. M1-M2 Flow cytometry gating strategies and individual plots of mice.
Figure A.4. Treg Flow cytometry gating strategies and individual plots of mice.
G. Basal Level Oscillation Patterns

Figure A.5. During 48-hour period of circadian time, basal levels of type I IFN oscillations.

All cells were treated with 0.1 μM dexamethasone for 2 hours for circadian clock synchronization. WT, STING<sup>−/−</sup>, cGAS<sup>−/−</sup>, TREX<sup>−/−</sup> and STING<sup>N154S</sup> Dual THP cells were plated at 10<sup>6</sup> cells/ml to 96-well plates with dexamethasone devoid fresh medium. Allowing 12 hour incubation for synchronization regulated response, cell supernatants were collected starting after 12 hour incubation in 4 hour time interval. Type I IFN production evaluated with Quanti Luc luciferase reporter assay. Relative luciferase units (RLU) are presented as ratio of individual values with respect to average of WT, time point 0 showed at upper panel. Two biological replicates were used and plots as representative two independent experiments, with similar results. NF-κB activity assessed with Quanti Blue SEAP reporter assay. Mean and SD represented at lower panel.
APPENDIX H

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