Nucleic acid sensing in CD8 T cells and NK cells during viral infections

Adham Abuelola Fathy Abeulola Mohamed

ORCID ID

0009-0008-5634-3551

from Giza, Egypt

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1. Supervisor: Prof. Martin Schlee
2. Supervisor: Prof. Andrew Brooks

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Institute of Clinical Chemistry and Clinical Pharmacology, Bonn University
Director: Prof. Günther Hartmann

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ABBREVIATIONS

Abbreviation	Meaning
-/-	Knockout
3p-dsRNA	Tri-phosphate double stranded ribonucleic acid
AF700	Alexa-flour 700
ANOVA	Analysis of variance
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cyanine 7
bp	Base pairs
BV510	Brilliant Violet 510
BV650	Brilliant Violet 650
Cas	CRISPR-associated
CCL	Chemokine ligand
CD	Cluster of differentiation
cGAMP	Cyclic guanosine monophosphate-adenosine monophosphate
cGAS	cGAMP synthase
CLs	Cytotoxic Lymphocytes
CMV	Cytomegalo Virus
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
Ctrl	RNA negative control
DMSO	Dimethyl sulfoxide
DNA	Deoxy Ribonucleic Acid
dsRNA	Double stranded RNA
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
fwd	Forward
gMFI	Geometric mean fluorescence intensity
h	Hour
HHV-A	Human Herpes Virus A
HIV	Human Immunodeficiency Virus
HPgV	Human Pegi Virus
HSV	Herpes Simplex Virus
HTLV-I	Hyman T Lymphocytic Virus I
IAV	Influenza A virus
IFIT1	Interferon Induced Protein with Tetratricopeptide Repeats 1
IFN-I	Type I interferons
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IFNAR	Interferon alpha/beta receptor

IH	Immuno-histochemistry
IKK	IκB kinase
IL-10	Interleukin 10
IL-15	Interleukin 15
IL-2	Interleukin 2
IL-7	Interleukin 7
IRF	IFN-regulatory factor
ISG	Interferon stimulated gene
ISH	In situ hybridization
IVT	*In vitro* transcription
JAK1	Janus kinase 1
KIR	Killer-cell immunoglobulin-like receptors
KO	Knockout
LCMV	Lymphocytic Choriomeningitis Virus
LGLs	Large Glanular Lymphocytes
LILR	Leukocyte immunoglobulin-like receptors
MAVS	Mitochondrial antiviral signaling protein
MDCK	Madin-Darby canine kidney cell line
Med	Medium control
MHC	Major histocompatibility
MOI	Multiplicity of infection
mRNA	Messenger RNA
MV	Measles virus
NCR	Nucleic acid receptor
NFκB	Nuclear factor kappa B
NK	Natural killer
NP	Nuclear protein
ns	Non-significant
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
pDNA	Plasmid DNA
PE	Phycoerythrin
PMA/Ion	Phorbol 12-myristate 13-acetate/Ionomycin
pNFκB -P65	Phospho p65 subunit of Nuclear factor κB
pSTAT2	Phospho Signal transducer and activator of transcription 2
pTBK1	Phospho TANK-binding kinase 1
qRT-PCR	Quantitative real-time PCR
Rel.	Relative
rev	Reverse
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute
RSV	Respiratory Syncytial virus
RT	Room temperature
SDS	Sodium dodecyl sulphate

SEM	Standard error or mean
SSC	Side scatter
ssRNA	Single stranded RNA
STAT2	Signal transducer and activator of transcription 2
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline
T_{CM}	Central memory T cells
TCR	T cell receptor
T_{EM}	Effector memory T cells
TEMED	Tetramethylethylendiamine
T_{EMRA}	Terminally differentiated effector memory t cells
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
T_N	Naïve T cells
TNF	Tumor necrosis factor
tracrRNA	Trans-activating crRNA
T_{Reg}	Regulatory T cells
TTV	Tornique Teno Virus
U/ml	Units per millilitres
v/v	Volume per volume
VZR	Varicella Zoster Virus
w/v	Weight per volume
WT	Wildtype
xg	Times gravity

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ABSTRACT

Pathogens can be detected by a variety of germline encoded pattern recognition receptors (PRR) that recognize highly conserved pathogen-associated molecular patterns (PAMPs). Among these receptors, Retinoic Acid-Inducible Gene I (RIG-I), a type I IFN (IFN-I) inducing sensor of cytosolic pathogen-derived RNA, plays a key role in the immune responses to RNA viruses in macrophages and dendritic cells. However, the specific impact of RIG-I activation on lymphocyte function has remained poorly understood. This *in vitro* study aimed to investigate the role of RIG-I in human NK and CD8 T cells. Activation of RIG-I by influenza A virus infection or the synthetic RIG-I ligand 3p-dsRNA demonstrated that both stimuli resulted in the production of IFN-I, which not only activated these cytotoxic lymphocytes but also significantly enhanced their degranulation and cytokine production. Pre-stimulation by 3p-dsRNA also significantly reduced the ability of influenza A virus to infect these cells. To further investigate the role of RIG-I receptors and the secreted IFN-I stimulated by influenza A infection and 3p-dsRNA, we employed CRISPR/Cas9-mediated gene editing in primary human lymphocytes. This approach demonstrated the involvement of RIG-I and STAT2 in the signalling pathway leading to the activation of cytotoxic lymphocytes. Altogether, the data shows that RIG-I activation not only protects lymphocytes from infection by inducing cell autonomous antiviral pathways in the lymphocytes themselves but also enhances lymphocyte effector function. This research contributes to our understanding of lymphocyte responses to viral infections, emphasizing the importance of RIG-I as a nucleic acid sensor in lymphocyte effector function and raise the possibility of activating RIG-I to enhance their effector function in cellular therapies.

Preface

The work of this thesis was carried out in the S2 laboratory, University Hospital Bonn, at the Institute for Clinical Chemistry and Clinical Pharmacology, Bonn, Germany and in the PC2 and laboratories, The University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, under the supervision of Prof. Martin Schlee, Prof. Andrew Brooks and Dr. Sanda Stankovic.

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LIST OF PUBLICATIONS

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1. LITERATURE REVIEW

Introduction

1.1 Cytotoxic Lymphocytes

Cytotoxic lymphocytes (CLs) are key players in the immune system's defense against virus-infected and cancer cells (Smyth and Trapani, 1995). They contribute to the clearance of virally-infected cells in many clinically common infections such as with hepatitis B virus (Schuch *et al.*, 2014; Zheng *et al.*, 2016) and influenza virus (Mbawuike *et al.*, 2007). These immune cells possess the ability to detect and eliminate target cells through different mechanisms, this diversity contributing to the capacity of the immune system to restore and/or maintain homeostasis following exposure to a diverse range of pathogens (Pfefferle *et al.*, 2020).

Cytotoxic lymphocytes activate cellular death pathways in target cells (Smyth and Trapani, 1998) through the secretion of the cytotoxic granules which contain perforin and granzymes. Perforin forms pores in the target cell membrane, allowing the entry of granzymes, which then induce target cell apoptosis (Trapani and Smyth, 2002). Additionally, cytotoxic lymphocytes can also induce target cell death through the engagement of the death ligands such as Fas ligand and/or TNF-related apoptosis-inducing ligand (TRAIL), as well as producing an array of cytokines and chemokines that can further either directly contain the spread of pathogens or through their impact on other cell types, modulate the broader immune response (Wajant, 2002; Kantarci *et al.*, 2004; Morvan and Lanier, 2015; Prager *et al.*, 2019). Of these, interferon-gamma (IFN-γ) has been shown to play a critical role in antiviral defence, enhancing phagocytic activity, and promoting the differentiation of CD4 T helper cells (Schroder *et al.*, 2004; Kang *et al.*, 2018) while TNF contributes to the recruitment and activation of other immune cells, such as macrophages and dendritic cells, further amplifying the immune response (Schroder *et al.*, 2004) and itself can induce apoptotic cell death (Ivashkiv, 2018).

Cytotoxic lymphocytes include different cellular subsets, each with distinct characteristics and functions that cooperatively contribute to the body's immune surveillance and immune-mediated cytotoxicity (Rosenberg and Huang, 2018). CD8 T cells, which recognise small peptides bound to MHC class I molecules via their T cell receptor, are perhaps the most prominent subset of cytotoxic lymphocytes and key drivers of cell-mediated immunity

(Jorgensen *et al.*, 1992; Rosenberg and Huang, 2018). Another crucial subset of cytotoxic lymphocytes are natural killer (NK) cells (Rosenberg and Huang, 2018). They lack the expression of CD3 but express CD56 surface marker. NK cells are different from CD8 T cells in that they do not require prior sensitization or activation to perform their cytotoxic functions (Uzhachenko and Shanker, 2019). Instead, NK cells retain inherent cytotoxicity and can promptly respond to infected or transformed cells (Uzhachenko and Shanker, 2019). NK cells are thought to be important in the early immune response against viral infections and certain types of tumours. Interestingly, like CD8 T cells, major histocompatibility class I (MHC-I) molecules play a central role in regulating the activation of NK cells forming ligands for inhibitory receptors expressed by NK cells (Andersen *et al.*, 2006).

The complementary functions of these two subsets of cytotoxic lymphocytes enhance defence mechanism against viral pathogens and abnormal cells and contributes to the clearance of infections and tumours and the regulation of immune responses.

1.1.1 CD8 T Cells

1.1.1.1 CD8 T Cells and Their Function

CD8 T cells, also known as cytotoxic T lymphocytes, develop in the thymus where their progenitors progress through a positive and negative selection to create a repertoire of T cells that recognise self-encoded MHC class I molecules but that are not overtly reactive to self-peptides presented in the thymus (Golubovskaya and Wu, 2016). CD8 T cells are a fundamental component of the adaptive immune system and play a significant role in eliminating infected or abnormal cells. They exhibit considerable functional diversity with an increasing number of subsets identified based on the expression of distinct combinations of cell surface receptors and transcription factors. Human CD8 T cells have commonly been sub-divided into four different subsets based on the expression of CD27 and CD45RA (Sallusto *et al.*, 1999). These subsets include naive CD8 T cells, effector CD8 T cells, effector memory CD8 T cells, and central memory CD8 T cells (Golubovskaya and Wu, 2016). Naive CD8 T cells (T_N) characterized with the high expression levels of CD45RA and CD27 have not experienced their cognate antigen (Nolz *et al.*, 2011) and predominantly reside in lymphoid tissues, such as lymph nodes and the spleen (Koch *et al.*, 2008). After recognition of cognate antigen, CD8 T_N cells undergo activation and differentiation into effector and/or memory CD8 T cells (Kaech *et al.*, 2002). Effector CD8 T cells downregulate CD45RA and CD27 but upregulate effector

molecules such as granzymes and perforin (Nolz *et al.*, 2011) and acquire strong cytotoxic potential and the capacity to secrete cytokines to respond to target cells. The exact origin of memory CD8 T cells, whether they differentiate directly from effector cells or develop from naive cells with effector-like characteristics while retaining antigen-specific information, remains a subject of ongoing investigation (Farber *et al.*, 2014). They can be further subdivided into central memory T cells T_{CM} ($CD27^+$ $CD45RA^-$) and effector memory CD8 T cells T_{EM} ($CD27^-$ $CD45RA^-$) and terminally differentiated effector memory cells T_{EMRA} ($CD27^-$ $CD45RA^+$) (Sallusto *et al.*, 1999). While T_{EM} and T_{EMRA} show immediate effector function and limited proliferative capacity, both T_{CM} and T_N populations have high proliferative potential and exhibit weak effector function (Gerritsen and Pandit, 2016). Critically CD8 T cells require prior sensitization through recognition of both cognate antigen expressed on the surface antigen presenting cells (APCs) as well as co-stimulation in order to differentiate into effector cells (Shah *et al.*, 2021). An additional population of memory cells has more recently been described which are localised and essentially retained within specific tissues, known as tissue-resident memory CD8 T cells (T_{RM} cells). As such, they are a specialized subset of memory T cells marked by the expression of CD69 and CD103 that offer localized immunity across both a range of mucosal tissues such as gut and skin but also even in some non-mucosal tissues such as the liver (Sasson *et al.*, 2020). In addition to the conventional subsets, there also evidence of populations of CD8 T cells with regulatory characteristics, known as regulatory CD8 T cells (T_{REG}). These cells are thought to play a role in immune tolerance and the suppression of excessive immune responses (Kondělková *et al.*, 2010). CD8 T_{REG} cells are characterized by the expression of regulatory markers, such as FoxP3, as well as the secretion of immunosuppressive cytokines, such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β) (Mishra, 2021).

The activation of CD8 T cells has been proposed to be achieved through a three-step mechanism (Mitchison and O'Malley, 1987; Vivier *et al.*, 2008). This involves the detection of specific antigens presented by MHC-I molecules via clonotypic TCR which triggers signalling via the CD3 complex (Davis and Bjorkman, 1988; Gao and Jakobsen, 2000), leading to the activation of downstream signalling cascades. In addition to TCR engagement, co-stimulatory signals provided by co-receptors, such as CD28 which binds to CD80 or CD86 expressed on APCs are required for the full activation of CD8 T cells (Sharpe, 2009). Finally, differentiation

and proliferation are also impacted by the release of cytokines including as IL-2, IL-7, IL12, and IL-15 (Schluns and Lefrançois, 2003; Geginat *et al.*, 2003; Pearce and Shen, 2007; Bevington *et al.*, 2017) **Figure 1**. Effector CD8 T cells can subsequently respond to foreign peptides presented on MHC-I molecules of the target cell, leading to the induction of the cytotoxic pathways and the secretion of cytokines such as IFN-γ.

CD8 T cells also express a broad range of additional surface receptors that can further regulate their activation, function, and effector responses. These receptors can be widely categorized into two groups: activating receptors and inhibitory receptors. Activating receptors, including NKG2D and CD94/NKG2C are able to recognize stress-induced ligands or specific antigens on target cells, triggering or augmenting signalling pathways that induce cytotoxicity and cytokine production (Van Bijnen *et al.*, 2011; Pump *et al.*, 2019). On the other hand, inhibitory receptors, such as programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), regulate T cell activation to avoid excessive immune response (Raskov *et al.*, 2020).

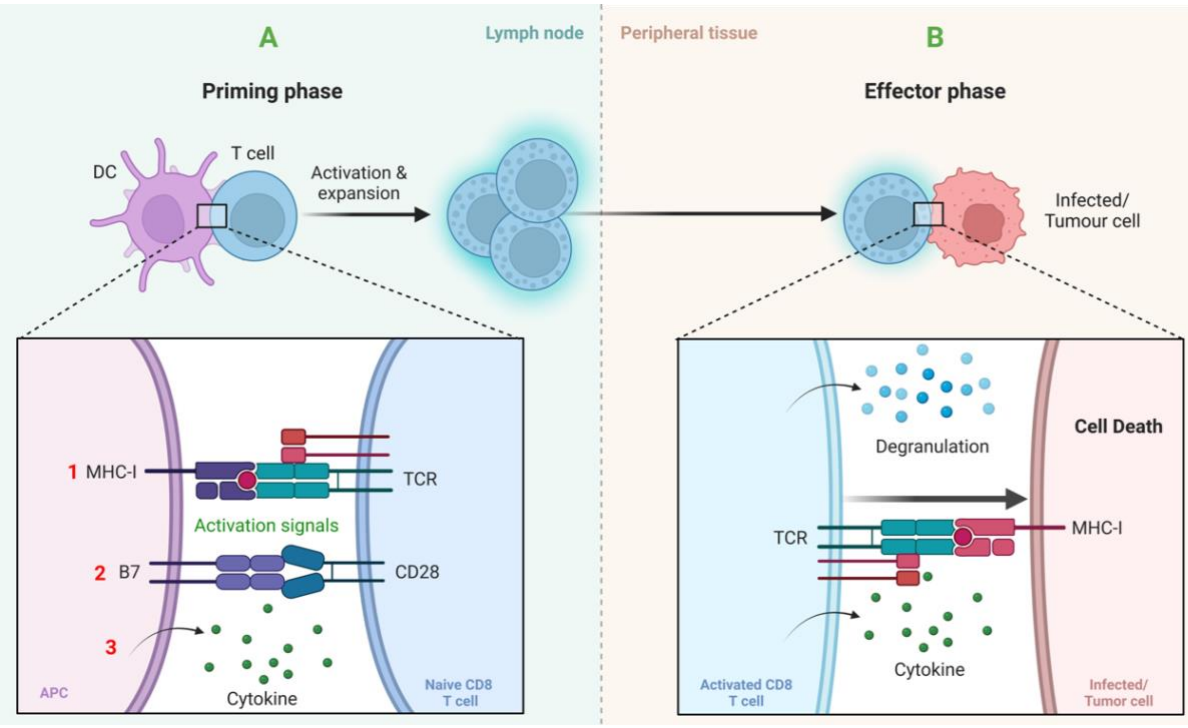

Figure 1. Cytotoxic T cell priming and activation

A) Cytotoxic T cell priming, this requires three different signals: signal 1, the presentation of the antigen on MHC-I molecules of the antigen presenting cell (APC), such as dendritic cell, to the TCR-CD8 complex; signal 2, the co-stimulation that involves the interaction between B7 on APCs and CD28 on CD8 T cells; signal 3 occurs via the cytokines such as IL2 secreted by APCs. B) Effector phase, the recognition of the specific antigen on the infected cells initiates the activation of TCR, which induces Fas mediated cell cytotoxicity and/or granule-dependent cytotoxicity. Abbreviations: MHC-I: major histocompatibility complex class I; TCR: T-cell receptor.

1.1.1.2 *In vitro* Stimulation and Proliferation of CD8 T Cells

Studies analysing the requirements for activation and proliferation of CD8 T cells *in vitro* have provided significant insights into the mechanisms underpinning the induction of T cell effector responses. This has typically been achieved *in vitro* through some form of TCR/CD3 engagement as well as other cell surface receptors. Commonly this has involved the use of monoclonal antibodies (mAbs) to crosslink the CD3 and CD28 receptors which triggers antigen-independent activation and proliferation of T cells *in vitro* (Onlamoon *et al.*, 2006) but a T cell mitogen such as phytohemagglutinin (PHA), (O'Flynn *et al.*, 1986) and superantigens, which all result in a degree of receptor crosslinking have also been used extensively (Arad *et al.*, 2000; Sundberg *et al.*, 2007). Phorbol 12-myristate 13-acetate (PMA) and ionomycin increase intracellular calcium ions through the activation of protein kinase C and calcineurin respectively (Kay, 1991), which lead to the activation and proliferation of T cells.

The expansion of T cells is supported by cytokines such as IL-2, IL-7 and IL-15 (Hombach *et al.*, 2001; Wilkie *et al.*, 2012; Ross and Cantrell, 2018). IL-2, a key cytokine produced by activated T cells, is a potent growth factor for CD8 T cells and supports their expansion and survival (Ross and Cantrell, 2018). IL-7 and IL-15, also contribute to CD8 T cell proliferation and maintenance of memory T cell populations (Hashimoto *et al.*, 2019). Collectively, these signals mimic many of the features of antigen presentation *in vivo*. This process involves intracellular signalling pathways, including the mitogen-activated protein kinase (MAPK) pathways ERK1 and ERK2 (D'Souza *et al.*, 2008), and phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Shah *et al.*, 2021), and nuclear factor of activated T cells (NFAT) (Macian, 2005). The activation and translocation of nuclear factor-kappa B (NF-κB) is also important in the immune response following TCR activation (Arima *et al.*, 1992). When NF-κB becomes active, it triggers the expression of essential cytokines such as IL-2 and IFN-γ which further perpetuates the loop of T cell activation and survival (Daniels *et al.*, 2023). These signalling pathways are critical for the regulation of the expression of genes involved in T cell activation, proliferation, and effector functions.

1.1.2 NK Cells

1.1.2.1 NK Cells and Their Function

NK cells are central components of the innate immune system and play a critical role in the defence against infections and the surveillance of malignant cells (Andoniou *et al.*, 2008). They are lymphocytes that belong to the innate lymphoid cell (ILC) lineage, which also includes lymphoid tissue-inducer cells and ILC1 (NK cells), ILC2, and ILC3 subsets (Cortez and Colonna, 2016). ILCs are characterized by the lack of recombination activating gene (RAG)-dependent rearranged antigen-specific receptors (Lopes *et al.*, 2023). Among ILCs, NK cells are distinct due to their strong cytotoxic response against infected or transformed cells without prior sensitization. Additionally, they can also have the capacity to secrete various cytokines and chemokines upon activation which can have both direct antiviral and anti-tumour effects as well more broadly impacting the nature of the response through their immunoregulatory properties (Abel *et al.*, 2018).

NK cells, commonly identified by the expression of CD56 paired with the lack of cell surface CD3, play a crucial role in the early immune response by detecting and eliminating abnormal cells without prior sensitization. (Lopes *et al.*, 2023). Upon target cell recognition, they can release cytotoxic molecules and secrete a range of cytokines and chemokines including IFN-γ, TNF and CCL4 contributing to immune regulation (Paul and Lal, 2017). NK cells also express characteristic combinations of activating/co-activating and inhibitory receptors which regulate their function (Shimasaki *et al.*, 2020; Björkström *et al.*, 2021). The activating receptors include natural cytotoxicity receptors (NCRs), such as NKp30 and NKp46, CD244, DNAM-1 as well as NKG2D, the latter of which recognizes stress-induced proteins such as the MHC class I chain-related polypeptide A (MIC-A) and MIC-B expressed on the surface of infected or stressed cells. Perhaps the best characterised inhibitory receptors are those that recognize MHC-I molecules such as members of the KIR, Ly49, CD94-NKG2 and LILR families of receptors (Paul and Lal 2017). The balance between activating and inhibitory signals is thought to regulate the activation of NK cells and their potential to respond to infected or transformed target cells. As such, NK cell activation is strictly regulated by a complex interplay between activating and inhibitory receptors. Diminished signalling from the inhibitory receptors that can ensue following virus- or transformation associated reductions in MHC-I expression allows for the propagation of activating signals and ultimately NK cell activation.

This phenomenon is known as "missing self-recognition" (Ljunggren and Kärre, 1990). However, NK cells can also be activated by increased signalling through activating receptors as a result of the induction of expression of stress-induced ligands such as MIC-A and MIC-B or exposure to innate cytokines (Paul and Lal, 2017) **Figure 2**. Several cytokines have been recognised as potent activators of NK cells with IL-2, IL-5, IL-12, and IL-15 playing important roles in NK cell development, survival, and activation. IL-2 is particularly important for NK cell expansion, while IL-12 and IL-15 enhance NK cell cytotoxicity and cytokine production (Wu *et al.*, 2017). Similarly interferon-alpha (IFN-α) and IL-18 can also contribute to NK cell activation and promote their effector functions (Walzer *et al.*, 2005).

As indicated above, upon activation, NK cells release a range of effector cytokines, including IFN-γ, TNF, and GM-CSF (Paul and Lal, 2017). IFN-γ is a key player in antiviral defence and immunity against intracellular pathogens. It activates macrophages, enhances antigen presentation, and promotes the differentiation of T cells into a Th1 phenotype, fostering cellular immunity (Schroder *et al.*, 2004). Furthermore, it plays a crucial role in cancer immunosurveillance by inhibiting tumour cell proliferation and promoting clearance (Shankaran *et al.*, 2001). On the other hand, TNF is implicated in the initiation of inflammatory responses against infections and other challenges, promoting immune cell activation and broadly contributing to host defence mechanisms (Aggarwal, 2003). GM-CSF promotes to the recruitment and activation of macrophages and dendritic cells (Schroder *et al.*, 2004). In addition to cytokine secretion, NK cells produce a range of chemokines that boost the immune response. These include CCL3 and CCL4, which are chemotactic factors that primarily attract monocytes, macrophages, and T cells to sites of inflammation or infection (Gismondi *et al.*, 2010). NK cells also produce CCL5, which is involved in the recruitment of various immune cells, including T cells, eosinophils, and basophils, playing a role in regulating immune responses and associated with the activation of specific immune cells (Mariani *et al.*, 2002). Additionally, IL-8, another chemokine produced by NK cells (Gismondi *et al.*, 2010), is responsible for recruiting and activating neutrophils (Van Damme *et al.*, 1988), a crucial component of the innate immune system. Thus, NK cells have the potential not only to directly combat infected or transformed cells but to play a significant role in orchestrating the nature of the broader immune response.

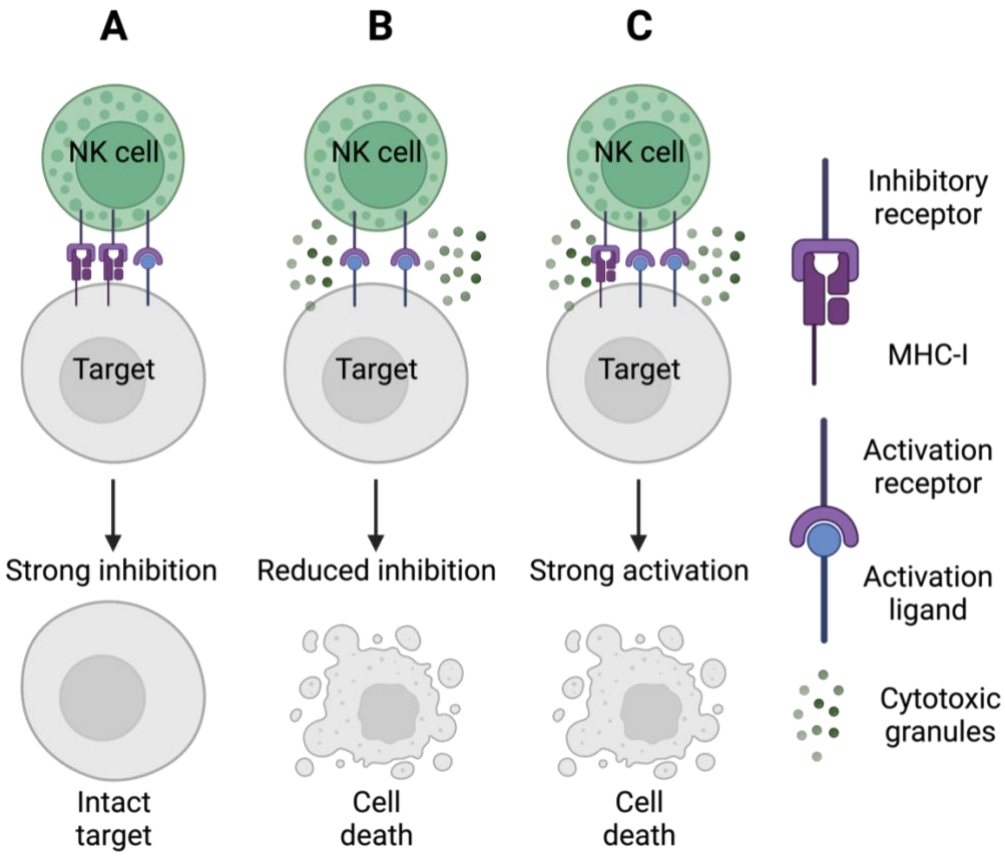

Figure 2. Target cell recognition by NK cells

A) Strong inhibition: NK cell response is not initiated if there is a less signal by the activating ligands than by the inhibitory MHC-I molecules. B) Reduced inhibition: the engagement of the activating ligands with activating receptors on NK cells in the absence of MHC-I molecule induces strong NK cell cytotoxicity, C) Strong activation: NK cell responds when the activating signals are stronger than the inhibitory signals by MHC-I molecules. Abbreviations: MHC-I: major histocompatibility complex class I; NK, natural killer.

1.1.2.2 *In vitro* Activation of NK Cells

In vitro activation of NK cells refers to the process of stimulating resting NK cells obtained from peripheral blood or other sources. This approach allows the study of NK cells function and evaluation of their effector activity under controlled conditions. Various methods have been established to activate NK cells *in vitro*, employing different stimulatory factors and techniques. Cytokine stimulation: One commonly used approach for NK cell activation involves the use of cytokines. Cytokines such as IL-2, IL-15, and IL-12 have been shown to effectively activate NK cells (Terme *et al.*, 2008). These cytokines can be added to NK cell cultures either individually or in combination, leading to the upregulation of activating receptors, enhanced cytotoxicity, and increased production of pro-inflammatory cytokines by

(Granzin *et al.*, 2017; Peighambarzadeh *et al.*, 2020). IL-2 has a potent effect on NK cell cytotoxicity (Trinchieri *et al.*, 1984; Phillips and Lanier, 1986) and can also stimulate the proliferation of a minority of NK cells (Trinchieri *et al.*, 1984; London *et al.*, 1986; Lanier *et al.*, 1988). Other interleukins such IL-4, IL-7, and IL-12 induce some NK cell proliferation but to a lesser extent than IL-2 (Robertson *et al.*, 1993). Antibody-mediated activation: Antibodies targeting specific activating receptors such as CD16 (FcγRIIIa), which mediates antibody-dependent cellular cytotoxicity (ADCC), can also be employed to induce NK cell activation (Uggla *et al.*, 1989; Capuano *et al.*, 2021). Typically, this has involved crosslinking monoclonal antibodies specific for specific activating receptors on the surface of NK cells, to mimicking the signals received during recognition of target cells. This approach can be used to activate NK cells and study their functional responses against tumour cells expressing the corresponding ligands (Lanier, 2008). Co-culture systems: NK cell activation can also be achieved through co-culture systems, where NK cells are cultured in the presence of target cells or accessory cells (Das *et al.*, 2001; Amakata *et al.*, 2001; Kim *et al.*, 2020). The interactions between NK cells and these target or accessory cells trigger activating signals, leading to the activation of NK cells. Co-culture systems can mimic the complex interactions that occur in the tumour microenvironment, providing valuable insights into the mechanisms underlying NK cell activation and their interactions with tumour cells. These systems have most commonly used cell lines, such as the human immortalized Epstein-Barr virus (EBV) transformed B-lymphocytes (721.221 cells) and the chronic myelogenous leukaemia cell line K-562 that do not express MHC-I molecules (Perussia *et al.*, 1987; Uggla *et al.*, 1989; Igarashi *et al.*, 2004; Saunders *et al.*, 2016; Moradi *et al.*, 2021).

1.2 Viral Infection and Cytotoxic Lymphocytes

Viral infections are a significant threat to the human body and consequently understanding the interactions between viruses and immune cells is crucial for developing effective strategies to develop better protective responses to such infections. In the immune response towards viral infections, NK cells and CD8 T cells assume distinct roles in identifying and eradicating infected cells. Consequently, direct infection of such cells by the virus, may have significant implications for their functions. Moreover, almost by definition, cytotoxic lymphocytes may be particularly susceptible to viral infection due to their requirement for direct exposure to virus-infected cells during the cellular conjugation processes involved in

their cytotoxic response. This exposure could potentially increase their risk of direct infection and ultimately impact their capacity to elaborate effector functions or their survival, proliferation and differentiation.

1.2.1 Indirect Effect of Viral Infection on Cytotoxic Lymphocytes

Viral infections have the capacity to impact the function of both CD8 T cells and NK cells. Most obviously, viral antigens expressed in the context of self-MHC class I molecules on the surface of APCs lead to engagement of TCR and the initiation of signalling events that prime them for effective targeting and elimination of virus-infected cells (Hilleman, 2004). Once activated, CD8 T cells undergo clonal expansion, resulting in the generation of expanded clones of antigen-specific CD8 T cells (Seder and Ahmed, 2003). This expansion allows for an amplified immune response against the viral infection. Unlike CD8 T cells, the activation of NK cells is typically not centred on a single clonotypic receptor but by modulation in signals received from an array of activating and inhibitory receptors (Paul and Lal, 2017). However infected cells can release pro-inflammatory cytokines, such as IL-12 and IFN-I, as part of their antiviral response (Dalod *et al.*, 2002). These cytokines act as alarm signals and play a crucial role in promoting the activation of both CD8 T cells and NK cells and shaping the nature of their response. IL-12 is a potent cytokine that stimulates the production of IFN-γ and TNF by CD8 T cells (Vacaflores *et al.*, 2017). Additionally, IL-12 can promote the differentiation of CD8 T cells into effector cytotoxic T cells, further boosting their antiviral functions (Bhardwaj *et al.*, 1996; Pearce and Shen, 2007). Moreover, both IL-12 and type I IFNs provide CD8 T cell signal 3 in the process of T cell activation, enhancing cell expansion (Keppler *et al.*, 2012). Type I interferons have pleiotropic effects and play a crucial role in activating CD8 T cells and NK cells. They enhance their effector function and promote their survival and proliferation (Kolumam *et al.*, 2005; Madera *et al.*, 2016; Kwaa *et al.*, 2019). The cytokines produced by infected cells create a positive feedback loop, further amplifying the immune response. The activation of CD8 T cells and NK cells by these cytokines leads to the elimination of infected cells, which in turn reduces viral loads. As a result, the cytokines produced by infected cells helps to strengthen the antiviral immune response mediated by CD8 T cells and NK cells (McNab *et al.*, 2015).

On the other hand, persistent exposure to antigens such in chronic viral infection can also lead to the exhaustion of CD8 T cell. This exhaustion is characterized by the continuous loss of effector function and an altered transcriptional profile, limiting their potential to effectively control viral infection or cancer (McLane *et al.*, 2015). Additionally, tissue damage can also create bystander effects on CD8 T cells which can cause excessive activation, leading to inflammation and bystander exhaustion of CD8 T cells and ultimately impair their function (Kim and Shin, 2019). Similarly, the high systemic levels of cytokines seen in cytokine storms such as those associated with severe SARS-CoV-2 infection have been shown to impair NK cell cytolytic function and survival (Osman *et al.*, 2020; Ghasemzadeh *et al.*, 2022).

Many viruses have developed strategies to evade the immune system by directly modulating the host immune response, affecting the recognition and activation of NK cells or T cells (Van Erp, Van Kampen, *et al.*, 2019). Viral proteins, like influenza HA, have the capacity to disrupt NK cell signalling pathways, including those involving NKp46 and NKp30, which can diminish their cytotoxicity and cytokine production (Mao *et al.*, 2010). Bystander effects on NK cells occur through the inability of blood circulating dendritic cells to properly respond to viral infections such as HIV, which are essential for initiating and regulating immune responses. Impaired activation and maturation of dendritic cells indirectly impact NK cell activation, thereby limiting their antiviral capabilities (Altfeld *et al.*, 2011).

1.2.2 Direct Viral Infection of Cytotoxic Lymphocytes

While cytotoxic lymphocytes, including NK cells and cytotoxic T lymphocytes, are primarily known for their specialized function to eliminate virus-infected cells, their inherent proximity to infectious organisms in processes associated with conjugation to target cells associated with the delivery of cytolytic granules, has the potential to elevate their susceptibility to direct infection. In theory, direct viral infection of cytotoxic lymphocytes may affect their cytotoxicity, cytokine production, or indeed impair target recognition by modulating the expression of key activating/inhibitory receptors manipulating their ability to eliminate virus-infected cells.

Various DNA viruses have been shown to infect NK cells, of which some resulted in manipulating the function of the NK cells. Viral DNA of Torque teno virus (TTV) was detected by real-time PCR in NK and T cells isolated from viraemic individuals suggesting *in vivo*

infection of human NK and T cells. During primary Epstein Bar Virus (EBV) infection in patients with infectious mononucleosis, a small number of latently infected nonneoplastic NK cells have been detected, demonstrating that NK cells can also be targets of EBV during early infection (Trempat *et al.*, 2002; George *et al.*, 2012). *In vitro*, NK cells cocultured with EBV-infected autologous CD21+ B cells during early EBV infection, acquired a weak CD21+ phenotype, allowing EBV binding to NK cells through a trans-synaptic acquisition of viral receptor (Tabiasco *et al.*, 2003). Further *in vitro* studies have confirmed EBV entry into cultured NK cells by establishing EBV-carrying NK cell clones (Isobe *et al.*, 2004). Moreover, a male patient with an acute hepatitis-like illness and increased peripheral blood large granular lymphocytes (LGLs) with an NK cell phenotype was found to have a malignant lymphoproliferative condition where EBV genomic DNA was identified within the clonal LGLs (Hart *et al.*, 1992). Additionally, in situ hybridisation (ISH), reverse transcriptase-polymerase chain reaction (RT-PCR), and immunohistochemistry (IH) analysis showed that both NK cell and T cell subtypes of nasal lymphomas expressed EBV proteins in a heterogeneous pattern (Chiang *et al.*, 1996).

In vitro infection of Herpes simplex virus (HSV) impairs NK cell function upon contact with infected fibroblasts, and this impairment is facilitated through cell-cell contact (Johnson and York, 1993). However, the exact mechanisms causing the modulation of NK cells by HSV are not yet clearly known. Similarly, *in vitro* experiments have demonstrated that Varicella zoster virus (VZV) is able to infect NK cells and resulted in the release of infectious particles. This infection upregulated CD57 and chemokine receptors, while downregulating CD56 and FcγRIII expression on the NK cells (Campbell *et al.*, 2018). NK cells co-cultured with Vaccinia virus (VV)-infected target cells also resulted in NK cell infection that both increased inhibitory KIR signalling and decreased cytotoxicity (Sánchez-Puig *et al.*, 2004; Kirwan *et al.*, 2006).

RNA viruses have also shown the ability to infect NK cells. For example, the viral RNA of human pegivirus (HPgV) from the Flaviviridae family was also detected in NK and CD8 T cells from virus-infected individuals (Chivero *et al.*, 2014). A number of studies have investigated the capacity of influenza A (IAV) to infect NK cells yielding somewhat contradictory findings. Casali et al. have shown that IAV infection of NK cells did not affect their cytolytic activity (Casali *et al.*, 1984). However, other studies showed that IAV infection of NK cells was associated with

increased apoptosis and therefore decreased cytotoxicity and cytokine production, but increased degranulation and CD69 expression, the early activation marker (Mao *et al.*, 2009; Guo *et al.*, 2009a; Mao *et al.*, 2010; Lin *et al.*, 2012; Mao *et al.*, 2017). Measles virus (MV) infection of NK cells does not alter their survival *in vitro*, however it did result in decreased cytotoxic activity (Casali *et al.*, 1984). Respiratory syncytial virus (RSV) has been shown to infect adult and neonatal NK cells *in vitro* and resulted in enhanced IFN-γ production and KIR expression, including inhibitory and activation KIRs, but reduced NK cell cytotoxicity (Krzyzaniak *et al.*, 2013; Van Erp, Feyaerts, *et al.*, 2019). Vesicular stomatitis virus (VSV) productively infected different NK cell lines and reduced their cytolytic function (Rosenthal *et al.*, 1986). In the context of HIV infection, despite not expressing surface CD4 there is some evidence to suggest that NK cells can be infected by the virus *in vitro*, (Chehimi *et al.*, 1991; Valentin *et al.*, 2002; Valentin and Pavlakis, 2003; Harada *et al.*, 2007; Bernstein *et al.*, 2009). A subset of NK cell express CD4 and HIV coreceptors CCR5 or CXCR4, allowing for virus entry that results in productive and persistent infection leading to reduced NK cell activity and apoptosis (Chehimi *et al.*, 1991; Valentin *et al.*, 2002; Valentin and Pavlakis, 2003; Harada *et al.*, 2007; Bernstein *et al.*, 2009). HTLV-I infection of NK cells *in vitro*, which can be achieved through activation with an anti-CD16 monoclonal antibodies and co-culture with HTLV-I-producing T cells, enhances IL-2-dependent proliferation without impairing NK cell cytotoxic functions. Interestingly, unlike T lymphocytes, HTLV-I-infected NK cells do not become immortal (Yamamoto *et al.*, 1982; Igakura *et al.*, 2003). Overall, infection of cytotoxic lymphocytes by several viruses has shown different effects on the cellular viability, effector function, and phenotype, however, the mechanisms underlying these changes are poorly understood.

In the realm of CD8 cells, there are relatively few studies discussing direct viral infection of these cells, whether *in vivo* or *in vitro*. Research involving both human and mouse models has demonstrated that CD8 T cells can be susceptible to viral infections. In individuals experiencing the prodromal stage of measles, measles virus (MV)-infected memory CD8 T cells were identified circulating in peripheral blood. Notably, there was a remarkable rise in infected cells within the CD8 effector memory cell subset, in part reflecting the prominent role of this subset in the direct control of infected cells (Laksono, de Vries, *et al.*, 2018). *In vitro* studies have confirmed that MV can undergo replication in T lymphocytes, including CD8

T cells (Laksono, Grosserichter-Wagener, *et al.*, 2018). *Ex vivo* infection of CD8 T cells by HHV-6 in lymphoid tissue was also associated with a significant depletion of these cells (Grivel *et al.*, 2003). Furthermore, both *in vitro* HHV-6 infection and stimulation of CD8 T cells led to the upregulation of CD4 protein on mature CD8 T cells, making them susceptible to HIV infection (Lusso *et al.*, 1991; Kitchen *et al.*, 1998). Viral RNA of EBV has been detected in CD8 T cells isolated from blood of patients with EBV-positive T-cell lymphoproliferative disease (Kim *et al.*, 2019). In addition, the EBV-2 strain efficiently infected purified CD8 T cells, leading to cell activation characterized by CD69 upregulation, increased proliferation, and altered cytokine expression (Coleman *et al.*, 2015). Studies in mouse models have also provided evidence that certain RNA viruses, such as lymphocytic choriomeningitis virus (LCMV) (Trapecar *et al.*, 2018) and Influenza A virus (IAV) (Manicassamy *et al.*, 2010), can directly target and infect CD8 T cells. Furthermore, while HTLV-I predominantly infects CD4 T cells, there is some evidence suggesting that CD8 T cells may also serve as an additional reservoir for HTLV-I in infected patients. This is shown by the detection of a high copy number of HTLV-I proviral DNA in purified CD8 T cells (Nagai *et al.*, 2001).

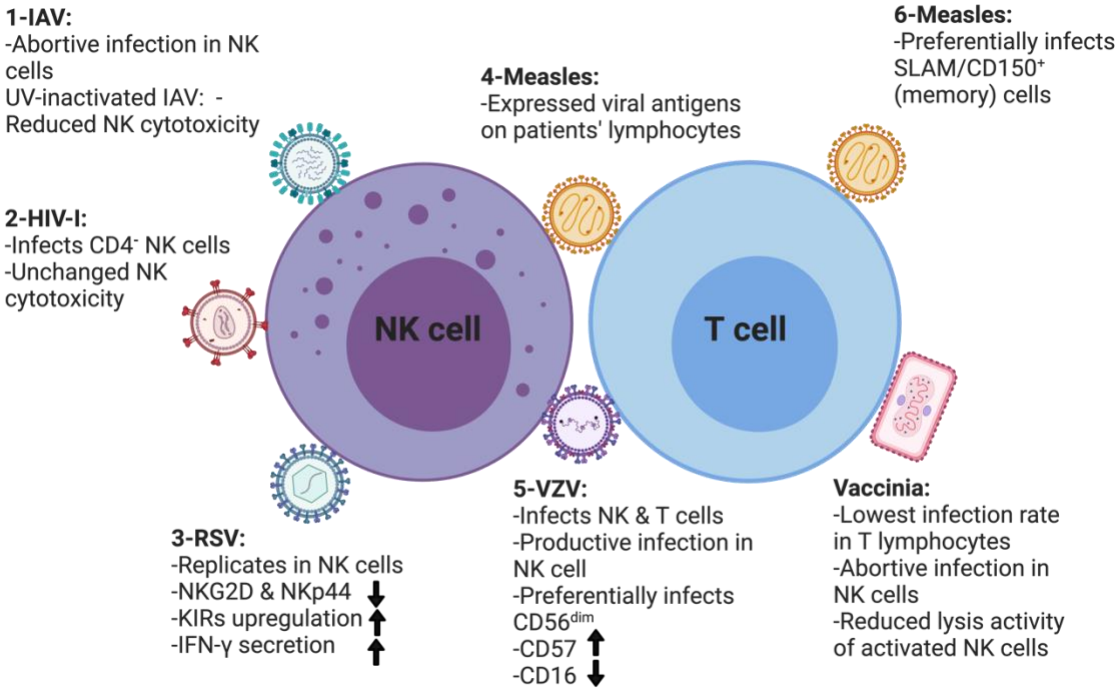

Figure 3.Examples for Viral Infection of Cytotoxic Lymphocytes

Graphical demonstration of some examples for viruses that are known to infect NK cells and T cells with the impact on their effector function and phenotype (Casali *et al.*, 1984; Sánchez-Puig *et al.*, 2004; Kirwan *et al.*, 2006; Krzyzaniak *et al.*, 2013; Chivero *et al.*, 2014; Laksono, de Vries, *et al.*, 2018; Van Erp, Feyaerts, *et al.*, 2019; Van Erp, Van Kampen, *et al.*, 2019).

1.2.3 Influenza A Virus (IAV)

Influenza A virus, also known as the flu, is a highly transmissible respiratory infection that cause a significant threat to public health worldwide. The virus belongs to the Orthomyxoviridae family and undergoes rapid genetic changes, making it a constant challenge for healthcare professionals to combat (Tong *et al.*, 2013). Influenza A virus is an enveloped, single-stranded RNA virus with eight segments (Tong *et al.*, 2013). The virus is classified into subtypes based on two proteins on its surface: hemagglutinin (HA) and neuraminidase (NA). Currently, there are 18 known HA and 11 NA subtypes, with the H1N1 and H3N2 subtypes being the most common in human infections (Tong *et al.*, 2013). The first line of defences against IAV are predominantly elements of the innate immune system. Upon infection, host cells recognize the viral components using pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) (Kato *et al.*, 2006). Viral RNA, especially the panhandle structure at the ends of the viral RNA segments, is a potent inducer of the antiviral response (Takeuchi and Akira, 2009). Once the PRRs detect the presence of viral RNA, they drive the of type I interferons (IFN-I) response and establish an antiviral state in cells (Ivashkiv and Donlin, 2014). However, Influenza A virus has evolved various strategies to evade the host's immune response, including interference with IFN-I production and signalling.

1.3 Nucleic Acid Sensing

1.3.1 Pattern Recognition Receptors

Pattern recognition receptors (PRRs) are a crucial component of the innate immune system, playing a pivotal role in the detection of invading pathogens and initiating the host's immune response (Amarante-Mendes *et al.*, 2018). PRRs are evolutionarily conserved receptors that recognize conserved molecular patterns associated with various microorganisms, including bacteria, viruses, fungi, and parasites (Amarante-Mendes *et al.*, 2018). Their ability to recognize pathogen associated molecular patterns (PAMPs) enables the host to quickly respond to potential threats, thereby providing the first line of defence and initiating an adaptive immune response against infections. PRRs are broadly expressed in various cell types, including immune and non-immune cells, allowing for a coordinated response to infections throughout the body (Li and Wu, 2021). They are categorized into several families,

including, NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and Toll-like receptors (TLRs), RIG-I-like helicases (RLHs) (Amarante-Mendes *et al.*, 2018). Each family of PRRs recognizes distinct PAMPs, and their activation triggers specific signalling pathways to initiate an immune response tailored to the invading pathogen. NOD-like receptors (NLRs) are mainly located in the cytosol and are involved in detecting bacterial PAMPs (Platnich and Muruve, 2019). NLR activation leads to the formation of inflammasomes, resulting in the proteolytic activation of Caspase 1 which cleaves and activates pro-forms of the pro-inflammatory cytokines IL-1β and IL-18 (Platnich and Muruve, 2019). C-type lectin receptors (CLRs) recognize carbohydrates on the surface of pathogens, such as fungi and parasites (Geijtenbeek and Gringhuis, 2016). These receptors are involved in phagocytosis, the induction of antimicrobial responses, and the shaping of adaptive immune responses (Geijtenbeek and Gringhuis, 2016). Toll-like receptors (TLRs) are among the most well-characterized PRRs and are primarily localized on the cell surface or within endosomes (El-Zayat *et al.*, 2019). TLRs detect various microbial components, such as lipopolysaccharides (LPS), lipoproteins, and nucleic acids, initiating signalling pathways that lead to the production of pro-inflammatory cytokines and type I interferons (IFNs) (McNab *et al.*, 2015). Some TLRs and receptors described in the following chapter recognize viral nucleic acids. The recognition of viral RNA by TLR3, TLR7, TLR8, and TLR9 leads to induction of signalling pathways which induce the expression of antiviral defence components (Lind *et al.*, 2021). RIG-I like receptors (RLRs) are cytosolic PRRs that recognize base paired RNA (Rehwinkel and Gack, 2020). Activation of the RLR RIG-I and MDA5 by dsRNA triggers the production of type I IFNs and pro-inflammatory cytokines, essential for the immune response and clearance of viral infections (Rehwinkel and Gack, 2020).

1.3.2 Nucleic Acid Receptors

Nucleic acid receptors are a critical component of the innate immune system, responsible for detecting the presence of viral and microbial nucleic acids in the cytoplasm and endosomes of cells. These specialized germline-encoded sensors are present in specific cellular compartments and have the ability to recognize unique nucleic acid sequences, nucleic acid modifications, or structures associated with viral infections (Schlee *et al.*, 2009; Schlee and Hartmann, 2016; Bartok and Hartmann, 2020). Nucleic acid receptors play a crucial role in initiating host defence mechanisms against invading pathogens and triggering antiviral

immune responses. Among the key nucleic acid sensors are RIG-I-like receptors (RLRs), cyclic GMP-AMP synthase (cGAS), and endosomal Toll-like receptors (TLRs), each recognizing distinct nucleic acid patterns and activating specific signalling pathways (Schlee *et al.*, 2009; Schlee and Hartmann, 2016; Bartok and Hartmann, 2020) **Figure 4**. RIG-I is a cytosolic sensor that specifically detects short double-stranded RNA (dsRNA) with 5' triphosphate or diphosphate termini, which are characteristic features of viral RNA genomes and nascent transcripts (Goubau et al. 2014; Hornung et al. 2006; Rehwinkel et al. 2010; Rehwinkel and Gack 2020; Schlee 2013; Schlee et al. 2009). Upon binding to viral RNA, RIG-I undergoes a conformational change that leads to release of its caspase activation and recruitment domains (CARDs) (Yoneyama *et al.*, 2015). The free RIG-I CARDs then interact with the mitochondrial antiviral-signalling protein (MAVS), which recruits and activate the kinases TBK1/IKKε and IKKα/IKKβ which activate IRF3/7 and nuclear factor kappa B (NF-κB) transcription factors, subsequently inducing the transcription and secretion of antiviral cytokines belonging to the type I interferon family (IFN-I), as well as pro-inflammatory cytokines and chemokines (Seth *et al.*, 2005; Rehwinkel and Gack, 2020; Bartok and Hartmann, 2020). cGAS is a sensor of cytosolic base paired DNA, a component of viral DNA genomes and replication intermediates of retroviruses (Sun *et al.*, 2013). Upon binding to cytosolic DNA, cGAS catalyses the synthesis of cyclic GMP-AMP (cGAMP), a second messenger that activates the stimulator of interferon genes (STING) (Sun *et al.*, 2013). Activated STING then recruits and activates TBK1 and IKKε, leading to the phosphorylation of IRF3 and subsequent production of type I IFNs and pro-inflammatory cytokines (Ishikawa and Barber, 2008). This cGAS-STING pathway serves as a key antiviral defence mechanism against DNA viruses and retroviruses (D. Gao *et al.*, 2013). TLR3, TLR7, and TLR8 are members of the Toll-like receptor family, primarily located in the endosomes of immune cells (Lind *et al.*, 2021). TLR3 recognizes double-stranded RNA, while TLR7 and TLR8 specifically recognize single-stranded RNA with GU-rich motifs (Lind *et al.*, 2021). Upon binding to viral RNA, these TLRs recruit MyD88 and trigger downstream signalling, leading to the activation of NF-κB and IRF7 (Kusiak and Brady, 2022), resulting in the production of type I IFNs and pro-inflammatory cytokines. Together with other nucleic acid sensors, such as TLR9 that recognizes unmethylated CpG motifs in DNA from bacteria and certain viruses (Dongye *et al.*, 2022), they play a crucial role in detecting viral and microbial nucleic acids, initiating potent antiviral

immune responses that inhibit viral replication, and coordinate the immune response against invading pathogens **Figure 4**.

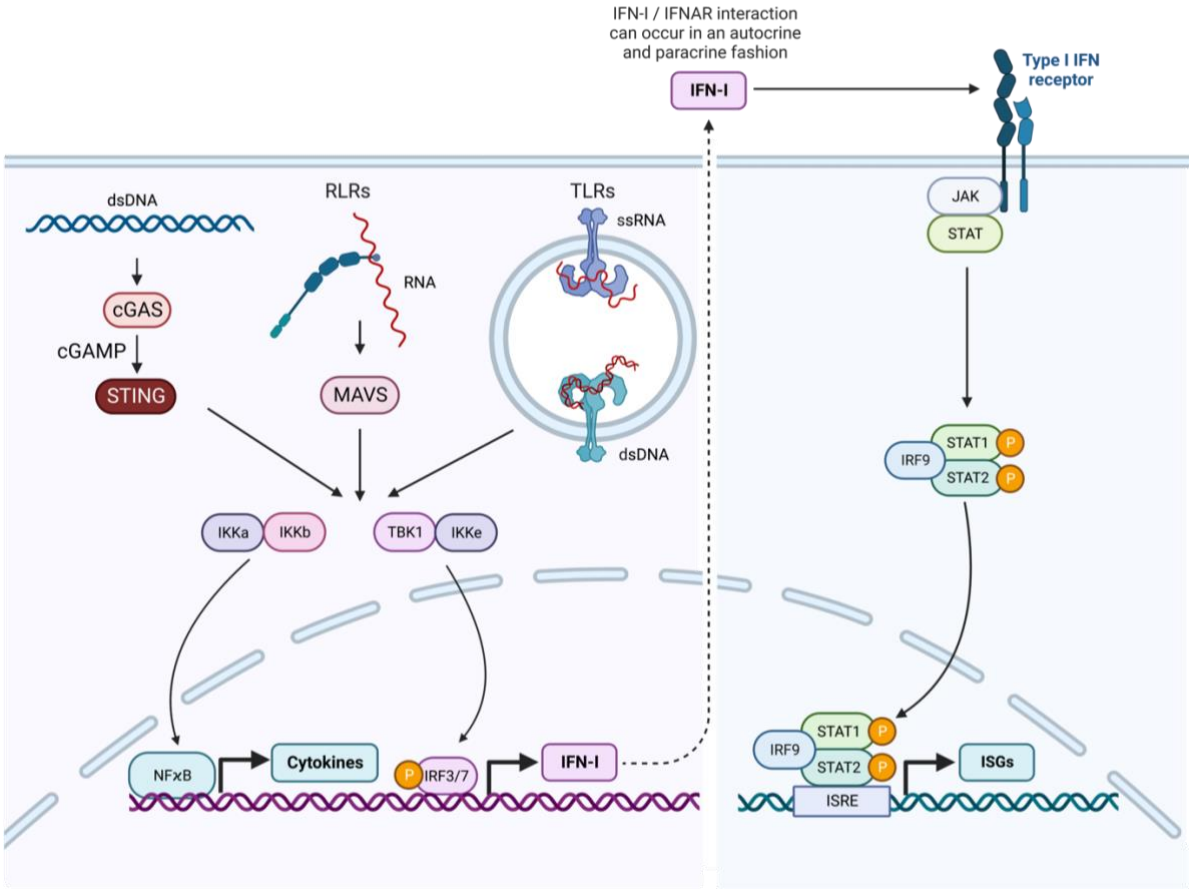

Figure 4. Nucleic acid sensing by different intracellular PRRs

Mammalian cells recognize nucleic acid derived by pathogens such as viruses. This occurs through different intracellular PRRs. Four different TLR family members TLR3, TLR7, TLR8 and TLR9 that are located in the endosomal compartment, where they detect RNA and DNA, leading to activation of TRIF- or MyD88-dependent pathways and consequently induce IRF and NF-κB pathways to release type I IFNs and pro-inflammatory cytokines. Cytosolic RLRs (RIG-I and MDA5) sense viral RNA, then signal through the mitochondria-localized signalling adaptor MAVS. Triphosphorylated double-stranded(ds) RNA (3p-dsRNA) is a strong ligand for RIG-I. Viral DNA is sensed by cGAS and signal via STING to activate the TBK1–IRF3 pathway. cGAS is known to synthesize the cyclic dinucleotide cGAMP after DNA sensing, which itself stimulates STING. In turn, secreted IFN-I can act in an autocrine manner to signal through IFNAR (interferon alpha/beta receptors) leading to the induction of the interferon stimulated genes (ISGs) via STAT1/STAT2/IRF9 complex.

1.3.3 Role of Type I Interferon in Innate Immunity

Type I interferon (IFN-I) cytokines play a vital role in orchestrating the immune response against viral infections and malignancies. In humans, the type I IFN family consists of multiple forms, including 13 IFN-α subtypes and a single IFNβ gene. Additionally, lesser-known family members such as IFN-ε, IFN-κ, and IFNω can also be induced (McNab *et al.*, 2015; Schoggins, 2018). Canonical type I IFN signalling via signal transducer and activator of transcription 1 and 2 (STAT1 & STAT2), STAT2 and IRF9 induce a broad antiviral program of interferon-stimulated

genes (ISG) devoted to restricting viral replication and spread (McNab *et al.*, 2015; Schlee and Hartmann, 2016; Schoggins, 2018; Rehwinkel and Gack, 2020), including nucleic acid receptors, such as RIG-I itself, and direct antiviral effectors, including the interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), which sequesters 5' tri-phosphorylated or cap0 bearing RNA. Specific agonists for such receptors have been explored as antiviral and antitumor agents (Van den Boorn and Hartmann, 2013; Coch *et al.*, 2017; Rehwinkel and Gack, 2020; Marx *et al.*, 2022; Y. Jiang *et al.*, 2023).

Beyond their well-established antiviral properties, IFN-I has emerged as a key regulator of immune cell function, with significant impacts on NK cells and CD8 T cells. Rapidly produced in response to pathogen encounters, IFN-I bridges the gap between the innate and adaptive immune responses, influencing various immune cell subsets (Stetson and Medzhitov, 2006; McNab *et al.*, 2015). IFN-I exposure boosts NK cell cytotoxicity and cytokine secretion, bolstering their capacity to eliminate infected or malignant cells. Furthermore, IFN-I aids in NK cell maturation and memory development, enabling sustained protection against recurring infections (Madera *et al.*, 2016; Müller *et al.*, 2017). IFN-I also enhances the differentiation of naïve CD8 T cells into potent effector cells, fortifying their cytotoxic potential and survival. Additionally, IFN-I contributes to memory CD8 T cell formation, conferring long term immunity (Welsh *et al.*, 2012). The potential of IFN-I, whether directly or by the induction of intrinsic pathways associated with nucleic acid sensing, to enhance immune cell function holds promise for immunotherapeutic strategies. Targeted IFN-I induction or IFN-I administration may bolster the immune response against challenging infections and cancers. Combining IFN-I with other immunomodulatory agents, such as immune checkpoint inhibitors, presents opportunities for improved efficacy and overcoming tumour immune evasion (Swann and Smyth, 2007). However, harnessing nucleic acid receptors in NK and CD8 T cells to induce IFN-I intrinsically for immunotherapy is yet to be investigated. Understanding the intricate crosstalk between nucleic acid sensing, IFN-I signalling and other immune function is essential for fine-tuning the cytotoxic response of NK and CD8 T cells to maximize the antiviral and antitumor therapeutic benefits.

1.3.4 Non-viral Stimulation of Nucleic Acid Receptors

Non-viral stimulation of pattern recognition receptors is an emmerging approach to study innate immune responses without actual viral infections. Exogenous and synthetic ligands serve as powerful tools to activate PRRs like RIG-I, cGAS, and TLR7/8 and investigate their signaling pathways (Cui *et al.*, 2008; Kato *et al.*, 2008; P. Gao *et al.*, 2013). For RIG-I activation, synthetic ligands like 3p-dsRNA (5'tripolyphosphorylated double-stranded RNA) closely mimic viral RNA structures and can be introduced into cells through transfection methods such as lipofection or electroporation (Ablasser *et al.*, 2009; P. Gao *et al.*, 2013; Chiang and Gack, 2017). Similarly, cGAS is triggered by exogenous pathogen-derived DNA or synthetic small molecules like cyclic dinucleotides, which efficiently activate cGAS's catalytic activity (Ablasser *et al.*, 2009; D. Gao *et al.*, 2013; Sun *et al.*, 2013; P. Gao *et al.*, 2013). These ligands can also be introduced into cells using transfection methods like lipofection or electroporation for pDNA (Orzalli *et al.*, 2012; Sun *et al.*, 2013), whereas small molecules can be directly added to the cell culture medium (Hall *et al.*, 2017; He *et al.*, 2022). TLR7/8 can be stimulated by synthetic small molecule ligands, such as imiquimod, TL8-506 and R848, which closely resemble viral single-stranded RNA (ssRNA) structures and effectively activate the receptors (Jurk *et al.*, 2002; Hemmi *et al.*, 2002). The use of these ligands offers valuable insights into PRR signaling and immune responses, with potential applications in developing antiviral and antitumour therapies as well as vaccine adjuvants, as they lead to the production of type I interferons and pro-inflammatory cytokines crucial for mounting potent antiviral immune responses.

1.4 Hypotheses and Aims

Although it is well understood how nucleic acid receptor stimulation in antigen presenting cells activate lymphocytes, a very limited literature exists on how a direct stimulation of such immune receptors in primary cytotoxic lymphocytes influences their functional response. Interestingly, lymphocyte populations such as NK and T cells express high basal levels of the nucleic acid receptors, such as RIG-I. The question is, if RIG-I activation only induces cell autonomous antiviral pathways in cytotoxic lymphocytes or if the intrinsic activation of these receptors impacts on their capacity to recognise and respond to target cells.

1.4.1 Hypotheses

1- Nucleic acid receptor stimulation in cytotoxic lymphocytes triggers endogenous antiviral response that modulates their effector functions.

2- Nucleic acid receptors in target cells can induce enhanced target recognition by cytotoxic lymphocytes.

1.4.2 Aims

Aim 1 (CD8 T cells): To investigate the functional role of nucleic acid receptors in cytotoxic lymphocytes

1) Assessment of the impact of viral infections on cytotoxic T cells and their effector functions:
    a) Determine if CD8 T cells are permissive to viral infections using IAV virus
    b) Evaluate the susceptibility to infection and develop *in vitro* models for this aim
    c) Determine the productivity of the infection
    d) Investigate if there is a cell subset tropism by IAV
    e) Assess the impact of the infection on the effector functions of cytotoxic T cells (degranulation and cytokine production)
    f) Determine involvement of RIG-I receptors in the cellular response towards IAV infection
2) Nucleic acid receptor activation by synthetic ligands to assess the impact on the effector functions of CD8 T cells
    a) Establish a model for mimicking viral infection using synthetic ligands for the nucleic acid receptors such as RIG-I agonists
    b) Introduce these ligands to the cytosol via any of the transfection methods, e.g. lipofection
    c) Evaluate the effector function and the susceptibility to infection of cytotoxic lymphocytes after nucleic acid receptor activation

Aim 2 (NK cells): To investigate the functional role of nucleic acid receptors in cytotoxic lymphocytes

1) Assessment of the impact of viral infections on NK cells and their effector functions:
    a) Determine if NK cells are permissive to viral infections using IAV virus

b) Evaluate the susceptibility to infection and develop *in vitro* models for this aim
c) Determine the productivity of the infection
d) Investigate if there is a cell subset tropism by IAV
e) Assess the impact of the infection on the effector functions of NK cells (degranulation and cytokine production)
f) Determine involvement of RIG-I receptors in the cellular response towards IAV infection

2) Nucleic acid receptor activation by synthetic ligands to assess the impact on the effector functions of NK cells
   a) Establish a model for mimicking viral infection using synthetic ligands for the nucleic acid receptors such as RIG-I agonists
   b) Introduce these ligands to the cytosol via any of the transfection methods, e.g. lipofection
   c) Evaluate the effector function and the susceptibility to infection of NK cells after nucleic acid receptor activation

Aim 3: To investigate the effect of nucleic receptor stimulation in target cells on NK cell effector function

1) Investigate the role of nucleic acid sensors in the infection susceptibility of target cells
2) Study the indirect impact of IAV infection of target cells on the effector function of NK cells
3) Determine whether the activation nucleic acid receptor in target cells using synthetic ligands would show similar indirect effects to viral infection on the effector function of NK cells

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment and Consumables

Table 1. Equipment

Item	Manufacturer
4D-Nucleofector® Core Unit	Lonza
4D-Nucleofector® X Unit	Lonza
12-channel pipette	VWR
12-channel pipette Proline Plus	Sartorius
Agarose electrophoresis system Biometra compact M	Analytik Jena AG
Autoclave dx-200	Systec
Autoclave vx-150	Systec
Automated cell counter TC20	Bio-Rad
Blue light transilluminator UVT-22-BE-LED	Herolab GmbH Laborgeräte
Centrifuge 5418	Eppendorf AG
Centrifuge 5425	Eppendorf AG
Centrifuge 5430	Eppendorf AG
Centrifuge 5430 R	Eppendorf AG
Centrifuge 5810	Eppendorf AG
Centrifuge 5810 R	Eppendorf AG
Centrifuge MiniSpin	Eppendorf AG
CO2 incubator ICO240med	Memmert
EasySep™ Magnet	STEMCELL Technologies
Electrophoresis cell Mini-PROTEAN tetra vertical	Bio-Rad
Electrophoresis power supply PowerPac HC highe-current	Bio-Rad
Electroporation device Neon	Thermo Fisher Scientific
Flow cytometer Attune NxT	Thermo Fisher Scientific
Flow cytometer LSRII	BD Biosciences
Freezer (-150 °C) MDF-C2156VANW-PE	PHCbi
Freezer (-20 °C)	Liebherr
Freezer (-80 °C) MDF-DU502VH-PE VIP ECO	PHCbi
Freezing container Mr. Frosty	Thermo Fisher Scientific
Hot plate stirrer ARE	Velp Scientifica
Imaging system Odyssey Fc	LI-COR Biosciences
Incubator Shaker New Brunswick Innova 42	Eppendorf AG
Inverted microscope Eclipse TS100	Nikon
Microplate spectrophotometer Epoch	BioTek
Multilabel reader EnVision 2104	PerkinElmer
NanoDrop One	Thermo Fisher Scientific
PCR cycler XT96	VWR
pH/mV bench meter FiveEasy plus	Mettler Toledo
Pipette controller Pipet-X	Mettler Toledo
Pipette controller PIPETBOY acu 2	Integra Biosciences
Precision balance MS6002TS/00	Mettler Toledo
Real-time PCR system QuantStudio 5	Thermo Fisher Scientific

Safety cabinet ScanLaf Mars	LaboGene
Scanner Epson perfection V370 photo	Epson
Single-channel pipette Eppendorf Research plus	Eppendorf AG
Single-channel pipette Pipet-Lite XLS	Mettler Toledo
ThermoMixer C	Eppendorf AG
Tube roller	VWR
Tube rotator	VWR
Vacuum pump 420312	ILMVAC GmbH
Vortex-Genie 2	Scientific Industries
Water bath LAUDA	GFL Technology
Wet transfer tank TE22 Mighty Small	Hoefer Inc.

Table 2. Consumable materials

Item	**Manufacturer**
Adhesive foil for qRT-PCR Opti-Seal	BIOplastics
Adhesive foil for ELISA Easyseal transparent	Greiner Bio-One
Blotting paper	Whatman GE Healthcare
Cell counting slides	Bio-Rad
Cell culture flasks (T25, T75, T175)	Sarstedt
Cell culture plates (96-well F- and U-bottom, 24-well, 12-well, 6-well)	TPP
Cell strainer 70 µm, nylon	BD Biosciences
Centrifuge tubes (15 ml, 50 ml)	Greiner Bio-One
Cryogenic storage vials	Greiner Bio-One
Flow cytometry tubes	Sarstedt
Gloves Peha-soft nitrile	Hartmann
Kimtech precision wipes	Kimberly-Clark
Micro reaction tubes (0.5 ml, 1.5 ml, 2.0 ml)	Sarstedt
Micro reaction tubes SafeSeal (2.0 ml)	Sarstedt
Micropipette filter tips	Axygen
Micropipette tips	Greiner Bio-One
Micropipette tips	Mettler Toledo
Microplate 96-well (F- and U-bottom)	Greiner Bio-One
Microplate 96-well high-binding (for ELISA)	Greiner Bio-One
Microplate 96-well white cell-star	Greiner Bio-One
Nitrocellulose membrane	Amersham Protran 0.45 µm
Optical reaction plate 384-well	MicroAmp Thermo Fisher Scientific
Parafilm M	Sigma-Aldrich
PCR single cap SoftStrips 0.2 ml	Biozym Scientific
PCR 8-cap strip	BIOplastics
PCR 8-tube strip 0.2 ml	BIOplastics
Reagent reservoirs	Cole-Parmer
Reagent reservoirs 12-well	Cole-Parmer
Scalpel	Pfm medical

Serological pipettes CELLSTAR	Greiner Bio-One
Syringe filters (0.2 µm, 0.45 µm)	GE Healthcare
Syringes Discardit II (10 ml, 20 ml)	BD Biosciences
Zymo-Spin IIICG columns	Zymo research

2.1.2 Reagents

Table 3. Chemicals and reagents

Item	Manufacturer
2-Propanol (> 99.5 % pure)	Carl Roth
5-Aza-2'-deoxycytidine (5-Aza)	Abcam
Acetic acid	Carl Roth
Adenosine-5'-triphosphate (ATP) disodium salt	Carl Roth
Agarose UltraPure	Invitrogen
Alt-R Cas9 electroporation enhancer	Integrated DNA Technologies
Alt-R CRISPR-Cas9 negative control crRNA #1	Integrated DNA Technologies
Alt-R CRISPR-Cas9 tracrRNA	Integrated DNA Technologies
Ammonium peroxydisulfate (APS)	Carl Roth
Ampuwa water (H2O)	Fresenius Kabi
Bacillol AF	Hartmann
Benzyl-ATPγS	Jena Bioscience
Calcium chloride (CaCl2)	Carl Roth
Coelenterazine native	Synchem
cOmplete Mini EDTA-free protease inhibitor	Roche
Cycloheximide	Carl Roth
Descogen liquid	Antiseptica
Dimethyl sulfoxide (DMSO)	Carl Roth
Disodium hydrogen phosphate heptahydrate (Na2HPO4 · 7 H2O)	Carl Roth
Disodium phosphate (Na2HPO4)	Carl Roth
Deoxynucleotide triphosphate (dNTP) mix	Thermo Fisher Scientific
Ethanol (> 96 % denatured)	Carl Roth
Ethanol (> 99.5 % pure)	Carl Roth
Ethanol (70 % denatured)	Otto Fischar
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth
EvaGreen QPCR-Mix II (ROX)	Bio-Budget
Fc receptor blocking solution	BioLegend
Ficoll-Paque plus	GE Healthcare
HEPES	Carl Roth
Intercept (TBS) blocking buffer	LI-COR Biosciences
Lipofectamine 2000 transfection reagent	Invitrogen
Magnesium chloride (MgCl2)	Carl Roth
N-ethylmaleimide	Sigma-Aldrich

Nuclease-free duplex buffer	Integrated DNA Technologies
PageRuler plus prestained protein ladder	Thermo Fisher Scientific
Paraformaldehyde	Carl Roth
PhosSTOP phosphatase inhibitor cocktail	Roche
Ponceau S	Carl Roth
Potassium chloride (KCl)	Carl Roth
Potassium dihydrogen phosphate (KH2PO4)	Carl Roth
Random hexamer primer	Integrated DNA Technologies
Red blood cell lysis buffer	Roche
RevertAid reaction buffer 5×	Thermo Fisher Scientific
RiboLock RNase inhibitor	Thermo Fisher Scientific
RLT lysis buffer	Zymo Research
RNA wash buffer	Zymo Research
RNase Zap	Thermo Fisher Scientific
RW1 wash buffer	Qiagen
Tetramethylethylenediamine (TEMED)	Carl Roth
Tris	Carl Roth
Triton X-100	Carl Roth
TRIzol Reagent	Thermo Fisher Scientific
Trypan blue solution 0.4 %	Gibco
Tween 20	Carl Roth
β-Mercaptoethanol	Carl Roth

Table 4. Kits

Description	**Manufacturer**
CellTrace™ Violet Cell Proliferation Kit	Thermo Fisher Scientific
EasySep™ Human NK Cell Isolation Kit	STEMCELL Technologies
EasySep™ Human CD8 T Cell Isolation Kit	STEMCELL Technologies
eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization Concentrate and Diluent	Thermo Fisher Scientific
Gel extraction kit	innuPrep Analytik Jena
Neon transfection system 10 µl kit	Thermo Fisher Scientific
P3 Primary Cell 4D-Nucleofector™ X Kit S	Lonza
Plasmid filter midiprep kit	PureLink HiPure Thermo Fisher Scientific
Plasmid miniprep kit	NucleoSpin Macherey-Nagel
Transcription kit TranscriptAid T7 High Yield	Thermo Fisher Scientific

Table 5. Recombinant proteins, enzymes, and inhibitors

Item	Manufacturer
Alt-R SpCas9 Nuclease V3	Integrated DNA Technologies
GolgiStop™ Protein Transport Inhibitor (Monensin)	BD Biosciences
GolgiPlug™ Protein Transport Inhibitor (Brefeldin A)	BD Biosciences
BD Biosciences	BD Biosciences
Human IL-2 IS	Miltenyi Biotec
Human IL-15	Miltenyi Biotec
IFNAR2 Monoclonal Antibody (MMHAR-2)	Thermo Fisher Scientific
IFNα2a (human)	Miltenyi Biotec
Purified NA/LE Mouse Anti-Human CD3	BD Biosciences
Purified NA/LE Mouse Anti-Human CD28	BD Biosciences
RevertAid reverse transcriptase	Thermo Fisher Scientific

Table 6. Cell culture media and supplements

Item	Manufacturer
DPBS	Gibco
EDTA 0.5 M UltraPure, pH 8.0	Invitrogen
Fetal calf serum (FCS)	Gibco
NK MACS® Medium	Miltenyi Biotec
Opti-MEM	Gibco
Penicillin-Streptomycin	Gibco
Roswell Park Memorial Institute (RPMI) 1640	Gibco

2.1.3 Buffers

Table 7. Buffers

Item	Composition
Annealing buffer	250 mM Tris/HCl, 250 mM NaCl, pH 7.4
ELISA assay buffer	10% (v/v) FCS, 100 U/ml Penicillin-Streptomycin, PBS
ELISA coating buffer	85 mM NaHCO3, 15 mM Na2CO3 in PBS, pH 9.5
Flow cytometry buffer	2% (v/v) FCS, 2 mM EDTA, PBS
Laemmle sample buffer 2×	120 mM Tris/HCl, 4% (w/v) SDS, 200 mM DTT, 10% (v/v) glycerol, Orange G, pH 6.8
FACS buffer	2% (v/v) FCS, 2 mM EDTA, PBS (sterile)
PBND buffer	50 mM KCl, 10 mM Tris/HCl, 2.5 mM MgCl2, 0.1 mg/ml gelatin, 0.45% (v/v) IGEPAL CA-630, 0.45% (v/v) Tween 20
Phosphate-buffered saline (PBS)	137 mM NaCl, 10 mM Na2HPO4, 2.7 mM KCl, 1.8 mM KH2PO4, pH 7.4
Ponceau S staining solution	0.1% (w/v) Ponceau S, 5% (v/v) acetic acid
RIPA lysis buffer	150 mM NaCl, 50 mM Tris/HCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, pH 8.0

SDS-PAGE running buffer	192 mM glycine, 25 mM Tris, 0.1% (w/v) SDS
SDS-PAGE resolving gel (8%)	375 mM Tris/HCl, 26.7% (v/v) rotiphorese gel 30 (37.5:1), 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.08% (v/v) TEMED, pH 8.8
SDS-PAGE stacking gel (3%)	125 mM Tris/HCl, 10% (v/v) rotiphorese gel 30 (37.5:1), 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.1% (v/v) TEMED, pH 6.8
Tris-buffered saline (TBS)	150 mM NaCl, 50 mM Tris/HCl, pH 7.5
TBS-T	0.1% (v/v) Tween 20, TBS
Transfer buffer	192 mM glycine, 25 mM Tris, 20% (v/v) ethanol

2.1.4 Antibodies

Table 8. Antibodies for flow cytometry

Description	Clone	Manufacturer	Dilution
Alexa-Fluor 647 anti-Influenza A M2 Antibody	14C2	Santa Cruz Biotechnology	1:200
Alexa-Fluor 700 anti-human IFN-γ	B27	BD Biosciences	1:100
APC anti-human CD8	RPA-T8	BD Biosciences	1:200
APC anti-human CD8 REAfinity™	REA734	Miltenyi Biotec	1:200
APC anti-human CD56	B159	BD Biosciences	1:200
APC anti-human CD56 REAfinity™	REA196	Miltenyi Biotec	1:200
APC-Cy7 anti-human CD3	SK7	BD Biosciences	1:200
BUV737 Mouse Anti-Human CD3	UCHT1	BD Biosciences	1:200
BUV395 Mouse Anti-Human CD8	RPA-T8	BD Biosciences	1:200
BUV395 Mouse Anti-Human CD56	NCAM16.2	BD Biosciences	1:200
BUV737 Mouse Anti-Human CD16	3G8	BD Biosciences	1:200
BV510 anti-human CD3	HIT3a	BD Biosciences	1:200
BV650 anti-human CD69	FN50	BD Biosciences	1:200
BV786 Mouse Anti-Human CD27	L128	BD Biosciences	1:200
FITC anti-human CD8	RPA-T8	BD Biosciences	1:200
FITC anti-human CD56	B159	BD Biosciences	1:200
FITC anti-influenza A Virus Nucleoprotein	D67J	Abcam	1:200
PE anti-human CD107a	H4A3	BD Biosciences	1:200
PE anti-human CD25	BC96	BD Biosciences	1:200
PE-Cy7 anti-human TNF	MAb11	BD Biosciences	1:100
PerCP-Cy™5.5 Mouse Anti-Human CD45RA	HI100	BD Biosciences	1:200
PE-C7 anti-human CD159a (NKG2A)	S19004C	BioLegend	1:200
PE Mouse Anti-Human CD158b (KIR2DL3)	CH-L	BD Biosciences	1:200
PE Mouse anti-Human CD314 (NKG2D)	1D11	BD Biosciences	1:200
APC Mouse Anti-Human CD94	HP-3D9	BD Biosciences	1:200
APC anti-human CD337 (NKp30)	P30-15	BioLegend	1:200
PE Mouse Anti-Human CD335 (NKp46)	9E2/NKp46	BD Biosciences	1:200
BV605 Mouse Anti-Human CD16	3G8	BD Biosciences	1:200

Mouse Anti-Human KIR (NKB1/KIR3DL1)	DX9	BD Biosciences	1:200

Table 9. Immunoblotting antibodies

Description	Clone/Code	Species	Manufacturer	Dilution
Primary antibodies				
anti-IFIT1	D2X9Z	Rabbit	Cell Signaling Technology	1:1000
anti-p-p65 (S536)	93H1	Rabbit	Cell Signaling Technology	1:1000
anti-p-TBK1 (S172)	D52C2	Rabbit	Cell Signaling Technology	1:1000
anti-p65	D14E12	Rabbit	Cell Signaling Technology	1:1000
anti-RIG-I	D14G6	Rabbit	Cell Signaling Technology	1:1000
anti-TBK1	D1B4	Rabbit	Cell Signaling Technology	1:1000
anti-STAT2	D9J7L	Rabbit	Cell Signaling Technology	1:1000
anti-p-STAT2	D3P2P	Rabbit	Cell Signaling Technology	1:1000
anti-RIG-I	D14G6	Rabbit	Cell Signaling Technology	1:1000
anti-β-Actin	926-42212	Mouse	LI-COR Biosciences	1:4000
Secondary antibodies				
anti-mouse IgG IRDye 680RD	anti-mouse IgG IRDye 680RD	Goat	LI-COR Biosciences	1:5,000
anti-mouse IgG IRDye 800CW	anti-mouse IgG IRDye 800CW	Goat	LI-COR Biosciences	1:5,000
anti-rabbit IgG IRDye 680RD	anti-rabbit IgG IRDye 680RD	Goat	LI-COR Biosciences	1:5,000
anti-rabbit IgG IRDye 800CW	anti-rabbit IgG IRDye 800CW	Goat	LI-COR Biosciences	1:5,000

2.1.5 Nucleic Acid Stimulants, Primers, CRISPR cRNAs

Table 10. Oligonucleotide and stimulants

Name	Sequence (shown 5' to 3')
RNA stimuli	
3pRNA, sense (FAM)	ppp-GGCCGAGACCUCGAAGAGAACUCU
3pRNA, antisense	ppp-AGAGUUCUCUUCGAGGUCUCGGCC
IVT4 template for *in vitro* transcription, sense	TTGTAATACGACTCACTATAGGGACGCTGACCCAGAAGATC TACTAGAAATAGTAGATCTTCTGGGTCAGCGTCCC
IVT4 template for *in vitro* transcription, antisense	GGGACGCTGACCCAGAAGATCTACTATTTCTAGTAGATCTT CTGGGTCAGCGTCCCTATAGTGAGTCGTATTACAA

Small molecules	
TL8-506	Benzoazepine compound, analogue of the TLR8 agonist VTX-2337 (Invovogen)
DNA stimuli	
Plasmid DNA	Bacterial plasmid DNA from stbl3 *E. coli*
G3-YSD, sense	GGGAAACTCCAGCAGGACCATTAGGG
G3-YSD, antisense	GGGTAATGGTCCTGCTGGAGTTTGGG
TL8-506	InvivoGen

Table 11. Primers

Gene	Forward primer (shown 5' to 3')	Reverse primer (shown 5' to 3')
DDX58	GAAAGACTTCTTCAGCAATGTCC	GTTCCTGCAGCTTTTCTTCAA
GAPDH	AAGGTGAAGGTCGGAGTCAA	AATGAAGGGGTCATTGATGG
IFN-γ	GAGTGTGGAGACCATCAAGGA	TGGACATTCAAGTCAGTTACCGAA
TRAIL	ATGGCTATGATGGAGGTCCAG	TTGTCCTGCATCTGCTTCAGC

Table 12. CRISPR crRNAs

crRNA	Sequence	Source
Hs.Cas9.DDX58.1.AA	/AlTR1/rGrGrArUrUrArUrArUrCrCrGrGrArArGrArCrCrCrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AlTR2/	IDT
Hs.Cas9.DDX58.1.AB	/AlTR1/rGrArUrCrArGrArArArUrGrArUrArUrCrGrGrUrUrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AlTR2/	IDT
Hs.Cas9.STAT2.1.AB	/AlTR1/rArArGrUrArCrUrGrUrCrGrArArUrGrUrUrCrCrArCrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AlTR2/	IDT
Alt-R® CRISPR-Cas9 Negative Control crRNA #1	N/A	IDT

2.1.6 Cell Lines

Table 13. Cell lines

Name	Description	Biosafety Level	Source
THP1-Dual	Reporter cell line detecting IRF and NF-κB activation	1	InvivoGen
721.221	Human lymphoma cells	2	Prof. Dr. Andrew Brooks, Melbourne University, Australia
MDCK	Madin-Darby canine kidney Permissive and support the growth of influenza	1	Prof. Dr. Patrick Reading, Melbourne University, Australia

2.1.7 Software

Table 14.Software

Name	Used for	Developer
Design and Analysis	qPCR analysis	ThermoFisher Scientitifc
FlowJo (version 10.5.3)	Flow cytometry data analysis	FlowJo, LLC
GraphPad Prism	Data visualization and statistical analysis	GraphPad Software
ImageStudio Lite	Immunoblot quantification	LI-COR Biosciences
Microsoft Excel	Calculation	Microsoft
Microsoft Word	Text editing	Microsoft
Mendeley Desktop	Bibliography	Mendeley Ltd.

2.2 Methods

2.2.1 Ethics Statement

Buffy coats were collected from anonymous, healthy donors who provided written informed consent, adhering to the principles outlined in the Declaration of Helsinki. This process was approved by the ethics committee responsible at both universities (Ethics Committees of the University of Bonn and the University of Melbourne).

2.2.2 PBMC Isolation and Cell Culture

For peripheral blood mononuclear cell (PBMC) isolation, a standard Ficoll-based isolation protocol was used. Blood from buffy coats was diluted 1:1 with RPMI or Saline (0.9% NaCl) and 30 ml was carefully overlayed onto 15ml of Ficoll. The tubes were then centrifuged at 700 rpm/min for 20 minutes (brakes off) to separate the PBMC layer which was then removed and washed with saline, followed by freezing in FCS 10% DMSO media for long term liquid nitrogen storage. For experiments, NK and CD8 T cells were enriched from PBMCs using negative selection kits (purity was >90%). Specifically, the EasySep™ Human NK Cell Isolation Kit and the EasySep™ Human CD8+ T Cell Isolation Kit were used. After isolation, the cells were cultured in NK MACS® Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 100 U/mL recombinant human IL-2. The cultured cells were maintained at 37 °C and 5% CO2 overnight before being used.

2.2.3 Proliferation of CD8 T Cells

U-shaped 96-well plates were coated with 100 µl of anti-CD3 (2 µg/ml) and anti-CD28 antibodies (2 µg/ml) overnight at 4°C in PBS. As a control, cells were cultured in wells that were incubated with PBS only overnight. The following day, purified CD8 T cells/well were labeled with cell trace violet (CTV) dye at 1µg/ml in PBS for 15 minutes, then washed and resuspended in fresh RPMI media containing 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 U/mL human IL-2, and 20 ng/mL IL-15. $1x10^5$ purified CD8 T cells/well were cultured for 3 days at 37°C at 5% CO2.

2.2.4 Infection of NK and CD8 T Cells

NK cells and CD8 T cells were exposed to a reassortant Influenza A virus (IAV) strain from PR8 and A/Brazil/11/1978: RG-PR8-Brazil78, NA (H1N1) at a multiplicity of infection of 10 (10 MOI) in serum-free medium. After one hour, the cells were washed twice with PBS to remove free viral particles.

2.2.5 Transfection of NK Cells and CD8 T Cells with Ligands

RIG-I ligands were generated through *in vitro* transcription using a Transcript Aid T7 *in vitro* transcription kit with annealed DNA oligonucleotides as a double-stranded DNA template (sequence:TTGTAATACGACTCACTATAGGGACGCTGACCCAGAAGATCTACTAGAAATAGTAGATC TTCTGGGTCAGCGTCCC) as described before (Goldeck *et al.*, 2014). For cGAS stimulation, bacterial plasmid DNA (pDNA), which was extracted from stbl3 *E. coli* bacteria using Plasmid miniprep kit, or G3-YSD synthetic ligands were used. Lipofectamine 2000 was used as transfection reagent to facilitate the delivery of the ligands to the CLs. TLR8 stimulation was achieved using TL8-506, without the need for a transfecting agent. For certain experiments, cells were treated with recombinant IFN-α2a (1000 U/mL). A single-stranded 3p-RNA, serving as the negative control for RIG-I-like receptor activation, was produced from the dsDNA template with the sequence CGCGCGTAATACGACTCACTATAGGGAGCGCAGACGCG AGCGCGGCACGGCCGCCAAGGCGAGAC.

2.2.6 IFN-I Reporter Assay

To assess the type I interferon amount in the cell culture supernatant, a reporter monocytic human, THP1 reporter cells (TBK1$^{-/-}$ IKKα$^{-/-}$ IKKβ$^{-/-}$ IKKε$^{-/-}$), (generated by Julia Wegner in our lab) were employed. This cell line lacks the expression of TBK1, IKKα, IKKβ, and IKKε, but retains interferon signaling, making detecting type I IFN specific. The THP1 dual knockouts were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C and 5% CO2. To measure the type I IFN reporter activity, 100 µl of cell-free supernatant was added to medium-free THP1 dual TBK1$^{-/-}$ IKKα$^{-/-}$ IKKβ$^{-/-}$ IKKε$^{-/-}$ cells and incubated for 24 hr. Luciferase activity was measured by mixing supernatant with water solution of coelenterazine (1 µg/ml) at 1:1 ratio and measuring the activity using an EnVision 2104 Multilabel Reader device.

2.2.7 RNA Extraction and Quantitative Real Time PCR

To extract cellular RNA, a minimum of 5×10^5 cells were pelleted and resuspended in 150 µl of RLT buffer. Equal volume of 70% (v/v) ethanol was added to the samples, and thoroughly mixed by pipetting. The solution was then loaded onto Zymo Spin IIICG columns and centrifuged at 10,000 ×g for 1 minute followed by two washes with 150 µl of RW1 buffer, and 150 µl of RNA wash buffer. After centrifugation at high speed for 2 minutes to dry the membranes, RNA was eluted in 20 µl of RNase-free water (H2O). RNA concentrations were determined using a NanoDrop One UV-Vis spectrophotometer. To synthesize cDNA, 100 - 500 ng of RNA was used, and random hexamer-primed reverse transcription was performed using the RevertAid first strand cDNA synthesis kit according to the manufacturer's instructions. The cDNA was diluted 1:4 with H2O. For SYBR green-based quantitative real-time PCR (qPCR), primers were designed spanning exon-exon junctions. In each qPCR reaction, 1 µl of diluted cDNA solution was mixed with 6.4 µl of H2O, 0.6 µl of primer solution (0.15 µM forward and reverse primer), and 2 µl of my-Budget 5× EvaGreen qPCR-Mix II. Reactions were run on 384-well qPCR plate using QuantStudio 5 real-time PCR system. The target cDNA levels (TRAIL and IFN-γ) were normalized to the cDNA levels of housekeeper gene GAPDH. The relative ratio was calculated using the delta delta Ct method: ΔCt = Ct (target gene) −Ct (GAPDH gene), and ΔΔCt = ΔCt (target sample) −ΔCt (reference sample). The final result of this method presents the fold change of target gene expression in the target sample relative to the reference sample, normalized to a reference gene.

Table 15. qPCR Program

Step	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	95	15 min	1
Denaturation	95	15 sec	40
Annealing	60	20 sec	40
Elongation	72	20 sec	40
Final Elongation	54	30 sec	1
End of Reaction	80	-	-

2.2.8 CRISPR-Cas9 Gene Knock Out in Primary Cells

Pre-designed crRNAs were utilized to delete genes in primary human CLs through the CRISPR/Cas9 system. The RIG-I gene (DDX58) was targeted on the negative strand (GGATTATATCCGGAAGACCC) and positive strand (GATCAGAAATGATATCGGTT) using two crRNAs, while STAT2 was targeted with one crRNA on the positive strand (AAGTACTGTCGAATGTCCAC). A pre-designed non-targeting control crRNA was employed as a negative control, referred to as "Wild type" in the results. The CRISPR/Cas9 mixture was prepared by adding 2 µL of 100 µM crRNA for each crRNA, equal amounts of tracrRNA (Integrated DNA Technologies, Leuven, Belgium, #1072534), 1.7 µL of Cas9 enzyme (Integrated DNA Technologies, Leuven, Belgium, #1081058), and 1 µL of 100 µM enhancer (Integrated DNA Technologies, Leuven, Belgium, #1075916) and electroporation buffer to the final volume of 25 µL per reaction. The electroporation buffer was prepared from the P3 Primary Cell 4D-Nucleofector X Kit S by mixing 16.65 µL P3 media to 3.65 µL supplement per reaction. Electroporation was performed using the CM137 program for NK cells and EH115 for CD8 T cells on the 4D Nucleofector system with the P3 Primary Cell 4D-Nucleofector X Kit S (Lonza, Cologne, Germany, #V4XP-3032). Up to 1.5×10^6 primary human NK cells/CD8 T cells were used per reaction. After electroporation, cells were left for 3 days at 37C at 5% CO2 before experiments.

2.2.9 Western Blot

Cells (at least 4×10^5) washed with PBS were pelleted (500× g for 5 minutes) and lysed with 1× Laemmle buffer containing phosphatase inhibitor (PhosStop) and protease inhibitor. The lysates were vortexed and incubated at 95 °C for 5-7 minutes in a thermomixer, shaking at 600 rpm, to denature the proteins. Following denaturation, equal volumes (equivalent to 4×10^5 cells/run) of the lysates together with protein ladder were loaded onto a 10% SDS-PAGE gel for electrophoresis. The protein electrophoresis was conducted for approximately 100 minutes at a constant voltage of 110V. The separated proteins were subsequently transferred to nitrocellulose membranes for approximately 90 minutes at a current of 0.45 A. These membranes were then sequentially stained using primary antibodies, including anti-β-actin mouse mAb, anti-IFIT1 rabbit mAb, anti-phospho-p65 rabbit mAb, anti-phospho-TBK1 rabbit mAb, anti-RIG-I rabbit mAb, anti-STAT2 rabbit mAb, and anti-phospho-STAT2 rabbit mAb.

Detection of these primary antibodies was accomplished using secondary antibodies conjugated with fluorophores, enabling the visualization and identification of the targeted proteins. The protein bands were then detected using the Odyssey Imaging system. The relative expression levels of the target proteins were quantified using Image Studio Lite software by normalizing the signal intensity of each target protein to the signal intensity of β-actin.

2.2.10 Flow Cytometry and Functional Assays

After washing with FACS buffer containing 2% FBS and 0.5 µM EDTA in PBS, the cells were incubated with specific antibodies targeting surface markers. For CD8 T cells, the antibodies used included anti-hCD3 BV-510, anti-hCD8 APC or anti-hFITC, anti-hCD69-BV650, anti-hCD107a-PE, and Fixable Viability Dye eFluor™ 780. Similarly, for NK cells, the antibodies used were anti-hCD3 APC-Cy7, anti-hCD56 APC or FITC, anti-hCD69-BV650, anti-hCD107a-PE, and Fixable Viability Dye eFluor™ 780. To perform the degranulation assay, both CD8 T cells and NK cells were subjected to stimulation. CD8 T cells were stimulated with 2 µg/ml anti-CD3 and 2 µg/ml anti-CD28 for 4 hr at 37°C, while NK cells were cocultured with 721.221 cells as target cells in a 1:1 ratio for the same duration. Throughout the assay, Golgi Stop, Golgi Plug, and CD107a-PE antibodies were added to the media. Following stimulation, the cells underwent intracellular protein staining. They were fixed and permeabilized using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set and then incubated with specific intracellular antibodies, including anti-hIFN-γ-AF700, anti-hTNF-PECy7, and anti-NP-FITC. Gating was performed as viable, single, CD3+ and CD8+ for CD8 T cells, while NK cells were defined CD3-CD56+ cells. The purity was determined to be greater than 90% for CD8 T cells and greater than 92% for NK cells for every donor **Figure 5**.

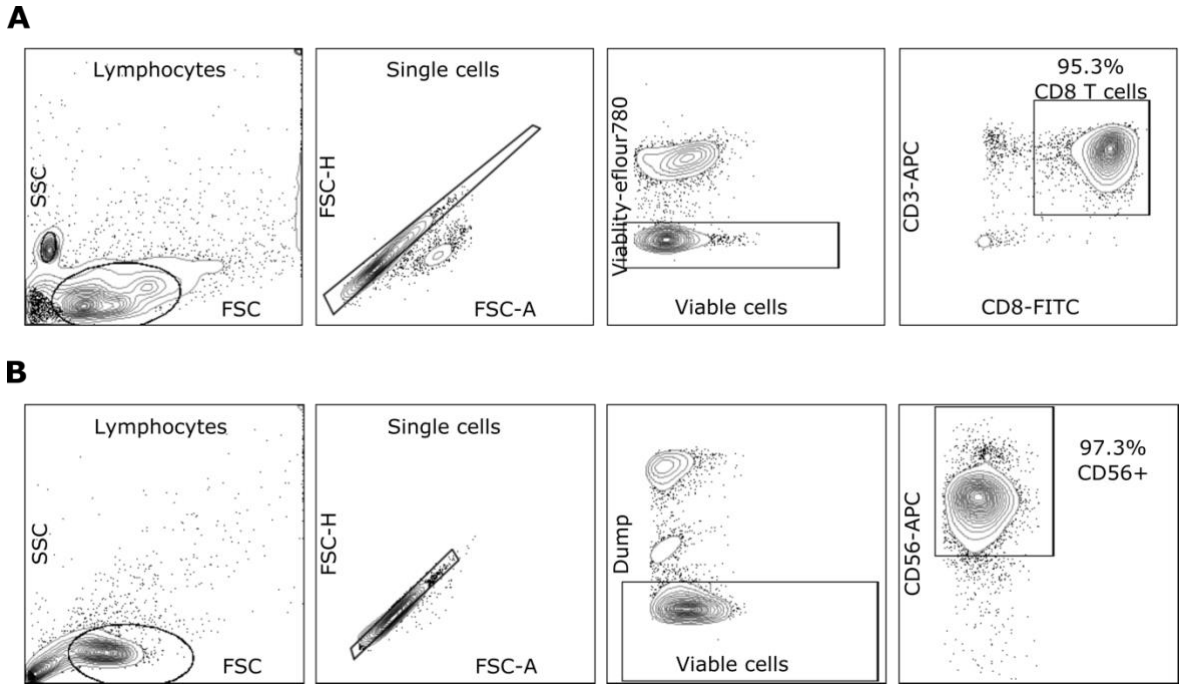

Figure 5. Gating strategy and NK purity post-enrichment.

Representative flow cytometry plots of PBMCs. The lymphocytes were gated using the forward and side scatter plot and by excluding doublets, dead cells. While the CD8 T cells were identified as $CD3^{+}$ $CD8^{+}$, the NK cells were identified as $CD56^{+}$ $CD3^{-}$ cells.

Samples were analyzed using the Attune NxT or LSR Fortessa flow cytometers, and FlowJo™ software was employed for data analysis. Technical controls such as unstimulated cells served as negative controls in all experiments. An example on gating controls used for NK cells is demonstrated in **Figure 6**.

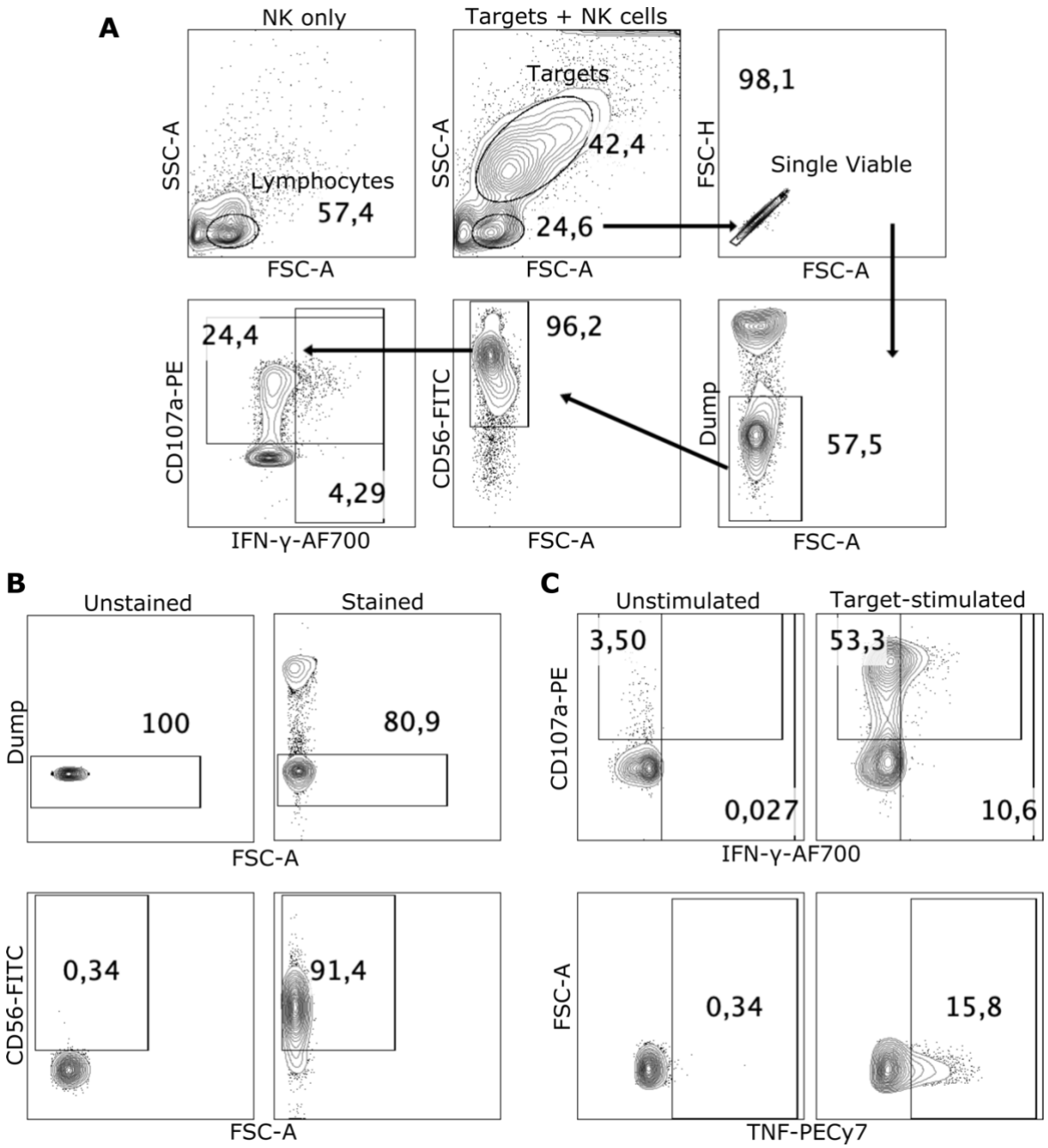

Figure 6. Flow Cytometry Plots for NK cells Demonstrating Gating Strategies for Positive Cell Identification.

A) The gating strategy applied to NK cells cultured with target cells involved: (i) Size-based selection, (ii) elimination of doublets, (iii) Gate based on Viability+CD3APC-Cy7 (Dump), (iv) Identification of NK cells (CD56+ CD3-), and (v) Identification of CD107a+ or IFN-γ+ cells. B) Exemplary scatter plots from flow cytometry illustrating the gating approach for Fixable viability dye-eflour780 and CD56-FITC staining after lymphocyte gating (FSC/SSC), alongside corresponding controls without staining. C) Illustrative scatter plots from flow cytometry demonstrating the gating strategy for CD107a-PE, IFN-γ-AF700, and TNF-PECy7 staining in target-stimulated cells and unstimulated controls.

2.2.11 Plaque Assay

CD8 T cells and NK cells cultured in 24-well tissue culture plates were incubated with Influenza A virus (IAV) at 10 MOI, diluted in serum-free media, for 1 hr at 37°C, after which the cells were washed to remove remaining virus and placed in serum-containing media. Cell supernatants were collected at 25 hr post-infection and centrifuged to collect cell-free supernatants. The infectious virus in the cell-free supernatants was quantified using a standard plaque assay. MDCK cells were cultured overnight in DMEM 10% FCS to 80% confluency, then cells were washed and exposed to 150 µL of serially diluted supernatant and incubated for 48-72 hr at 37°C under a carboxymethylcellulose overlay. After that, number of plaques were counted to calculate the PFU/ml of each sample and compared with supernatant from MDCK infected at 10MOI as a positive control.

2.2.12 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 9 (California, USA). Each symbol in the figures represents an individual donor, and different colors distinguish between donors. The data presented in the figures are obtained from a minimum of three independent experiments, involving three or more donors, as specified in the figure legends. The bars represent the mean ± standard error of the mean (SEM) across all donors unless otherwise stated. "Normal distribution of data was confirmed using the Shapiro-Wilk test. Paired t-tests were utilized to assess differences between two paired groups. For comparisons involving more than two paired groups of healthy donors, one-way ANOVA for repeated measures followed by Dunnett's correction was applied. Paired/repeated measures tests were chosen due to donor-dependent variabilities. Ordinary one-way ANOVA was applied to evaluate differences between different THP1 cell lines. For multiple comparisons, two-way ANOVA followed by Bonferroni's correction was employed. Statistical significance is indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$).

3. Aim 1: Investigating The Role of Nucleic Acid Receptors in CD8 T Cell Effector Function

3.1 Introduction

Cytotoxic CD8 T cells are a cornerstone of the adaptive immune system, wielding significant influence in responses to viral infections and cancer in particular (Sun *et al.*, 2023). Upon activation, CD8 T cells are transformed into potent effectors, capable of releasing cytotoxic substances including perforin and granzymes, which can directly eliminate infected or malignant cells (Golstein and Griffiths, 2018). This activation relies on two critical signals: the recognition of invading pathogens through TCRs interacting with antigens presented on MHC-I, along with co-stimulatory signals (Kumar *et al.*, 2018). Beyond their cytotoxic abilities, CD8 T cells play a pivotal role in shaping the immune response by releasing signaling molecules like TNF and IFN-γ (Zhang and Bevan, 2011). Interestingly *in vivo*, the process of responding to virus-infected cells implicitly requires CD8 T cells to make direct contacts with such cells and potentially rendering them more suscpetible to infection either productive or or non-productive, which in turn may impact their function (Schmidt and Varga, 2018).

A substantial body of research, involving both human subjects and murine models, has shed light on the vulnerability of CD8 T cells to viral infections. This susceptibility extends to various viruses, including DNA viruses like EBV, HTLV-I, HHV-6A, and HIV (Lusso *et al.*, 1991; Kitchen *et al.*, 1998; Grivel *et al.*, 2003; Kim *et al.*, 2019). Moreover, CD8 T cells have been found to be susceptible to (-) ssRNA viruses such as measles virus (Laksono, Grosserichter-Wagener, *et al.*, 2018) and Influenza A virus (Manicassamy *et al.*, 2010). However, the relationship between viral infection, nucleic acid receptor stimulation, and their impact on CD8 T cell effector function remains unexplored. Therefore, this study aimed to investigate whether RIG-I activation induced by either viral infection or exposure to specific RIG-I ligands affects the effector function of CD8 T cells. The findings indicated that activated CD8 T cells were more susceptible to IAV infection and that RIG-I activated by IAV infection, resulted in the initiation of the TBK1 and NF-κB pathways and the release of type I IFN. Moreover, IAV infection enhanced the functions of CD8 T cells, such as degranulation, along with the secretion of IFN-γ and TNF. Similarly, targeted triggering of RIG-I using 3p-dsRNA led to an increased IFN release and boosted the functions of CD8 T cells. Additionally, this RIG-I activation provided protection from subsequent IAV infection and enhanced the proliferative capacitty of CD8 T cells. In summary, these findings highlight that RIG-I activation, whether

induced by IAV or 3p-dsRNA, elevates the effector functions and cytokine release of CD8 T cells.

3.2 Results

3.2.1 Influenza A Virus (IAV) Infection

3.2.1.1 IAV Infection Activates CD8 T Cells and Downregulates CD8 Expression

To study the impact of Influenza A virus (IAV) infection on CD8 T cells, enriched human blood CD8 T cells were cultured with IAV at multiplicities of infection (MOI) of 1, 5, and 10 and the proportion of infected cells assessed by detecting the intracellular expression of viral nuclear protein (NP) via flow cytometry. Notably, NP-positive cells (NP+) were observed with an MOI of 10 (**Figure 7A,B**), peaking at 9h post-infection and dropping by 25 hr (**Figure 7B**). Interestingly, following infection, CD8 expression was downregulated specifically on NP+ cells not NP- cells at 25 hr post infection **(Figure 7C,D**). To determine if infection with IAV was associated with early or partial activation of CD8 T cells, the expression of CD69 was assessed. A significant and consistent increase in CD69 expression was observed on CD8 T cells in the presence of IAV compared to control cells. Among IAV-exposed CD8 T cells, NP+ cells showed a higher increase compared to NP- cells in the expression of this activation marker **(Figure 7E,F**), indicating a direct association with IAV infection. In summary, IAV infection induced the expression of a lymphocytic surface marker that is known to be associated with early activation (**Figure 7F**).

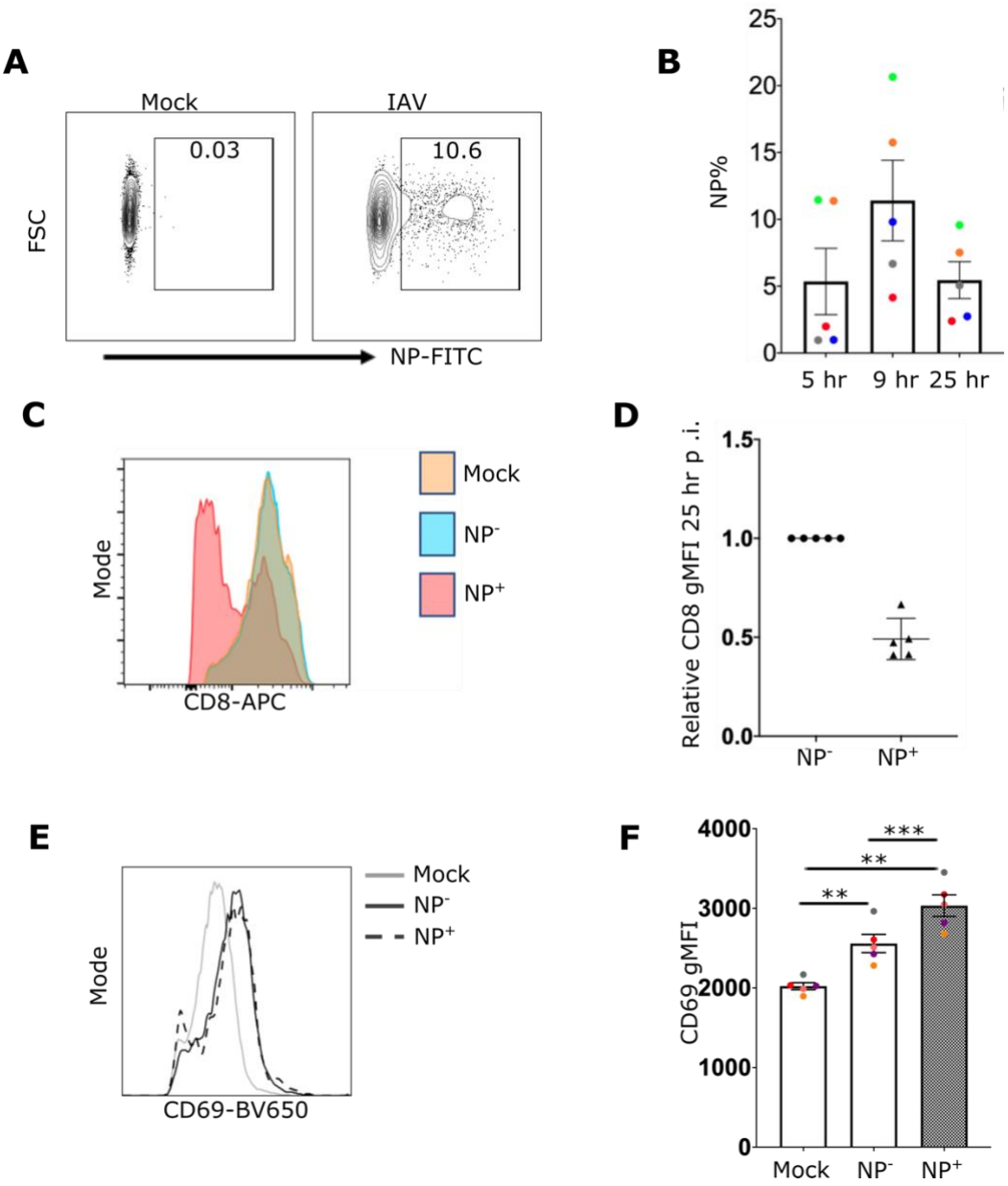

Figure 7. Effect of IAV infection on surface markers of CD8 T cells

A) Representative flow cytometry plot after IAV exposure, showing mock treated and IAV-infected CD8 T cells incubated for 8 hr after 1 hr of viral exposure followed by washing. **B)** Bar graph of NP+ cell proportion at different timepoints post-infection. **C)** Histogram showing the surface expression level (gMFI) of CD8 on mock, NP- and NP+ CD8T cells (gated on CD8+CD3+) 25 hr post-infection. **D)** Dot plot graph showing gMFI of CD8 on NP+ and NP- CD8 T cells. **E)** Histogram showing CD69 expression (gMFI) at 25 hr post-infection. **F)** Quantification of CD69 gMFI from **E**. Each symbol represents an individual donor and bars show mean ± SEM. Statistical significance was tested using One-way ANOVA followed by Dunnett's correction for more than two groups (**$p < 0.01$, and ***$p < 0.001$).

3.2.1.2 Activated CD8 T Cells Are More Susceptible to IAV Infection

The impact of IAV exposure on resting and activated CD8 T cells was next compared. IAV was added either to resting CD8 T cells or those pre-activated with anti-CD3/anti-CD28 antibodies for 3 days. Following 8 hr, cells were again stained for NP and assessed by flow cytometry (**Figure 8A**). Notably, the proportion of NP+ cells was significantly higher in the antibody-activated group compared to the resting group (a mean of 16% vs. 7%) **(Figure 8B**). This suggests a potential connection between the activation state or differentiation of CD8 T cells and their susceptibility to IAV infection. Alternatively, these findings could suggest variations in the inherent antiviral responses between activated and resting cells, or that the mechanisms associated with T cell activation inadvertently facilitate viral replication.

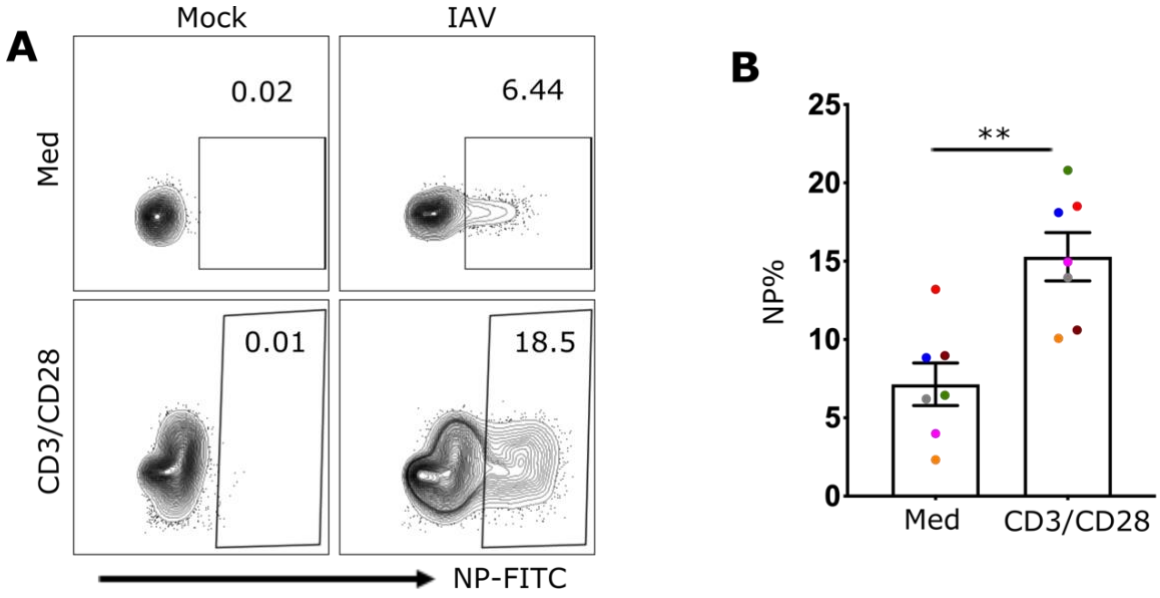

Figure 8. Activated CD8 T cells Are More Susceptible to IAV Infection

A) Flow cytometry plots showing FSC on the Y axis and viral nuclear protein (NP) on the X axis for gated CD8 T cells. Cells were incubated for 3 days in media alone (Med) or with anti-CD3/CD28 antibodies, followed by exposure to 10 MOI of IAV (or mock) for 1 h then incubated for 8 hr. **B)** A bar chart demonstrating the quantification of infection for 7 different donors treated as mentioned in A. Each symbol represents individual paired samples and bars show mean ± SEM. Paired t-test was used for two group comparison (**$p < 0.01$).

3.2.1.3 Unbiased Infection of CD8 T Cell Subsets by IAV

Numerous subpopulations of CD8 T cells have been identified based on the differential expression of a range of cell surface receptors and transcription factors. Amongst these, the co-expression patterns of CD45RA and CD27 are commonly used to identify cells of 4 subsets: naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}) and effector memory re-expressing

CD45RA (T_{EMRA}) cells. Since activated CD8 T cells exhibited higher proportions of infection compared with resting cells, the distribution of NP+ cells following IAV infection was assessed across these subsets. At 5 hr post infection, there was no significant bias towards any particular subset suggesting that they were similar in terms of their capacity to support initial infection (**Figure 9A**). However, after 25 hr post-infection the effector cells (T_{EM} and T_{EMRA}) demonstrate consistently higher NP+ population compared with T_N and T_{CM} (**Figure 9B,C**) although this did not reach statistical significance, possibly due to variation in infection efficiencies between donors.

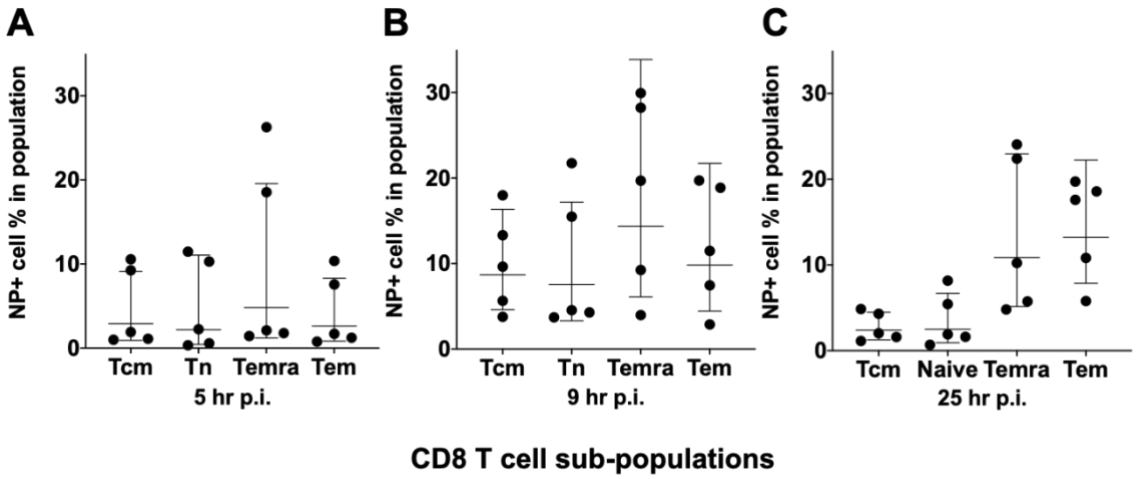

Figure 9. Susceptibility of Different CD8 T Cell Subsets at Different Time Points

Different CD8 T cell subsets infected for 1 hr then washed cultured until various time-points post-infection: 5 hr (**A**), 9 hr (**B**), and 25 hr (**C**). Infection is represented as the proportion of NP+ cells (n=5, mean±SD). Subset are defined as: $CD27^+$ $CD45RA^-$ (T_{CM} Central Memory), $CD27^+$ $CD45RA^+$ (T_N Naïve), $CD27^-$ $CD45RA^+$ (T_{EMRA} Terminally differentiated Effector Memory cells), $CD27^-$ $CD45RA^-$ (T_{EM} Effector Memory).

3.2.1.4 IAV Infection of CD8 T Cells Is Abortive and Does Not Lead to Rapid Cell Death

The drop in the proportion of NP+ CD8 T cells after 9 hr (**Figure 7**) suggested that either infected cells undergo cell death or they inhibit viral replication. When cell viability was evaluated, no significant difference in the percentage of viable cells after exposure to IAV was observed between 5 and 25 hr. Both the mock group and the group exposed to IAV showed similar viability, with 79% viability in the mock group and 76% viability in the IAV-exposed group at 8 hr post-infection (**Figure 10A**). Furthermore, to investigate if these cells were able to support viral replication, plaque assays were performed on supernatants from 25 hr post

exposure to IAV. Infectious IAV was identified from all cells exposed to IAV at both 2 and 24 hr. Critically however, while there was a 10-fold increase in viral titre between 2 and 24 hr post infection from MDCK cell infection indicative of productive infection, there was no increase in viral titre from supernatants of infected CD8 T cells 24 hr post infection compared to the 2 hr control. Thus, virus obtained from CD8 T cells most likely represents input virion that had adhered to the cell surface rather than bone fide productive infection (**Figure 10B**). The data supports the conclusion that IAV-infection of CD8 T cells was non-productive and that while able to enter the cells, the virus is unable to complete replication in these cells.

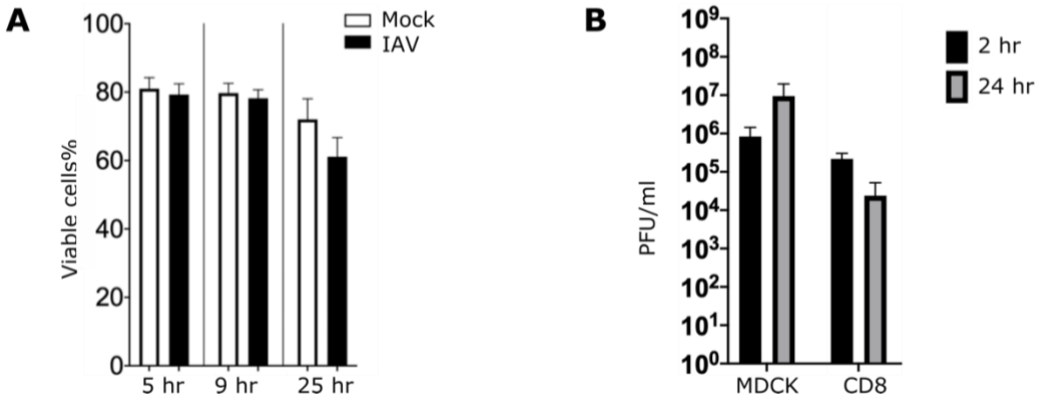

Figure 10. Outcomes of Influenza A infection of CD8 T cell

A) Bar graph illustrating the viability of CD8 T cells from mock or IAV-exposed cells (MOI10) at 5, 9, and 25 hr post-exposure. **B)** Bar graph showing IAV titre (PFU/ml) measured from supernatants collected from CD8 T cells and MDCK cells (positive control) at 2 and 24 hr post-infection (n= 5 donors, mean ± SEM).

3.2.1.5 Infection Activates Nucleic Acid Receptors and Promotes the Type I IFN Response in CD8 T Cells

IAV infection has been shown to activate nucleic acid sensors such as RIG-I and MDA5 in cells of myeloid origin. To determine whether it similarly stimulates nucleic acid receptor signalling in CD8 T cells, the activation of downstream pathways was initially assessed by determining the proportion of NF-κB and TBK1 that was phosphorylated by Western blot. IAV infection induced significant phosphorylation of both NF-κB.p65 and TBK1 and suggested that nucleic acid receptors, which are known to induce type I IFN secretion, may have been activated (**Figure 11A,B**). To confirm this, a type I IFN reporter cell line, which secretes luciferase enzymes upon exposure to type I IFN, was used. The THP1 reporter cells showed that type I

IFN was detected in the supernatant of infected CD8 T cells 24 hr post-infection (**Figure 11C**). Thus, the activation of NF-κB, TBK1 and type I IFN secretion suggests the presence of functional nucleic acid receptors in CD8 T cells, likely RIG-I or MDA5 receptors that are able to detect the presence of viral RNAs.

Since CD8 T cells express IFNAR on their cell surface, type I IFN production by CD8 T cells can lead to the activation of the interferon response by both neighbouring cell types in a paracrine manner but also potentially by the cells that produce type I IFN in an autocrine manner (Kolumam *et al.*, 2005). The potential for type I IFN signalling was assessed by determining the extent of phosphorylation of transcription factor STAT2 downstream of type I IFN receptor (IFNAR) activation. Western blotting showed that exposure to IAV led to the phosphorylation of STAT2 in CD8 T cells (**Figure 11D**). This suggested that secreted type I IFN also leads to signalling in CD8 T cells in response to viral infection. Furthermore, the induction of IFIT1, an interferon-induced protein, provided additional evidence for the type I IFN response (**Figure 11E**).

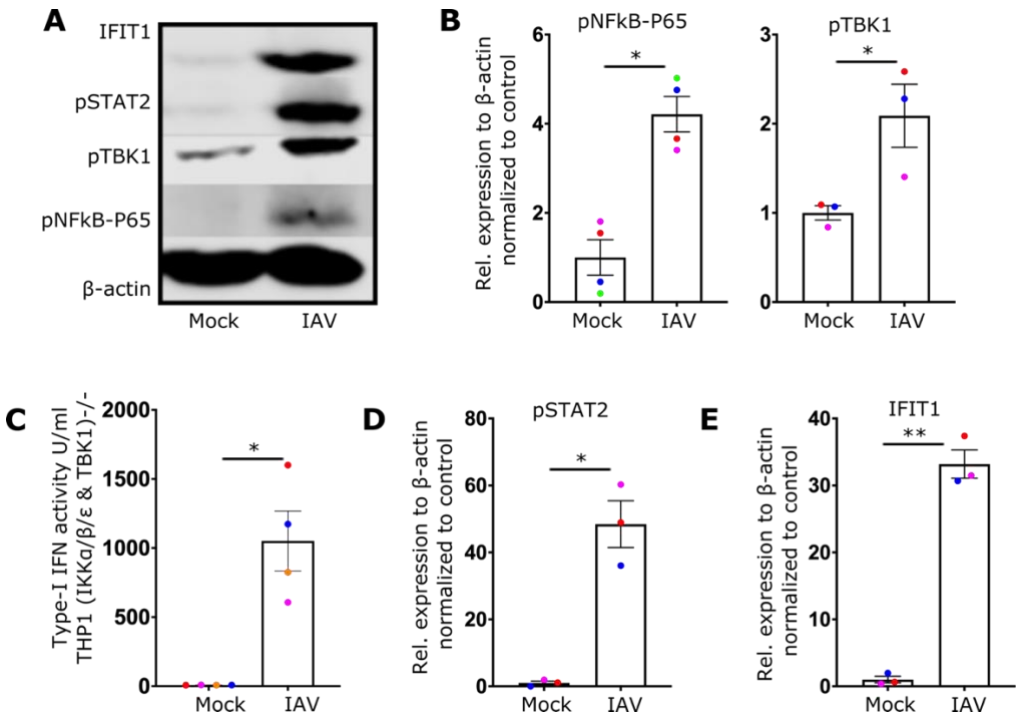

Figure 11. Activation of Nucleic Acid Receptors and Type I IFN Response by IAV

A) Representative Western blot of CD8 T cells with media alone (mock) or IAV (MOI10). The membrane shows bands for different proteins of interest. **B)** Quantification of the proteins pNF-κB-p65 and pTBK1. **C**) Bar graph showing the activity of secreted IFN-I detected by TBK1$^{-/-}$ IKKα$^{-/-}$ IKKβ$^{-/-}$ & IKKε$^{-/-}$ THP1 dual reporter cells. Quantification of pSTAT2 (**D**) and induction of IFIT1 (**E**) (n=3-4 donors). (n=4 experiments). Each symbol represents individual donor, bars show mean ± SEM. Paired t-test was used for two group comparison (*$p < 0.05$, and ** $p < 0.01$).

3.2.1.6 IFN-I Response is RIG-I- and STAT2- dependent

To address whether the interferon response of CD8 T cells during IAV infection was dependent on RIG-I, CRISPR-Cas9 editing was employed to delete the RIG-I and STAT2 genes from primary human CD8 T cells. The efficacy of the knockout was evaluated using Western blot (**Figure 12A**) and protein quantification demonstrated that the expression of both RIG-I (**Figure 12B**) and STAT2 (**Figure 12D**) was reduced by more than 50% in the CRISPR-Cas9 treated cells. Importantly, IAV-induced expression of IFIT1, downstream of type I IFN signalling was significantly reduced in cells lacking RIG-I or STAT2, indicating the essential role of both RIG-I and STAT2 in the type I IFN signalling (**Figure 12C,E**).

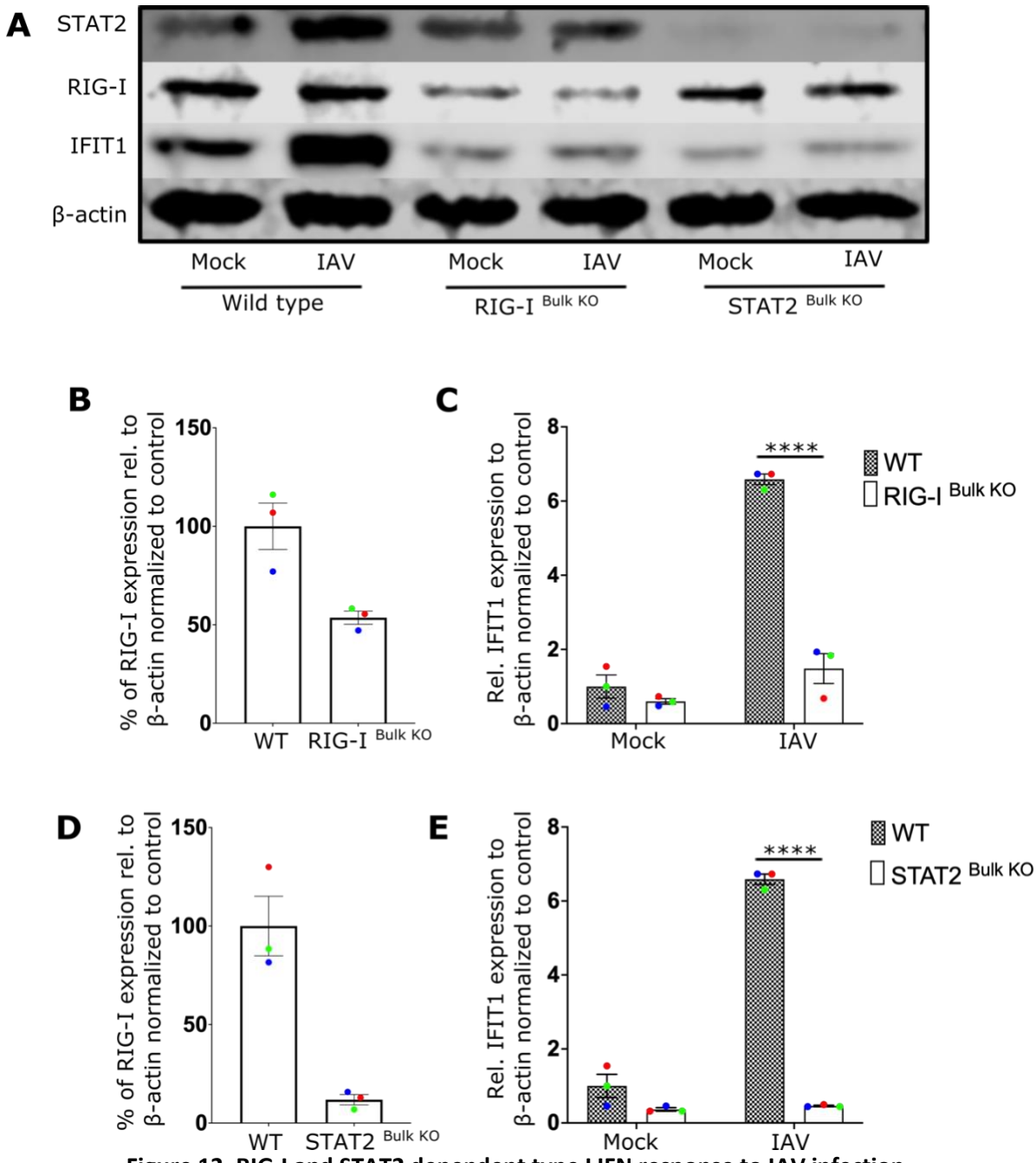

Figure 12. RIG-I and STAT2 dependent type I IFN response to IAV infection

A) Representative Western blot showing wildtype (WT), RIG-I Bulk Knock Out (KO), and STAT2 Bulk KO CD8 T cells incubated with media alone (Mock) or IAV. Quantification of the relative expression level of RIG-I (**B**) and STAT2 (**D**) in WT and CRISPR-Cas9 edited CD8 T cells. The relative expression level of IFIT1 (**C**) in WT and RIG-I bulk KO CD8 T cells is or STAT2 bulk KO CD8 T cells (**E**) is shown. Data were normalized to mock treated WT control. Each symbol represents an individual donor bars show mean ± SEM. Two-way ANOVA followed by Bonferroni's correction for more multiple comparison (****$p < 0.0001$).

3.2.1.7 IAV Infection of CD8 T Cells Increases Their Effector Function

After confirming the involvement of RIG-I and IFNAR in IAV response, the next aim was to evaluate the impact of IAV infection on effector function of CD8 T cells. IAV-exposed (or mock treated) CD8 T cells were incubated for 4 hr with plate-bound anti-CD3/anti-CD28 and their activation measured by staining for CD107a and IFN-γ. In the absence of antibody stimulation, neither IAV-exposed nor mock-treated CD8 T cells showed significant increase in the degranulation or IFN-γ production (**Figure 13A-C**), although a significant difference was observed between groups, with NP+ cells exhibiting highest level of degranulation. Although significant, the levels were still low when compared with the degranulation levels in antibody-stimulated CD8 T cells. Stimulation by crosslinking CD3/CD28 resulted in robust degranulation and IFN-γ responses. Critically, these were much more evident in NP+ cells relative to either NP- or mock treated cells (**Figure 13D-F**). This data suggests an intrinsic effect of IAV infection in enhancing CD8 T effector function, most probably due to the activation of immune pathways within the infected cells that did not simply reflect the response to the secreted type I IFN.

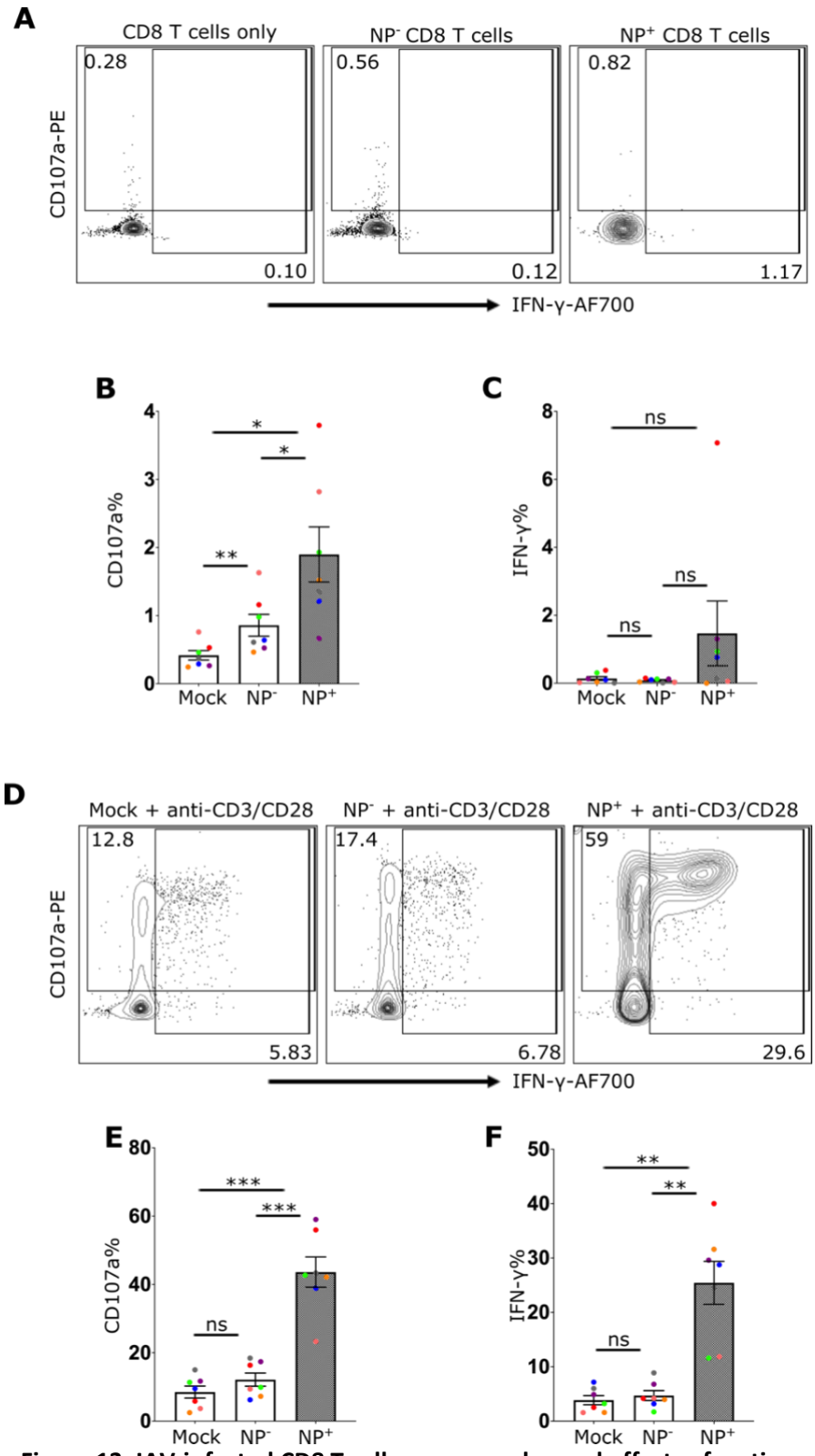

Figure 13. IAV-infected CD8 T cells possess enhanced effector function

A) Representative flow cytometry plots of mock-treated, NP- and NP+ CD8 T cells at 5 hr post-infection showing CD107a and IFN-γ. The proportions of CD107a+(**B**) and IFN-γ+ (**C**) CD8 T cells in the absence, and in the presence of anti-CD3/CD28 activation (**D-F**). Each symbol represents an individual donor and bars show mean ± SEM. Repeated measure one-way ANOVA followed by Dunnett's correction for more than two groups (ns= not significant, $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$,).

3.2.2 Transfection of RIG-I Receptor Ligands

3.2.2.1 Activation of CD8 T Cells and Induction of NF-κB and TBK1 Pathways by 3p-dsRNA Leading to Type I IFN Response

The increased effector function of infected CD8 T cells suggested that RIG-I-dependent signalling may act to enhance CD8 T cell effector responses. To more directly assess the impact of RIG-I signalling on T cell activation in the absence of potentially confounding effects associated with IAV-infection, CD8 T cells were transfected with 3p-dsRNA, a synthetic RIG-I ligand or control RNA (3p-ssRNA) and their effector function was then evaluated. For comparison, cells exposed to IFN-α were also assessed. Similar to IAV infection, exposure to the RIG-I ligand induced the upregulation of CD69 (**Figure 14A,B**) and Western blot analyses showed that it activated NF-κB and TBK1 pathways which was shown by the significant increase in the phosphorylation of NF-κB and TBK1 relative to control treated cells (**Figure 14C,D**). Somewhat surprisingly but similar to 3p-dsRNA treated cells, IFN-α treatment resulted in activation of NF-κB and TBK1 pathways, which are not known to be part of IFNAR signalling.

Building on the finding that IAV infection has shown that RIG-I stimulation causes secretion of IFN-I, the next step was to evaluate if 3p-dsRNA will lead to the same effect. The treatment of CD8 T cells with 3p-dsRNA had a similar effect to IAV infection with type I IFN detected in supernatants of transfected CD8 T cells (**Figure 14E**). Consistent with this leading to IFNAR signalling, Western blot analyses of 3p-dsRNA-transfected cells showed clear increases in the levels of both IFIT1 and phosphorylated STAT2 (**Figure 14C,D**). Furthermore , pre-treatment of CD8 T cells with 3p-dsRNA or IFN-α both resulted in a significant reduction in the proportion of infected cells suggesting that ligand recognition by RIG-I was sufficient to initiate intrinsic innate responses that limit direct viral infection of CD8 T cells(**Figure 14F,G**).

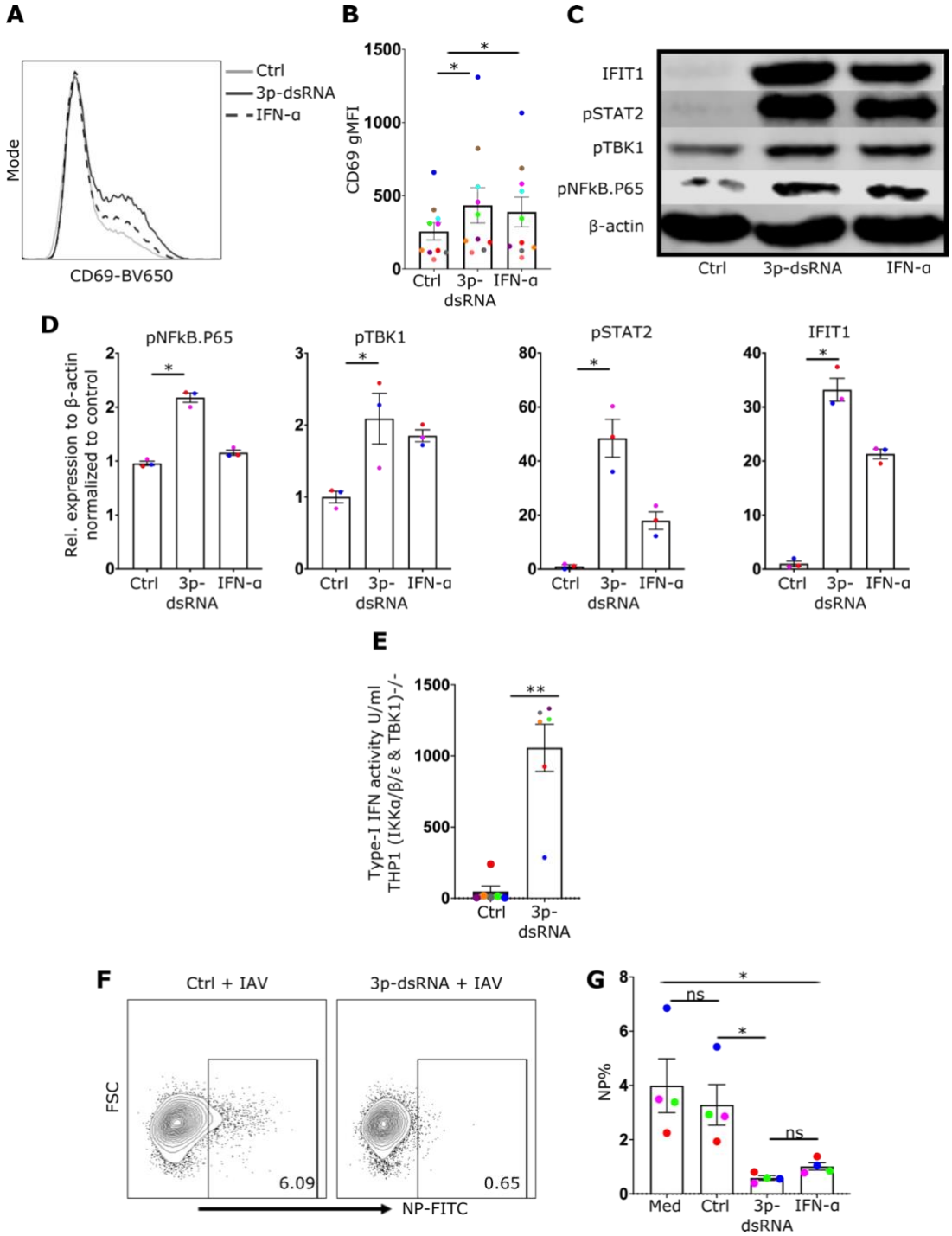

Figure 14. Stimulation of RIG-I receptors activates NF-κB, TBK1 and IFN-I response

(**A**)Representative histogram showing CD69 expression on CD8 T cells in the presence control RNA (Ctrl), RIG-I ligand (3p-dsRNA) or IFN-α. (**B**) Pooled data for CD69 gMFI from 10 donors. (**C**) Western blot for proteins from the three treatment conditions. **D**) Bar graphs showing relative expression or phosphorylation of different proteins relative to β-actin (n=3). **E**) Bar graphs of type I interferon activity in the supernatants of CD8 T cells cultured in two different conditions. **F**) Flow cytometry plots of CD8 T cells treated as above followed by influenza A infection (IAV) for 8 hr. **G**) Bar graph of NP+ CD8 T cells in medium alone (Med), control RNA (Ctrl), 3p-dsRNA and IFN-α pre-treatment. Each symbol represents an individual donor, bars show mean ± SEM. Paired t-test was used for two group comparison and repeated measures one-way ANOVA followed by Dunnett's correction for more than two groups (ns= not significant, * $p < 0.05$).

3.2.2.2 3p-dsRNA Induces IFN-I via the RIG-I and IFNAR/STAT2 Axis

Secreted type I IFN acts in an exocrine fashion to induce signalling via IFNAR as demonstrated in the experiments with IAV. Therefore, the next aim was to assess whether following stimulation with a specific RIG-I agonist, events preceding IFNAR-engagement impacted signalling. When neutralising antibodies to IFNAR2 were used to block IFNAR signalling, following RIG-I receptor stimulation, a reduction of the phosphorylation of STAT2 and IFIT1 induction was observed (**Figure 15A**). Moreover, using a CRISPR-Cas9 gene knock out approach to reduce expression of RIG-I and STAT2, there was a significant reduction in IFIT1 expression in bulk knockout of RIG-I and STAT2 coding genes in CD8 T cells compared with WT cells (**Figure 15B-F**). This indicated that interferon response induced by 3p-dsRNA ligands was both RIG-I and STAT2 dependent.

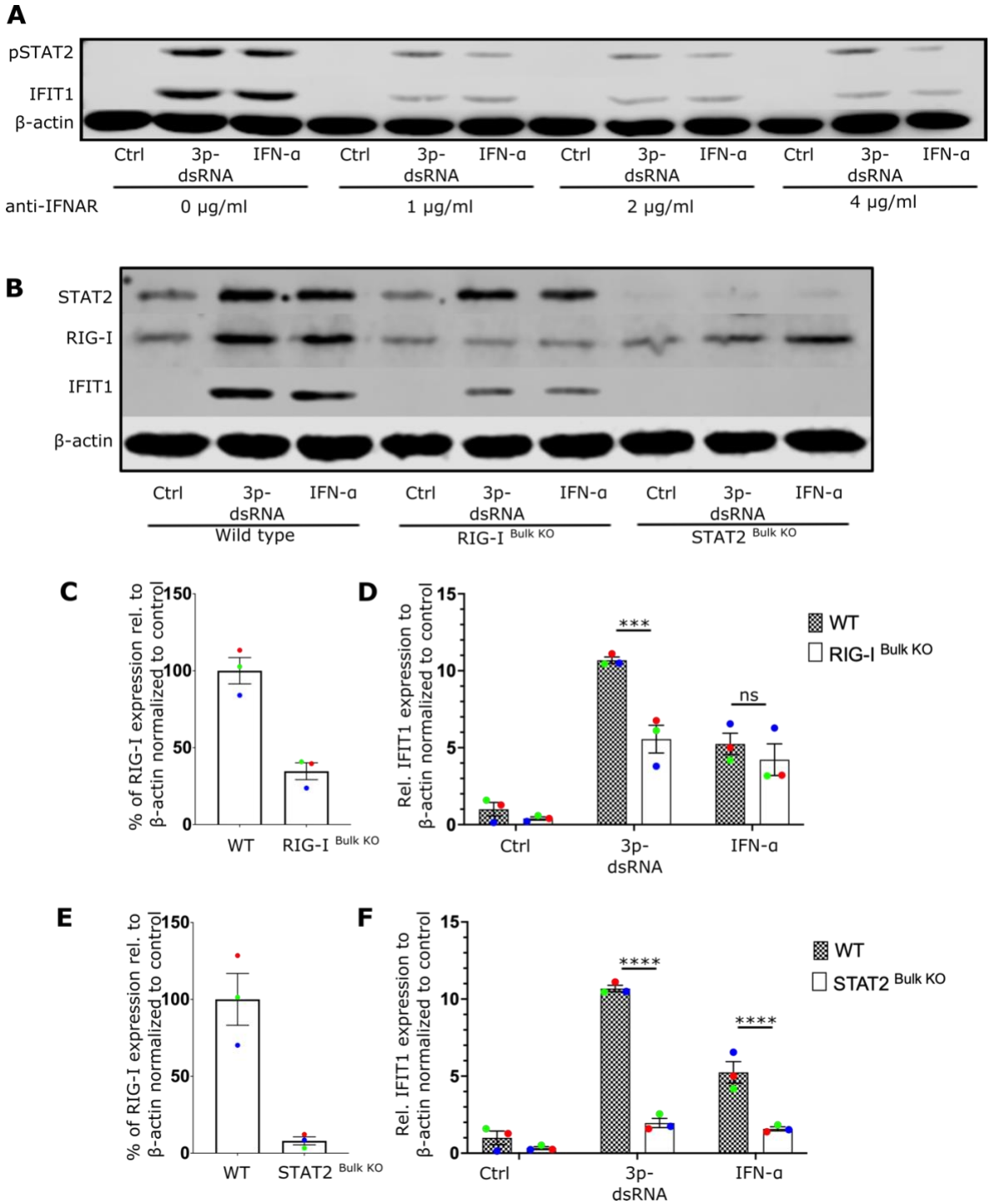

Figure 15. 3p-dsRNA induces type I IFN response RIG-I, IFNAR and STAT2 dependent mechanism

A) Purified CD8 T cells were pre-treated with anti-IFNAR2 antibody at 1, 2, or 4 μg/ml for 1 hr before culture with control RNA (Ctrl), 3pdsRNA, or IFN-α. Western Blot of pSTAT2 and IFIT1 is shown. **B**) The representative Western blot of IFIT1, RIG-I, and STAT2 from WT, RIG-I$^{Bulk\ KO}$ and STAT2$^{Bulk\ KO}$ CRISPR/Cas9-edited CD8 T cells with pooled results for expression of RIG-I (**C**) and STAT2 (**E**). Bar graph of relative expression of IFIT1 from RIG-I bulk KO (**D**) and STAT2 bulk KO (**F**). Each symbol represents an individual donor and bars show mean ± SEM, n=3. Repeated measures two-way ANOVA followed by Bonferroni's correction for multiple comparisons (ns= not significant, ***p < 0.001, and ****p < 0.0001).

3.2.2.3 3p-dsRNA Enhances CD8 T Cell Effector Function

To confirm whether the increased effector function of IAV-infected CD8 T cells was directly driven by the activation of the RIG-I receptors, CD8 T cells were treated with either control RNA, IFN-α or 3p-dsRNA and their activation in response to anti-CD3/anti-CD28 stimulation assessed. In line with IAV infection findings, a significant increase in degranulation (**Figure 16A,B**), as well as cytokine production including IFN-γ (**Figure 16A,C**) and TNF (**Figure 16D**), was detected following RIG-I stimulation which was comparable to that induced by IFN-α stimulation.

Moreover, to exclude the effect of any contaminating non-CD8 T cells that are known to produce type I IFN, the responses of sorted CD8 T cells (>98% purity) (**Figure 17A**) were also assessed. Again, enhanced degranulation (**Figure 17B,C**), IFN-γ (**Figure 17B,D**) and TNF (**Figure 17B,E**) production from CD8 T cells was observed following 3p-dsRNA treatment as noted following IAV infection. These findings confirm that the enhanced activation of CD8 T cells following RIG-I receptor stimulation results from the intrinsic activation of RIG-I pathways within CD8 T cells themselves, more efficiently than the effects induced by exogenous or bystander sources of type I IFN. This conclusion is based on the observation that IAV exposure enhanced the function of NP+ CD8 T cells but not NP- cells. In summary, it can be concluded that the effector function of CD8 T cells is significantly enhanced by IAV and 3p-dsRNA in a RIG-I dependent mechanism.

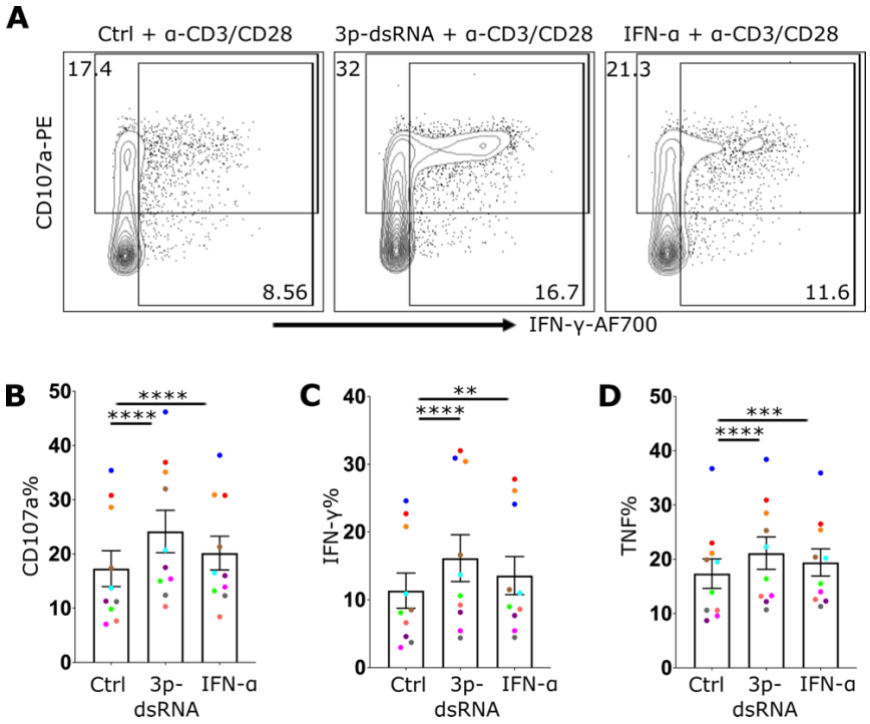

Figure 16. Stimulation of RIG-I enhances the effector function of CD8 T cells

A) Flow cytometry plots representing CD8 T cells activated with anti-CD3/CD28 antibodies, after the overnight incubation with control RNA (Ctrl), 3p-dsRNA or IFN-α. The plots show the expression of CD107a and IFN-γ. B-D) Bar charts demonstrating the proportions of CD107a+ **(B)**, IFN-γ+ **(C)** and TNF+ **(D)** CD8 T cells. Every donor is represented by a coloured dot, bars show mean ± SEM, n=10. Repeated measures one-way ANOVA followed by Dunnett's correction for more than two groups (**$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

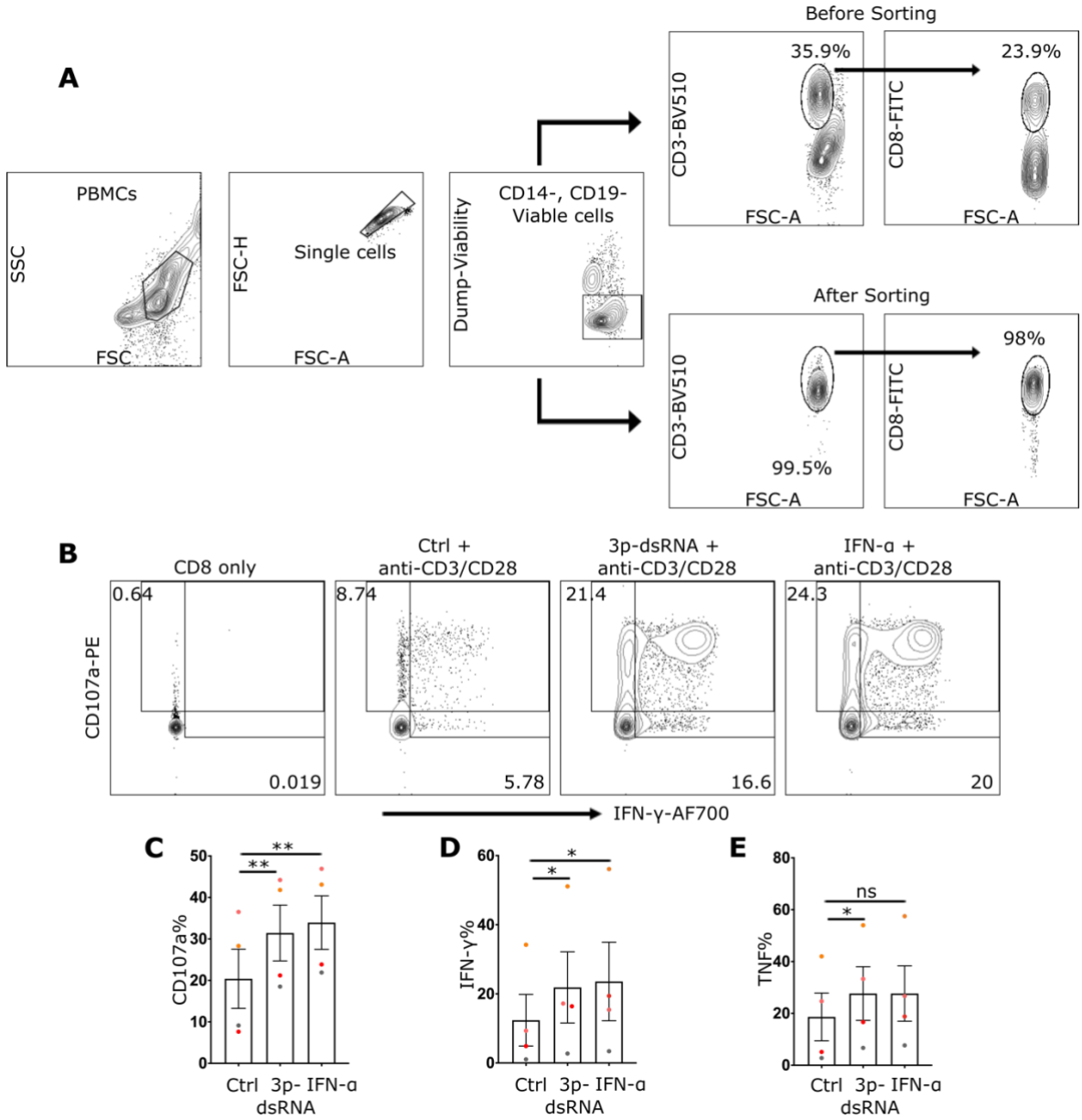

Figure 17. Confirmed effects by RIG-I stimulation using sorted CD8 T cells

A) Flowcytometry plots representing the sorting strategy used to purify CD8 T cells. **B)** Demonstrative flow cytometry plots showing sorted CD8 T cells treated with control RNA (Ctrl), 3p-dsRNA or IFN-α showing CD107a on the x axis and IFN-γ on the y axis. Quantification of CD107a+% **(C)**, IFN-γ+% **(D)**and TNF+%**(E)**. Each donor is shown by a colored dot, bars show mean ± SEM, n=4. Repeated measures one-way ANOVA followed by Dunnett's correction for more than two groups (ns= not significant, *$p < 0.05$, and **$p < 0.01$).

3.2.2.4 3’ Sequencing Indicates Predominant Interferon Signature by Both IAV and 3p-dsRNA

To further examine the effects of RIG-I ligands and IAV infection on CD8 T cells, gene expression profile of isolated CD8 T cells was analyzed 4 hr after stimuli with IAV, RIG-I and IFN-α. The antiviral interferon stimulated genes, as listed in **Figure 18** were found to be significantly upregulated. Other genes which are associated with the activation of CD8 T cells were also upregulated such as Lymphocyte activating 3 (*LAG3*), *IFNG* (IFN-γ) and *TNFSF10* (TRAIL) (**Figure 18**). The data confirms the potential of nucleic acid sensing to modify the transcriptional landscape of CD8 T cells even in the absence of overt TCR receptor stimulation.

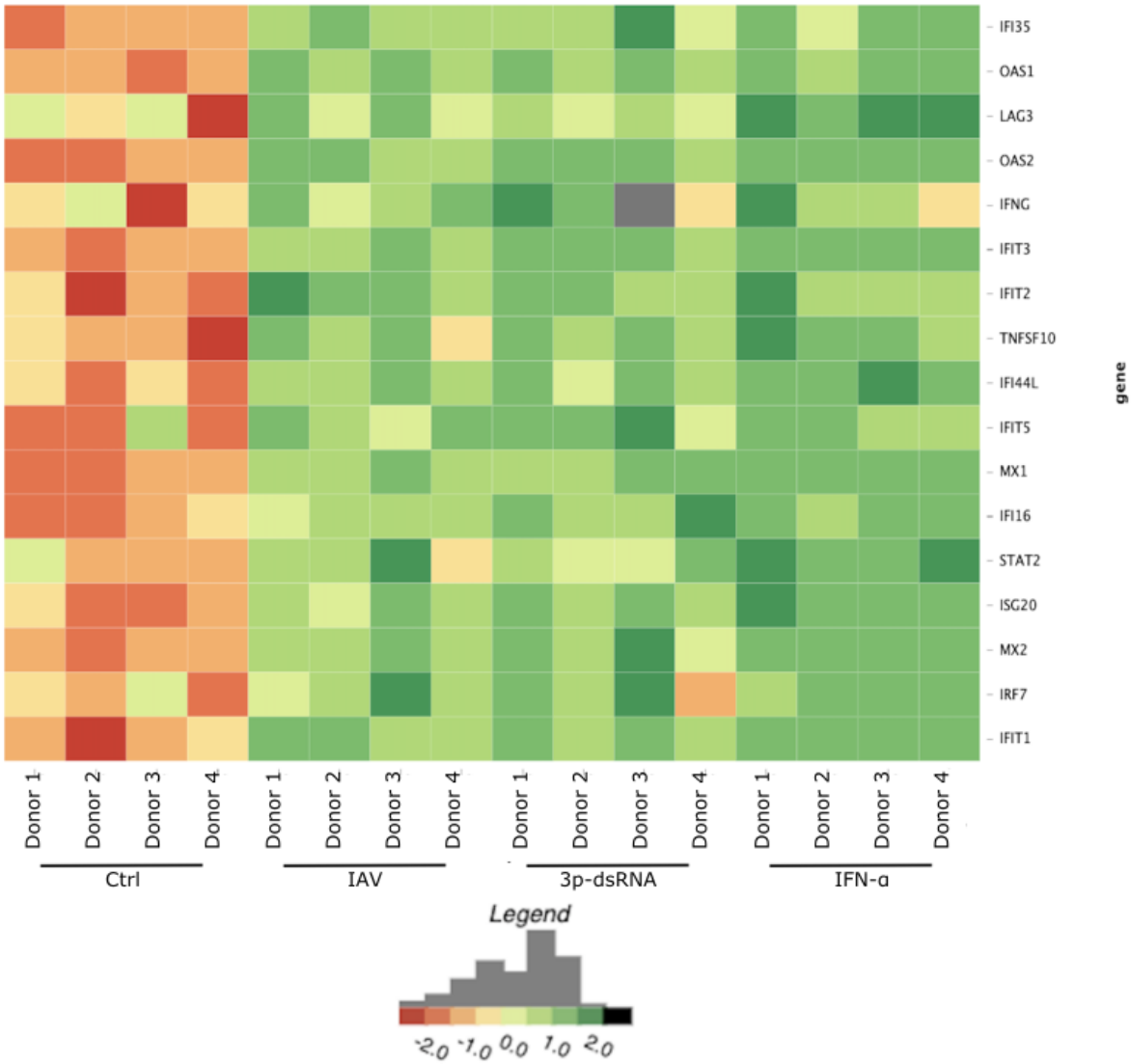

Figure 18. 3' Sequencing Confirms Similarities Between IAV Infection and RIG-I Stimulation in CD8 T Cells

Purified CD8 T cells were infected with IAV or transfected with 3p-dsRNA complexed with lipofectamine, or treated with IFN-α and RNA was purified using the RLT buffer. The heatmap shows significant upregulation of different type I IFN stimulated genes, including Interferon Induced Protein 35 (*IFI35*), 2'-5'-Oligoadenylate Synthetase 1 (*OAS1*), 2'-5'-Oligoadenylate Synthetase 2 (*OAS2*), Interferon Induced Protein With Tetratricopeptide Repeats 1 (*IFIT1*), *IFIT2*, *IFIT3*, Interferon Induced Protein 44 Like (*IFI44L*), MX Dynamin Like GTPase 1 and 2 (*MX1* & *MX2*), Gamma-Interferon-Inducible Protein (*IFI16*), *STAT2*, Interferon Stimulated Exonuclease Gene 20 (*ISG20*), and *IRF7,* as well as activation-associated genes such as TRAIL genes (*TNFSF10*), as well as IFN-γ (*IFNG*) genes in CD8 T cells treated with IAV virus, 3p-dsRNA, and IFN-α compared with control RNA. The RNA was isolated from cells treated as listed above for 4 hr (n=4 donors). Total RNA was used to generate libraries of sequences close to the 3’ end of polyadenylated RNA. The libraries were sequenced on an Illumina HiSeq1500 device. The reads were aligned to the human reference genome using STAR (Dobin *et al.*, 2013). The transcripts were quantified using HTSeq (S Anders, T P Pyl, W Huber: HTSeq — A Python framework to work with high-throughput sequencing data. bioRxiv 2014). Differential expression analysis was performed using EdgeR (McCarthy *et al.*, 2012). The colour scale represents the transcription levels, ranging from (-2.5, shown in red) to (+2.5, shown in black).

3.2.2.5 RIG-I Stimulation Has a Stronger Effect on Functional CD8 Cell Enhancement Than cGAS and TLR8

Since RIG-I stimulation enhanced functional responses of CD8 T cells, the capacity of other nucleic acid receptors that also drive type I IFN responses and may have similar roles was assessed. Specifically, the roles of cGAS a cytosolic DNA sensor, and TLR8 an endosomal RNA sensor, both of which are abundantly expressed by CD8 T cells were addressed by exposing CD8 T cells to either plasmid DNA (pDNA) introduced into the cytosol or the exogenous TLR8 ligand, TL8-506. These ligands did not induce significant upregulation of CD69 (**Figure 19A,E**), although exposure to pDNA cGAS resulted in a significant, although marginal increase in both the degranulation response (**Figure 19B**) and cytokine production (**Figure 19C,D**), particularly IFN-γ. In contrast coculture with TL8-506 did not have any observable effect on CD8 T cell function suggesting that TLR8 engagement had little impact on the effector responses of CD8 T cells, at least as assessed *in vitro* (**Figure 19F-H**). Nevertheless, it has to be considered that transfection conditions for pDNA might not be as optimal as observed in transfection of RIG-I ligands. In summary, RIG-I ligand has shown the capacity to enhance the effector function of CD8 T cells more than cGAS and TLR8 ligands in the applied conditions and therefore appears to be the preferable approach to enhance the effector function of CD8 T cells.

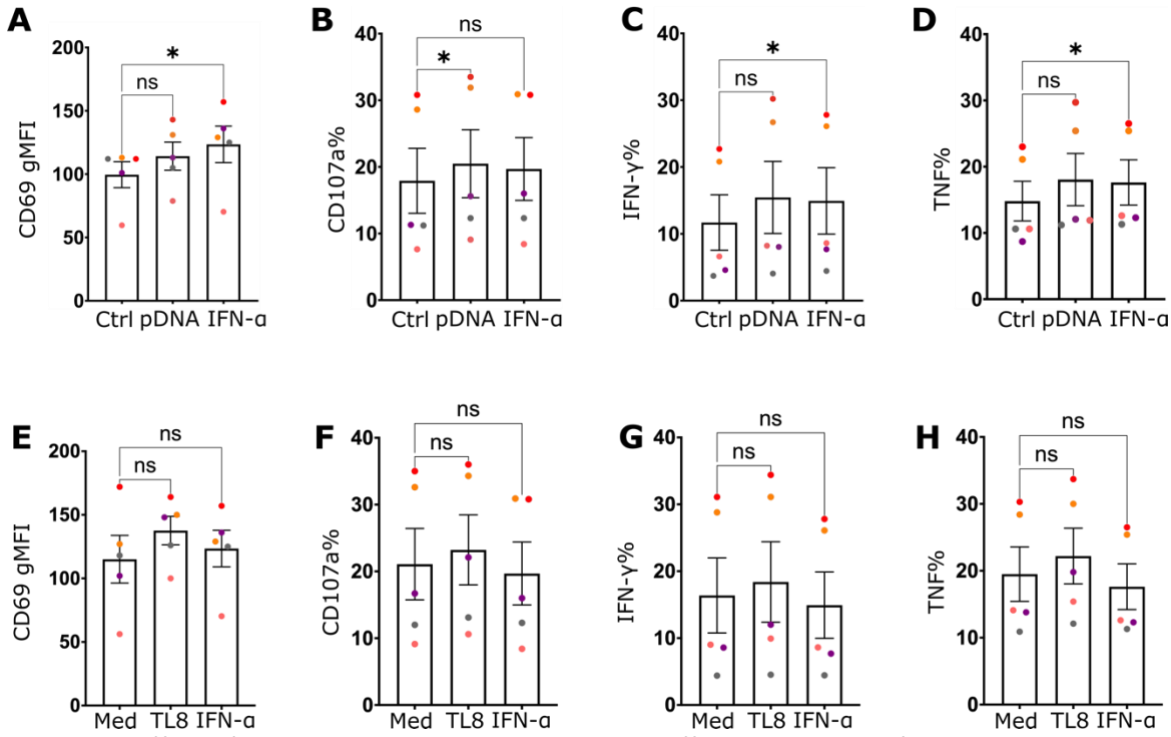

Figure 19. Effect of cGAS and TLR8 stimulation on The Effector Function of CD8 T Cells

CD8 T cells activated with anti-CD3/CD28 antibodies, after the overnight incubation with cGAS ligand plasmid DNA (pDNA) and TLR8 ligand TL8.506. Demonstrated bar charts shows analysis of **A,E**) CD69 gMFI, **B,F)** CD107a%, **C,G)** IFN-γ% and **D,H)** TNF% of CD8 cells in response to pDNA (A-D) and TL8.506 (E-H). Each data point is an individual donor (n=5). The bars show mean ± SEM. The numbers are cell frequencies. Repeated measures one-way ANOVA followed by Dunnett's correction for more than two groups (ns= not significant, * p <.05).

3.2.2.6 Establishment of CD8 T Cell Proliferation Assay

Since exposure to RIG-I ligands enhanced anti-CD3/anti-CD28-mediated activation of CD8 T cells the next aim was to explore whether these stimuli also influenced additional activation driven parameters such as proliferation. CD8 T cells were stimulated with anti-CD3 and anti-CD28 (1 µl/ml) antibodies and 100 U/ml IL-2 for 3, 5, and 7 days or PHA, a non-specific mitogen. In addition, rhIL-2 alone served as negative control. Interestingly, treatment with anti-CD3/CD28 promoted a faster cell proliferation of enriched CD8 T cells than PHA which induced some initial cell death. Antibody-mediated proliferation with antibodies to CD3 and CD28 was used for subsequent experiments. To define which time points would be most appropriate for assessing effector function *in vitro*, cell viability and cell growth (log phase) were the major concerns. CD8 cells show logarithmic phase during day 3 to day 5, however the cell viability at day 5 was slightly lower than at day 3 (**Figure 20**). Therefore, 3-4 days after inducing the cell proliferation was chosen as the most optimal activation period prior to infection.

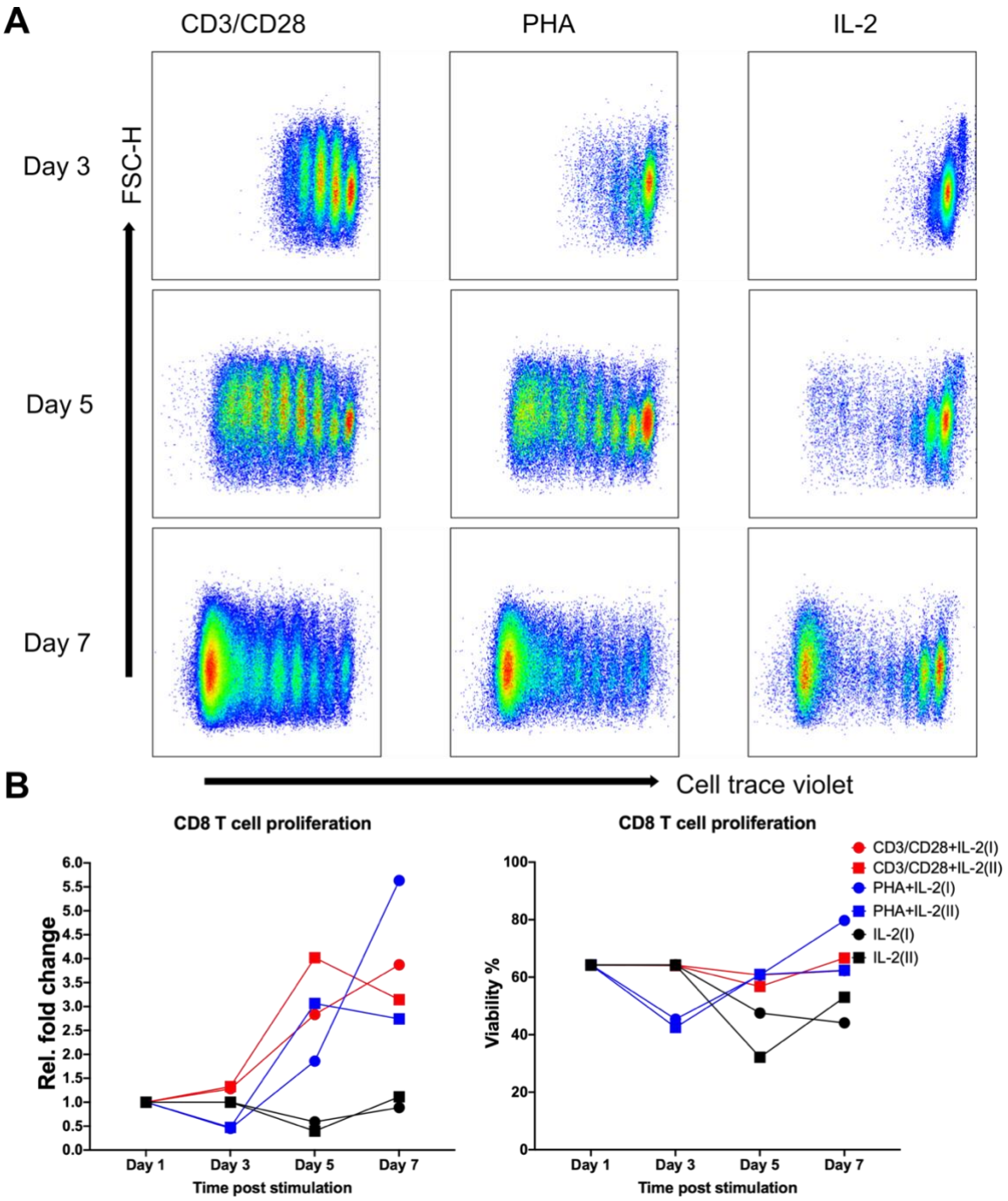

Figure 20. *In vitro* Proliferation of CD8 T cells

A) Representative flow cytometry plots showing stained CD8 T cells with cell trace violet proliferation dye after stimulating them with CD3/CD28 or PHA+IL-2 or IL-2 only at 3, 5 and 7 days. **B)** The relative cell count after stimulating CD8 T cells with the previously mentioned stimulants (left) and their cell viability (right) over time (n=1 donor, 2 replicates).

3.2.2.7 3p-dsRNA Enhance CD8 T Cell Proliferation Capacity

While stimulation of RIG-I has been documented to trigger apoptosis in tumour cells (Besch *et al.*, 2009) the data herein suggested that RIG-I stimulation actually boosted TCR-dependent activation and hence might also impact T cell proliferation and expansion. To explore this, the impact of control RNA, RIG-I ligand or IFN-α on anti-CD3/28-induced proliferation was assessed, tracking cellular division with cell trace violet (**Figure 21A**).The data showed that CD8 T cells stimulated through RIG-I exhibited an enhanced level of proliferation compared to IFN-α treated groups (**Figure 21B**). Unexpectedly, IFN-α treated CD8 T cells did not show a significant difference in total number of cells compared with control group. This might be resulted from the excessive stimulation by two direct signals, IFN-α and CD3/CD28 antibodies, which can cause early cell death affecting the total cell count (**Figure 21A**). Interestingly, while CD25 expression remained relatively consistent across all conditions, the activation marker CD69 exhibited a significant increase in expression specifically within the RIG-I stimulated CD8 T cells (**Figure 21C,D**). This observation suggests that RIG-I stimulation enhanced CD8 T cell activation, to promote enhanced cellular expansion. In essence, the findings indicate that RIG-I stimulation had the capacity to augment expansion of CD8 T cells beyond simply exposure to IFN-α, again consistent with a direct contribution of RIG-I signalling to cellular activation.

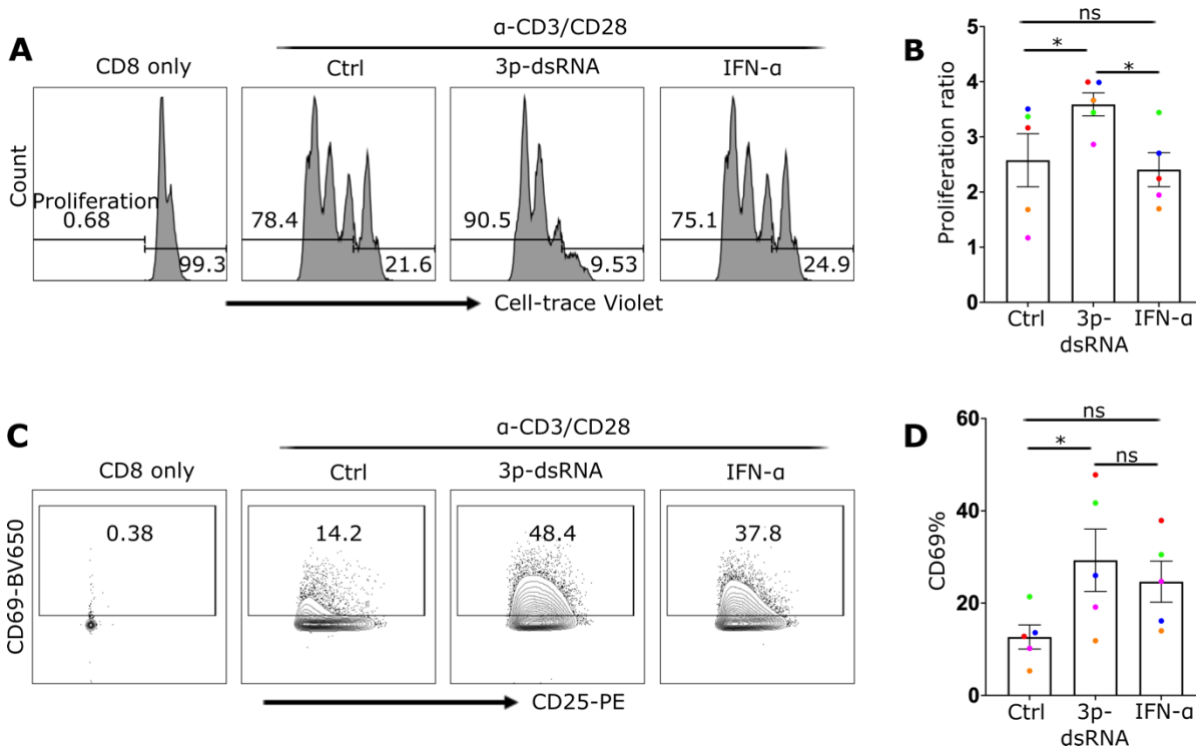

Figure 21. RIG-I enhanced CD8 T cell proliferation more than IFN-α

A) Shown are histogram plots representing CD8 T cells under various stimulation conditions. The first histogram depicts the control group without RIG-I stimulation or anti-CD3/CD28 antibodies. The remaining histograms display cells cultured overnight with control RNA (Ctrl), 3p-dsRNA, or IFN-α, followed by a 4-day incubation on plates coated with CD3/CD28 antibodies. The histograms exhibit the tracking dye for proliferation (Cell-trace Violet), with each peak of lower fluorescence indicating a newly divided cell population. **B)** The proliferation ratio, calculated by dividing the cell count of anti-CD3/CD28 stimulated cells by the cell count of the control group with no anti-CD3/CD28 antibodies, was computed for each condition. **C)** Flow cytometry plots were generated for the same conditions mentioned earlier, plotting CD69 on the Y-axis and CD25 on the X-axis. **D)** The proportion of CD69+ cells was quantified, with each donor represented by a coloured dot. The bars represent the mean ± SEM, n=5. A repeated measures one-way ANOVA was conducted, followed by Dunnett's correction for cases involving more than two groups. (ns= not significant, * $p < 0.05$).

3.3 Discussion

In the process of targeting infected cells, CD8 T cells are often directly exposed to infectious virions in particular at synaptic junctions, potentially rendering them susceptible to infection. Numerous studies have demonstrated that ligand recognition by nucleic acid receptors can have profound effects on CD8 T cell responses (Pufnock *et al.*, 2011; Kandasamy *et al.*, 2016; X. Jiang *et al.*, 2023). Most commonly, this is achieved through engagement of such receptors expressed in antigen presenting cells which triggers both the secretion of immunomodulator cytokines as well as elevation of cell surface expression of molecules that have the capacity to co-stimulate T cell responses (Sköld *et al.*, 2018). However, there is also evidence that T cell intrinsic expression and activation of nucleic acid receptors can modulate T cell function. Notably, following exposure to their ligands, endosomal TLRs have been shown to enhance various aspects of CD8 T cell function, including cytotoxicity, proliferation, and cytokine production (Tabiasco *et al.*, 2006; Li *et al.*, 2019). The potential of cytosolic nucleic acid receptors such as RIG-I and cGAS to impact CD8 T cell effector function during viral infections has remained relatively unexplored. While CD8 T cells are not the primary cell type targeted by most viruses, there is evidence in both human and mouse systems (Lusso *et al.*, 1991; Kitchen *et al.*, 1998; Grivel *et al.*, 2003; Trapecar *et al.*, 2018; Laksono, de Vries, *et al.*, 2018; Kim *et al.*, 2019) that they can be susceptible to infection by viruses, including IAV (Manicassamy *et al.*, 2010).

The analyses of RIG-I activation and its downstream signaling in primary human CD8 T cells uncovered several key findings- namely that both IAV infection and RIG-I ligand stimulation induced CD69 expression, phosphorylation of TBK1 and NF-κB, and the secretion of type I interferons, all of which were dependent on RIG-I signaling. These cellular patterns of activation were also observed in transcriptional analyses which showed similar patterns of induced gene expression following IAV infection, transfection with RIG-I ligand or exposure to type I IFN. Furthermore, immunoblotting experiments of CRISPR/Cas9-edited cells indicated that the induction of interferon stimulated proteins such as IFIT1 was amplified via STAT2 and hence on the production of type I IFN. Moreover, the induction of the type I IFN response in CD8 T cells following either IAV infection and 3p-dsRNA transfection was largely dependent on activation of RIG-I. On a functional level, IAV infection and transfection of CD8 T cells with 3p-dsRNA enhanced degranulation and cytokine production following CD3/CD28

crosslinking. Notably, flow cytometric analyses revealed that this increased activation was primarily observed in infected cells (NP+), while non-infected bystander cells (NP-) did not display the same elevated responses. This suggests that the augmented responses were at least in part associated with intrinsic responses to the infection as opposed to being purely a result of the response to secreted type I IFN. Consistent with this, culture with IFN-α had a weaker impact on CD8 T cell effector function compared to the effects induced by both IAV and RIG-I ligands, again suggesting that RIG-I stimulation in CD8 T cells triggers signals that are distinct from those purely induced by exposure to type I IFN. These findings underscore the potential for RIG-I receptor signaling to enhance CD8 T cell function, making it a target for improving CD8 T cell-based therapies.

The analyses of the impact of RIG-I stimulation on CD8 T cell proliferation paralleled those centred on effector function. Again, exposure to IFN-α did not lead to enhanced CD8 T cell proliferation compared with RIG-I ligands. In contrast stimulation by 3p-dsRNA, which requires initial RIG-I signaling and while leading to the production of type I IFN, was associated with increased proliferation capacity of CD8 T cells. This might be explained by pathways that RIG-I receptor stimulates that are distinct from those induced by type I IFNs, with NF-κB being an obvious candidate as it is activated by both RIG-I- (Zhang *et al.*, 2013) and TCR- (Cheng *et al.*, 2011) signaling but not by IFNAR. The direct comparison between IAV infection and RIG-I stimulation in terms of CD8 T cell responses highlights the specific and congruent role of RIG-I activation in augmenting CD8 T cell function and conferring protection against subsequent viral infections.

This work provides insights into the role of intrinsic RIG-I pathway activation in modulating cellular responses during viral infections, particularly in the context of IAV. The data demonstrate that RIG-I activation can enhance TCR dependent activation of CD8 T cells including degranulation, proliferation and cytokine release as well as conferring protection against subsequent IAV infection. Collectively these hint at potential therapeutic applications of RIG-I activation for fortifying protective immune responses against viral pathogens.

Ligands such as pDNA and TL8.506 have also shown some promising effects on the function of CD8 T cells; however, there considerable optimization of the transfection/stimulation

settings are required in order to increase the feasibility of such approaches. Further research is necessary to fully explore the therapeutic implications and *in vivo* effects of RIG-I activation in the context of antiviral immunity. Understanding the interplay between viral infections and the adaptive immune response, particularly for CD8 T cell-mediated immunity, may pave the way for the development of innovative immune therapeutic strategies to combat viral infections and enhance immune responses against pathogens and tumors.

4. Aim 2: Investigating The Role of Nucleic Acid Sensing in NK Cell Effector function

4.1 Introduction

Natural killer cells are innate lymphocytes that have key roles in immunosurveillance and the control of both transformed and virus-infected cells. They express a diverse array of activating and inhibitory receptors which are critical for their capacity to discriminate between healthy and aberrant cells (Shimasaki *et al.*, 2020; Björkström *et al.*, 2021). Following activation, they can degranulate releasing perforin and granzymes across the synapse formed between NK cell and target, or alternatively trigger programmed cell death in the target through the deployment of FAS-Ligand and TNF-related apoptosis-inducing ligand (TRAIL (Morvan and Lanier, 2015; Prager *et al.*, 2019). Furthermore, NK cells are capable of influencing other immune cells by secreting a multitude of cytokines and chemokines (Morvan and Lanier, 2015; Shimasaki *et al.*, 2020; Björkström *et al.*, 2021). Notably, they release TNF and IFN-γ, which not only promote Th1- responses but also activate antigen-presenting cells and enhance MHC-II expression, an important facet of immune activation (Ivashkiv, 2018). During many viral infections, NK cells can have the capacity to direct contact infected cells, potentially exposing themselves to mature virions (Smyth *et al.*, 2007; Van Erp, Van Kampen, *et al.*, 2019). Interestingly, certain -ssRNA viruses such as Influenza A (IAV) and Respiratory Syncytial Virus (RSV) (Van Erp, Feyaerts, *et al.*, 2019) have demonstrated an ability to infect primary NK cells.. Interestingly while RSV infection has been linked to increased IFN-γ secretion by NK cells, in mouse models IAV appears to induce apoptosis and dampen NK cell cytokine production and cytotoxicity (Guo *et al.*, 2009b; Mao *et al.*, 2010) (Mao *et al.*, 2009). Prolonged exposure of human NK cells to IAV virions and hemagglutinin has also been associated with impaired NK cell effector responses (Mao et al. 2010). Despite the high basal expression of RIG-I in NK cells (Daßler-Plenker *et al.*, 2019), which could potentially recognize IAV-derived RNA (Schlee *et al.*, 2009; Rehwinkel *et al.*, 2010) and trigger an antiviral response, the role of RIG-I stimulation in shaping NK cell responses to IAV has remained largely unexplored. The data herein assessed the impact of RIG-I activation, triggered either by IAV infection or specific synthetic RIG-I ligands (3p-dsRNA), on NK cell effector function. It showed that IAV indeed activated RIG-I in NK cells, leading to the activation of the TBK1 and NF-κB pathways and the secretion of type I IFN. Moreover, IAV infection heightened NK cell effector functions, including degranulation and the release of IFN-γ and TNF. Similarly, targeted activation of RIG-I with 3p-dsRNA bolstered IFN release and NK cell effector functions while also providing protection against

subsequent IAV infection. These findings collectively underscore the potential of RIG-I activation to augment NK cell function.

4.2 Results

4.2.1 IAV Infection

To investigate the effects of Influenza A virus infection of NK cells, they were initially isolated from human buffy coats and exposed to IAV at MOI's of 1, 5, and 10 (**Figure 22A,B**). The proportion of cells that were infected with virus was assessed by flow cytometry following staining for IAV nucleoprotein (NP). Notably, a significant percentage of NP+ cells was observed with an MOI of 10 (**Figure 22A,B**), and viral infection peaked at 9h post-infection. Similar to CD8 T cells, at 25 hr post-infection, the proportion of NP+ cells decreased (**Figure 22A,B**). To gain further insights into the changes associated with viral infection, the expression of NK cell surface markers was assessed (**Figure 22C**). CD56 expression was downregulated after 25 hr post-infection only on NP+ cells (**Figure 22C**). The subsequent aim was to determine whether NK cells undergo activation upon IAV infection, using CD69 expression as an early indicator of cell activation. We observed a significant and consistent increase in CD69 expression on NK cells that were exposed to IAV compared to unexposed cells (**Figure 22E,F**). Furthermore, there was a difference in CD69 expression between NP+ and NP- cells, which was statistically significant, indicating a direct association with IAV infection (**Figure 22F**). In summary, IAV infection activated NK cells and was associated with the downregulation of CD56.

Viral infections can impact expression of NK cell receptors and modify their functional potential (Van Erp, Van Kampen, *et al.*, 2019). The expression of a range of cell surface markers on infected NK cells was analysed and compared with uninfected cells. The expression of activating NKG2D, NKp30, NKp46, CD16 or inhibitory receptors NKG2A/CD94, KIR3DL1 and KIR2DL3 did not change in response to IAV infection(**Figure 22G,H**).

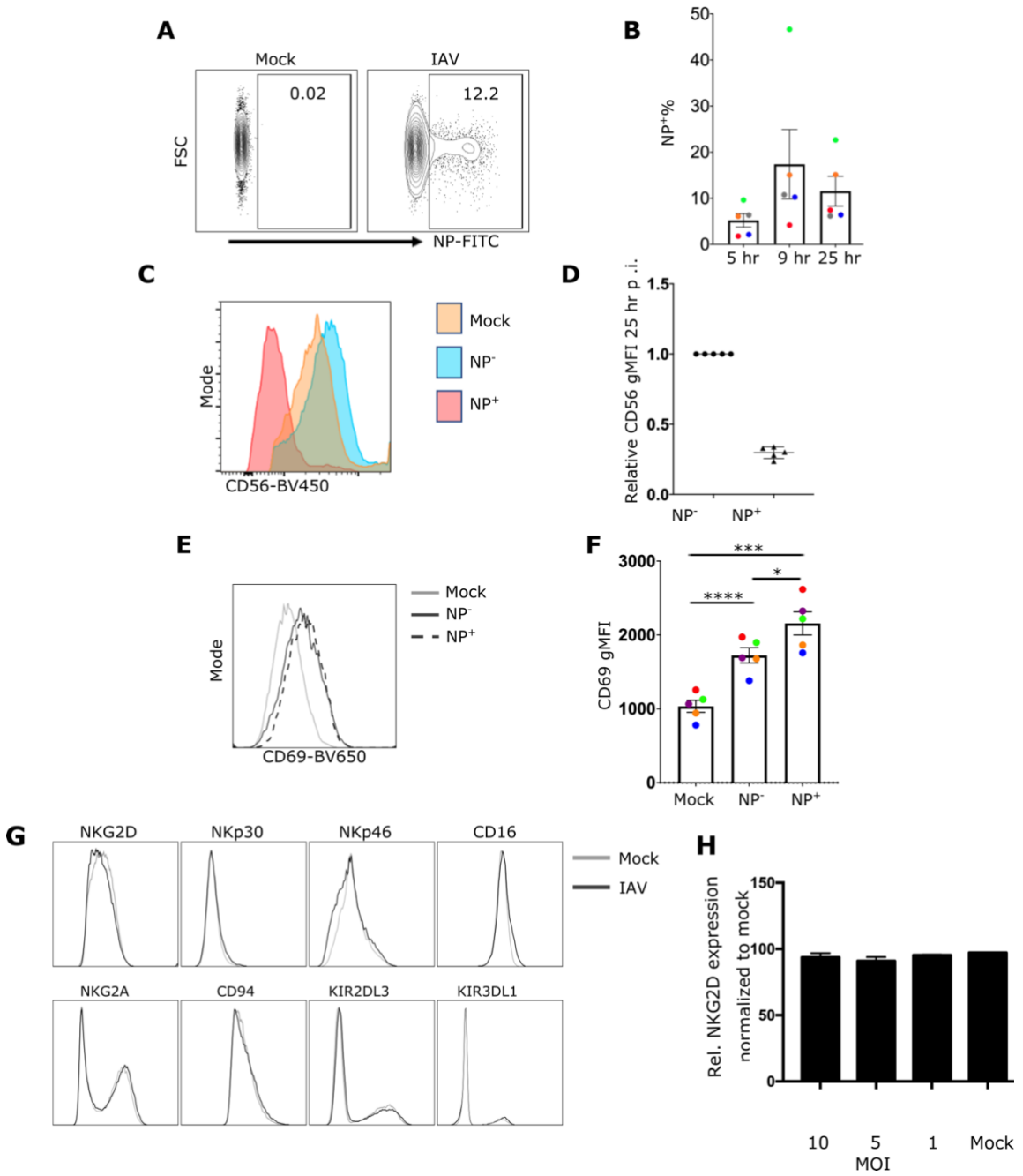

Figure 22. Effect of IAV infection on surface markers of NK cells

A) Flow cytometry plots of mock infection and IAV-exposed NK cells cultured for 8 hr after washing the one-hour virus coculture. **B)** Quantification of infection at different timepoints as labelled. **C)** Histogram demonstrating the expression level of the surface marker CD56 on mock, NP- and NP+ cells. **D)** Quantification of CD56 relative expression level on infected (NP+) and non-infected (NP-) NK cells normalized on NP- cells. **E)** Histogram demonstrating CD69 expression of the same conditions in **C**. **F)** Quantification of CD69 gMFI. Every donor is represented by a dot/triangle, bars show mean ± SEM. Paired t-test was used for two group comparison and one-way ANOVA followed by Dunnett's correction for more than two groups ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$).**G**) The histograms demonstrate the expression levels of activation receptors (NKG2D, NKp30, NKp46, and CD16) and inhibitory receptors (NKG2A/CD94, KIR2DL3, and KIR3DL1) on NK cells following a one-hour exposure to IAV (influenza A virus), followed by thorough washing and an 8-hour incubation period. **H)** Quantification of the NKG2D relative expression on infected (MOI 10, 5, and 1) and mock treated cells normalized to mock control, n=3 donors.

4.2.1.1 Activated NK Cells Are More Susceptible to IAV Infection

To investigate the extent to which IAV infection and impacts their functional response, NK cells were incubated with IAV MOI 10 for 1 hr after which they were incubated with the HLA class I-deficient target cell line 721.221 for a further 4 hr. IAV as reflected by staining for NP, could be detected in a proportion of cells pre-incubated with IAV (**Figure 18A**). However, increased NP+ cell proportions were observed in activated NK cells suggesting an increased susceptibility or an increased survival and replication of viral particles in activated cells (**Figure 23**).

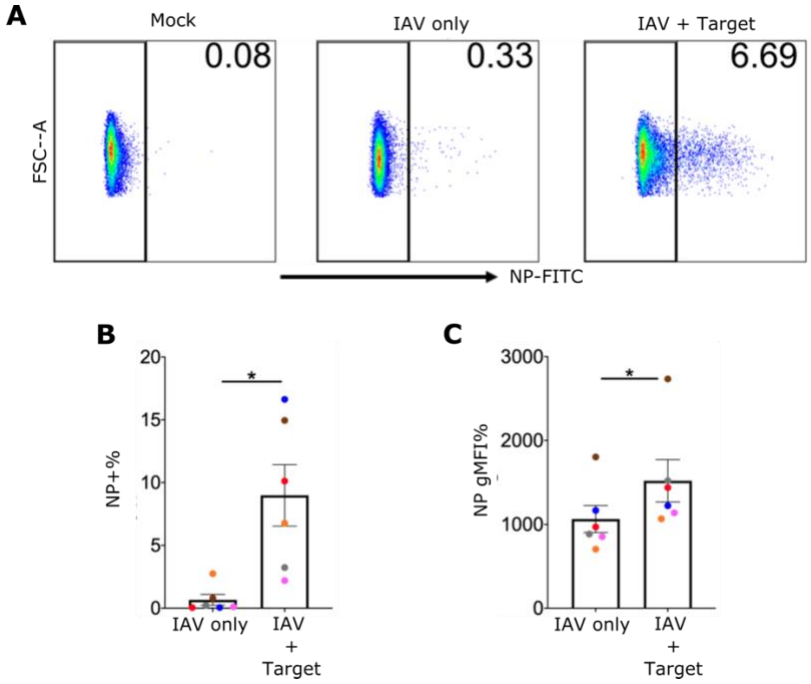

Figure 23. Activation of NK cells is associated with increased infection

NK were infected with influenza virus for 1 hr, washed then activated for 4 hr. For activation, NK cells were cocultured with target cells at 1:1 ratio in the presence of Glolgistop and Golgiplug. After that, cells were examined by flow cytometry for frequency of infected cells. **A)** representative flow cytometry plots for NK showing mock infection, infection without (IAV only) or with target activation (IAV + Target). **B,C)** Frequencies of infected cells (left), means of fluorescence intensity of viral nucleoprotein with demonstrative histogram are shown (right). Each data point is an individual donor (n=6). The bars show mean ± SEM. The numbers are cell frequencies. Paired t-test was used for two group comparison (*$p < 0.05$).

4.2.1.2 IAV Infection of NK Cells Is Abortive and Does Not Lead to Rapid Cell Death

To further explore the impact of exposure of NK cells to IAV, cell viability was assessed over a 25hr period. Somewhat surprisingly, at this time point there was no difference in cell viability in the presence of IAV with those that were mock-infected, albeit that there was significant variation in viability at 25hrs that appeared independent of exposure to IAV (**Figure 24A**). This

variation in NK cell viability between different donors did not occur after shorter incubation times of 5 or 9 hours.

The capacity of IAV to undergo productive infection in NK cells was also assessed. Plaque forming assays measuring the viral titre in the cell-free supernatants 25 hr after infection showed that while infectious virus was recovered from NK cells at both 2 hr post-infection and 25 hr post infection, the titres at 25 hr post-infection were lower than at 2 hr post-infection indicating that there was little evidence of the production of newly-made viruses were formed, indicating that IAV infection of NK cells is non-productive (**Figure 24B**). This contrasted with infection with MDCK cells which are known to be productively infected with IAV where increased amounts of virus were observed 25 hr post infection.

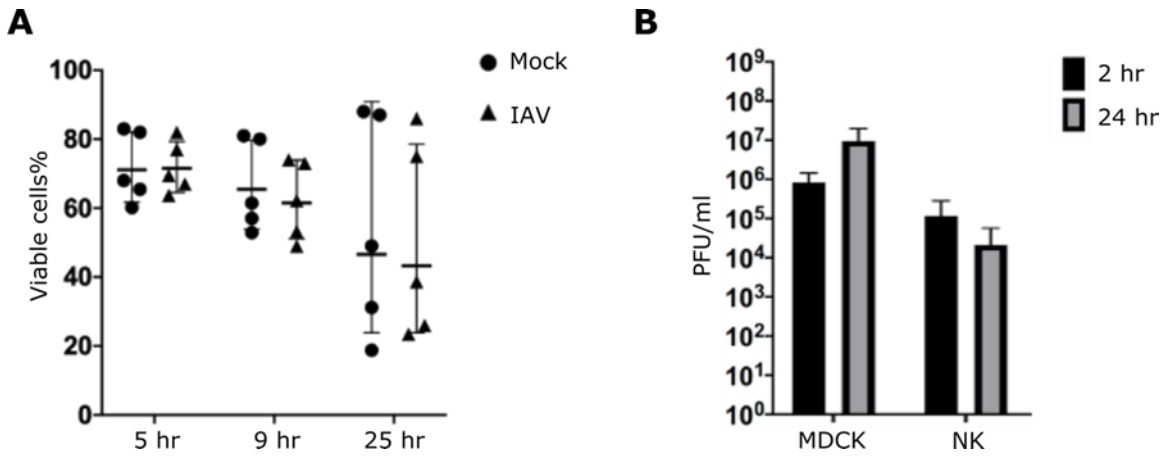

Figure 24. NK Cell Viability and Infectious Particles in The Supernatant IAV-exposed Cells

A) Dot plot represents the viability of mock-treated and IAV-infected NK cells (10 MOI) at 5, 9, and 25 hr post infection Each symbol represents a unique sample. Data are shown as mean± SD, n=5. **B)** Bar graph of viral titre (PFU/ml), measured by plaque assay, from cell-free supernatants of NK cells and MDCK cell cultures at 2- and 24-hr post-incubation with IAV. Bars show mean± SEM, n=3 donors.

4.2.1.3 IAV Infection Activates Nucleic Acid Receptors in NK cells and Induces an IFN-I Response

To investigate the potential of IAV infection to modulate nucleic acid receptors of NK cells, the activation of proteins associated with RIG-I signalling including NF-κB complex and TBK1 was assessed. Western blot analyses showed increased levels of phosphorylation of both NF-κB p65 and TBK1 in NK cells exposed to IAV relative to mock-treated cells (**Figure 25A,B**). Additionally, analyses of supernatants of IAV-exposed NK cells at 24 hr showed clear induction of type I IFN relative to mock treated controls (**Figure 25C**). Similar to what was observed for

CD8 T cells, there was also increased levels of phosphorylated of STAT2, indicative of IFNAR signalling in IAV-exposed NK cells (**Figure 25D**). Moreover, the induced expression of IFIT1, an interferon-induced protein, further confirmed the induction of type I IFN (**Figure 25E**).

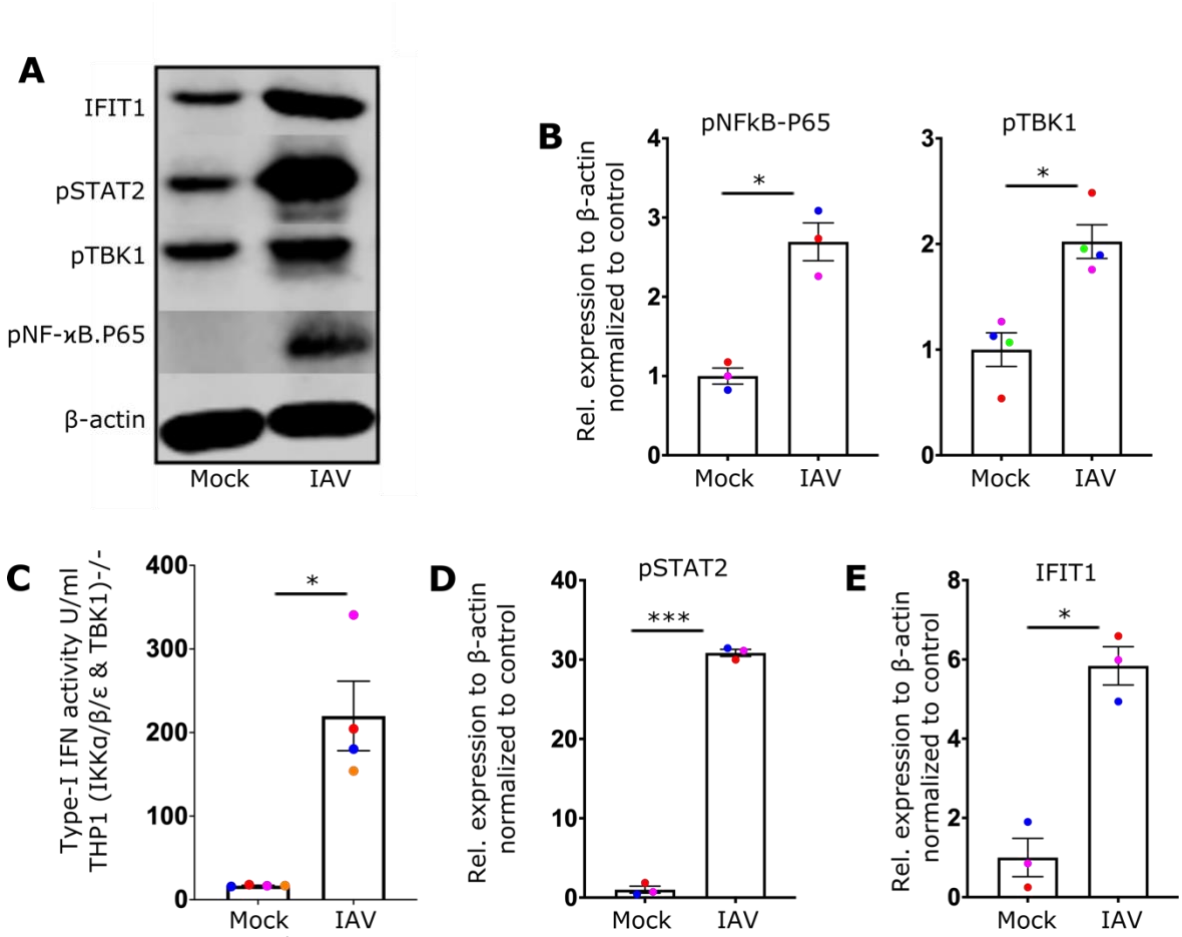

Figure 25. Exposure of NK cell to IAV activates nucleic acid receptors and induce IFN-I response

A) Representative NK cell Western blot for the mentioned proteins obtained from NK cells after overnight culture in media (Mock) or exposure to IAV. **B)** Quantification of Western blot bands of pNF-κB.p65 and pTBK1. **C)** Bar chart demonstrating IFN-I activity detected by TBK1$^{-/-}$ IKKα$^{-/-}$ IKKβ$^{-/-}$ & IKKε$^{-/-}$ THP1 dual reporter cells after 24 hr of incubation with supernatants from infected or mock treated NK cells (n=4 donors). Quantification of pSTAT2 **(D)** and IFIT1 expression **(E)** (3–4 donors). Each colored dot represents an individual donor (color is representative of paired samples), and bars show mean ± SEM. Statistical significance was tested using paired t-test for two group comparisons and one-way ANOVA with Dunnett's correction for more than two groups (* $p < 0.05$, and *** $p < 0.001$).

To ascertain the role of RIG-I receptor signalling in initiating the type I IFN response, CRISPR/Cas9 gene editing was employed to abrogate the expression of RIG-I and STAT2 proteins in primary human NK cells. As an indicator of this approach's success, the expression levels of IFIT1, which reflects the extent to which the type I IFN response was activated

following exposure to IAV was assessed by Western blot. In CRISPR/Cas9 control guide RNA treated cells, IAV infection but not mock treatment resulted in substantial IFIT1 induction, indicative of a robust type I IFN response. In cells treated with CRISPR/Cas9 guide RNA targeting RIG-I and STAT2, where RIG-I and STAT2 was knocked out in 60% of the cells (**Figure 26A,B,D**), induction of IFIT1 was still evident but nevertheless, significantly diminished indicating these responses were at least partially dependent on both RIG-I and STAT2 (**Figure 26A,C-E**).

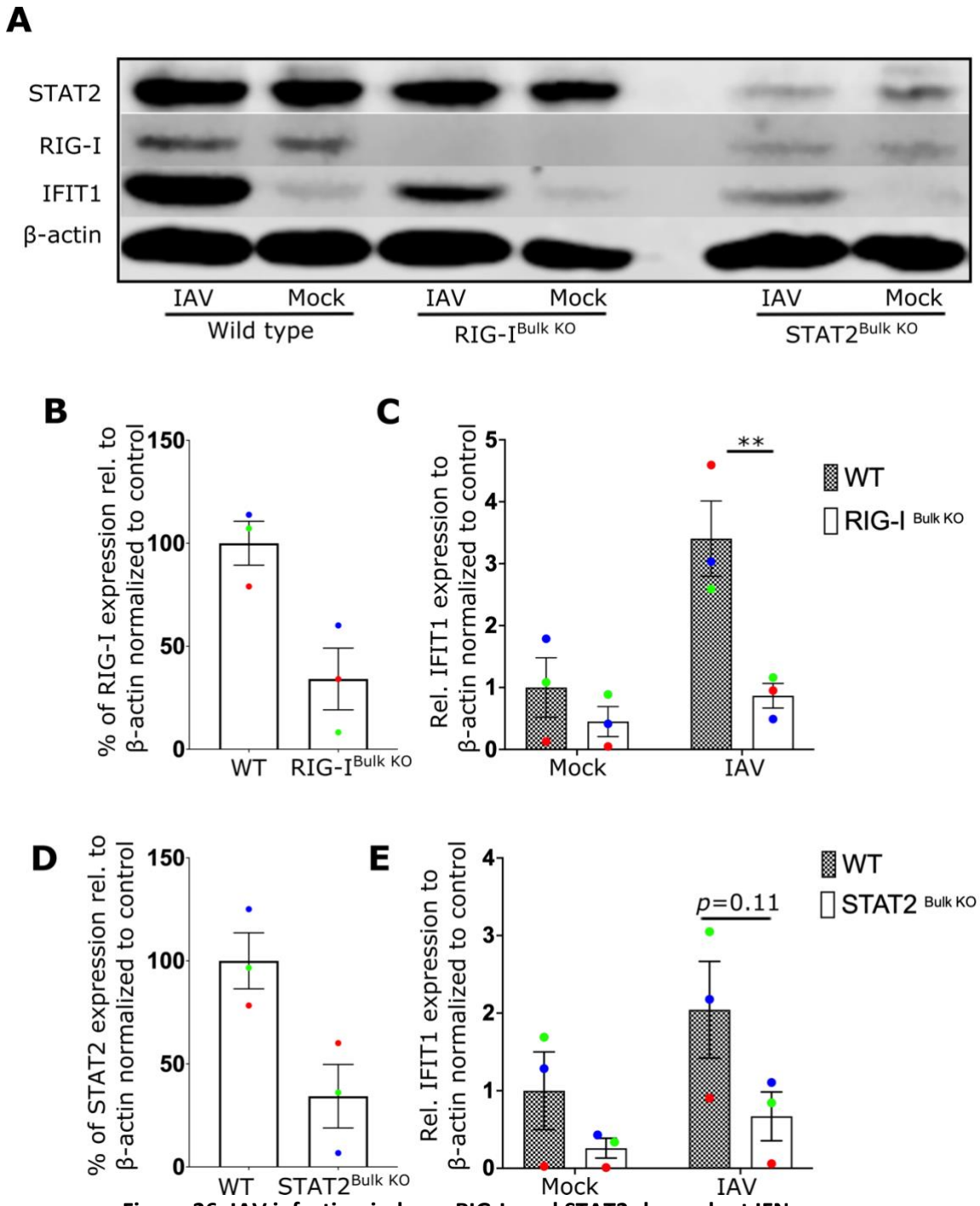

Figure 26. IAV infection induces RIG-I- and STAT2-dependent IFN response

A) Representative Western image showing STAT2, RIG-I, and IFIT1 proteins after overnight incubation with IAV in wildtype controls (WT), or cells treated to delete RIG-I (RIG-I bulk KO) or STAT2 (STAT2 bulk KO) genes. Relative RIG-I expression in % in WT vs RIG-I $^{\text{Bulk KO}}$ cells **(B)** and WT vs STAT2$^{\text{Bulk KO}}$ cells **(D)** are shown. IFIT1 relative induction in WT vs RIG-I bulk KO cells **(C)** and WT vs STAT2$^{\text{Bulk KO}}$ cells **(E)** are quantified**.** Each donor is represented as a coloured dot (colour indicates paired samples), n=3. Bars represent mean ± SEM. Statistical significance was tested using two-way ANOVA followed by Bonferroni's correction for multiple group comparisons (** $p < 0.01$).

4.2.1.4 IAV-infected NK Cells Possess Increased Effector Function

Given that IAV infection induced the secretion of type I IFN after direct infection of NK cells, its impact on the effector function of NK cells was then assessed. While there was little evidence of constitutive expression of CD107a or IFN-γ in mock treated NK cells, exposure to IAV was sufficient to induce low levels of CD107a, IFN-γ and TNF expression both of which were markedly higher on NP+ compared with NP- cells. The impact of IAV infection on the response of NK cells to HLA-deficient 721.221 cells was then assessed. Co-culture of untreated NK cells with target cells induced strong degranulation and cytokine production (**Figure 27E**). However, both the degranulation and cytokine responses of IAV-exposed NK cells (both NP+ & NP-) to 721.221 cells was markedly elevated. In particular the proportion of degranulating or cytokine producing NK cells was significantly higher in NP+ cells than either mock treated cells. Furthermore the response of cells that were exposed to the virus but remained NP- were also elevated relative to mock treated controls albeit not to the extent of the NP+ population suggesting there was an impact of both direct infection as well as an indirect effect, most likely the result of exposure to type I IFN (**Figure 27E-H**).

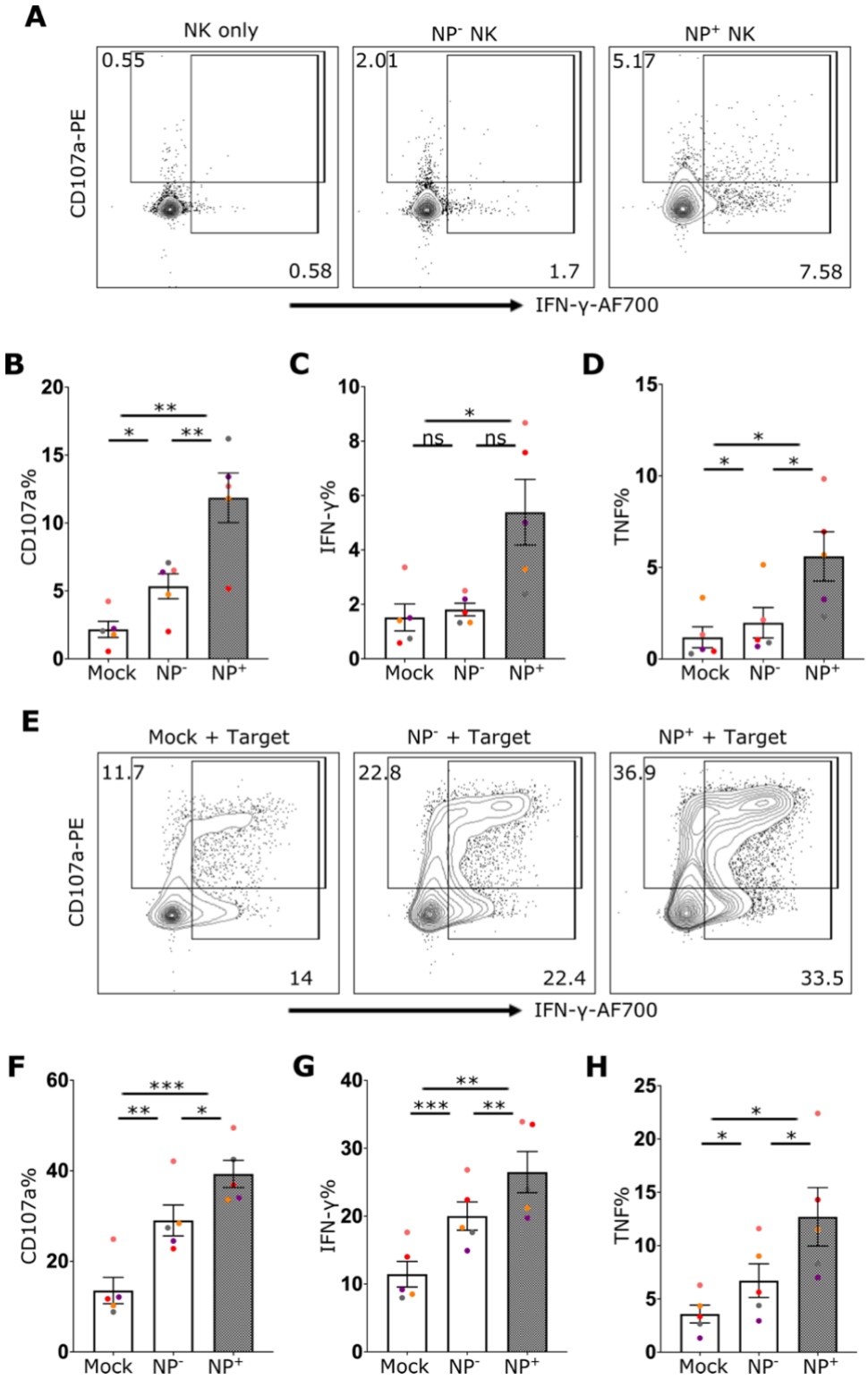

Figure 27. IAV infection increases degranulation and cytokine production of NK cells

Flow cytometry plots for NK cells showing CD107a on the Y axis and IFN-γ on the X axis from media treated cells (mock) or NP⁻ (uninfected IAV-exposed) and NP⁺ (infected) cells after incubation with IAV in the absence **(A)** or the presence **(E)** of target cells. The proportion of cells expressing CD107a+ **(B,F)**, IFN-γ+ **(C,G)**, and TNF+ **(D,H),** cultured without or with target cells respectively, is represented (n=5). Each colored dot represents an individual donor (color matching indicates paired donors). Bars show mean ± SEM. Statistical significance was tested using repeated measure one-way ANOVA with Dunnett's correction (ns= not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

4.2.2 Transfection of RIG-I Receptor Ligands

4.2.2.1 3p-dsRNA Activates NK Cells and Induces NF-κB and TBK1 Pathways

While the data showed that IAV infection of NK cells leads to the activation of RIG-I receptors, the impact of IAV infection on NK cell function may have been caused by additional effects of the infection rather than cellular activation via RIG-I *per se* (Guo *et al.*, 2011). To specifically evaluate the effect of RIG-I activation, the synthetic RIG-I ligand 3p-dsRNA was transfected into NK cells and its effects on NK cell activation assessed. Similar to IAV infection, flow cytometric analyses revealed that cells transfected with3p-dsRNA showed increased CD69 expression that was essentially identical to the levels induced by culture of NK cells with IFN-α (**Figure 28A,B**). Similarly transfection of 3p-dsRNA induced the phosphorylation of NF-κB p65, and TBK1 proteins downstream of RIG-I along with IFIT1 expression relative to that observed in control treated cells (**Figure 28C,D**). Moreover, the levels of phosphorylated NF-κB p65, and TBK1 and IFIT1 closely resembled those obtained by treating NK cells with IFN-α. Taken together, the data demonstrated that transfection of NK cells with 3p-dsRNA induced a pattern of activation of RIG-I pathway components similar to what was observed following IAV infection as well as treatment with type I IFN.

4.2.2.2 RIG-I stimulation Induces IFN-I Secretion and Reduces NK Cell Susceptibility to IAV

Since RIG-I receptor stimulation in other cell types had been shown to drive the production and secretion of type I IFN, supernatants of both control and 3p-dsRNA stimulated NK cells were assessed for the presence of type I IFN by adding them to a TBK1/IKKα/IKKβ/IKKε-deficient THP1 dual reporter cell line (**Figure 28E**). Consistent with RIG-I activation, 3p-dsRNA-treated but not control treated cells stimulated type IFN production (**Figure 28E**).

While RIG-I stimulation clearly was associated with elevated functional responses from NK cells, its activation in other cell types has also been critical in inducing intrinsic anti-viral responses. To monitor RIG-I induced intrinsic antiviral effects, the capacity of IAV to infect RIG-I-stimulated NK cells was also assessed. Consistent with their effects on other cell types, pre-treatment of NK cells with 3p-dsRNA or IFN-α reduced IAV infection significantly compared to control cells exposed to either media alone or a control RNA that cannot activate RIG-I (**Figure 28F,G**).

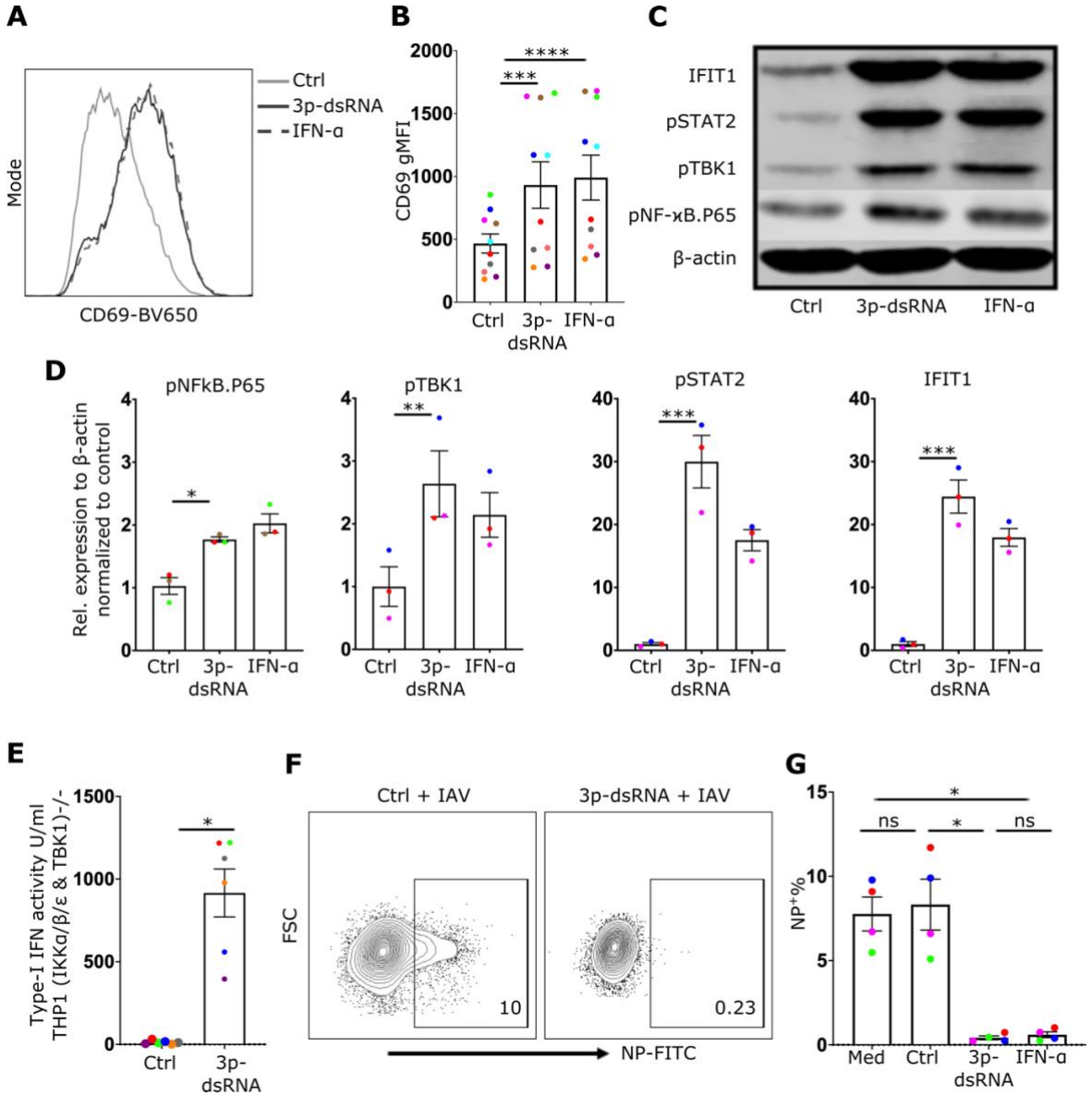

Figure 28. 3p-dsRNA stimulates downstream of nucleic acid receptors and leads to IFN-I response

A) Histogram of gMFI of CD69 after overnight treatment with 3p-ssRNA (Control, Ctrl), 3p-dsRNA, or IFN-α. **B)** quantification of CD69 gMFI (n=10). **C)** Western blot from NK cells stimulated as in A. **D)** Relative protein expression (n=3). **E)** Bar chart representing IFN-I activity of TBK1/ IKKα/IKKβ/IKKε-deficient THP1 dual reporter cell line after 24-hr incubation with cell-free supernatants from NK cells. **F)** Representative flow cytometry plots from NK cells and **G)** proportion of infected (%NP$^+$) cells (n=4) after overnight treatment with RIG-I ligands (or control) followed by incubation with IAV for 8 hr. Med=media only control. Each symbol represents an individual donor (color is representative of paired samples), n=4. Bars show mean ± SEM. Statistical significance was tested using paired t-test for two group comparison and repeated measures one-way ANOVA with Dunnett's correction for more than two groups (ns= not significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

4.2.2.3 3p-dsRNA Induces IFN-I via RIG-I and IFNAR/STAT2 Axis

To better define the contribution of secreted type I IFN to the enhanced activation of NP+ NK cells relative to intrinsic signalling stemming from RIG-I activation, neutralizing IFNAR2-specific mAbs were used to block IFN signalling. As expected, cells treated with control RNA did not show evidence of the induction of IFIT1 expression or the presence pSTAT2 whereas treatment with both 3p-RNA or IFN-α resulted in STAT2 phosphorylation and the induction of IFITI1 expression. Addition of anti-IFNAR2 mAb at 1, 2 and 4 μg/ml resulted in a marked diminution of both responses. Strikingly, at a concentration of 4μg/ml, the IFNAR2 mAb completely abrogated the production of pSTAT2 and IFIT1 following 3p-RNA treatment while these proteins were both detected following IFN-α treatment suggesting that the generation of pSTAT2 and IFIT1 in 3p-dsRNA treated cells may in part be due to an intrinsic response to IFN-α. However, The results demonstrated a clear reduction in the phosphorylation of STAT2 and the expression of IFIT1 proteins (**Figure 29A**) in the presence of blocking IFNAR2 antibodies, at 4μg/ml the mAbs did not completely neutralize the effect by exogenously added type I IFN. The molecular basis for these observations was further explored by using CRISPR-Cas9 technology to delete RIG-I (**Figure 29B,C**) and STAT2 (**Figure 29B,E**) in primary NK cells. As expected, knockout of RIG-I resulted in a marked reduction of 3p-RNA-induced STAT2 phosphorylation and IFIT1 upregulation. Similarly, NK cells lacking STAT2 expression showed a reduced upregulation of IFIT1 upon RIG-I stimulation. Taken together the reduced expression of RIG-I (**Figure 29D**) and STAT2 (**Figure 29F**) was associated with a diminished interferon response, evident in the reduced IFIT1 expression in NK cells treated with RIG-I ligands. Indeed, the induction of IFIT1 by IFN-α in the cells lacking RIG-I demonstrated no significant difference. However, the deletion of STAT2 proteins lowered the induction of IFIT1 by both RIG-I ligands and IFN-α. This confirms that the induction of IFIT1 is due to the autocrine/paracrine effect of the secreted type I IFN which can be reproduced using exogenous IFN-α.

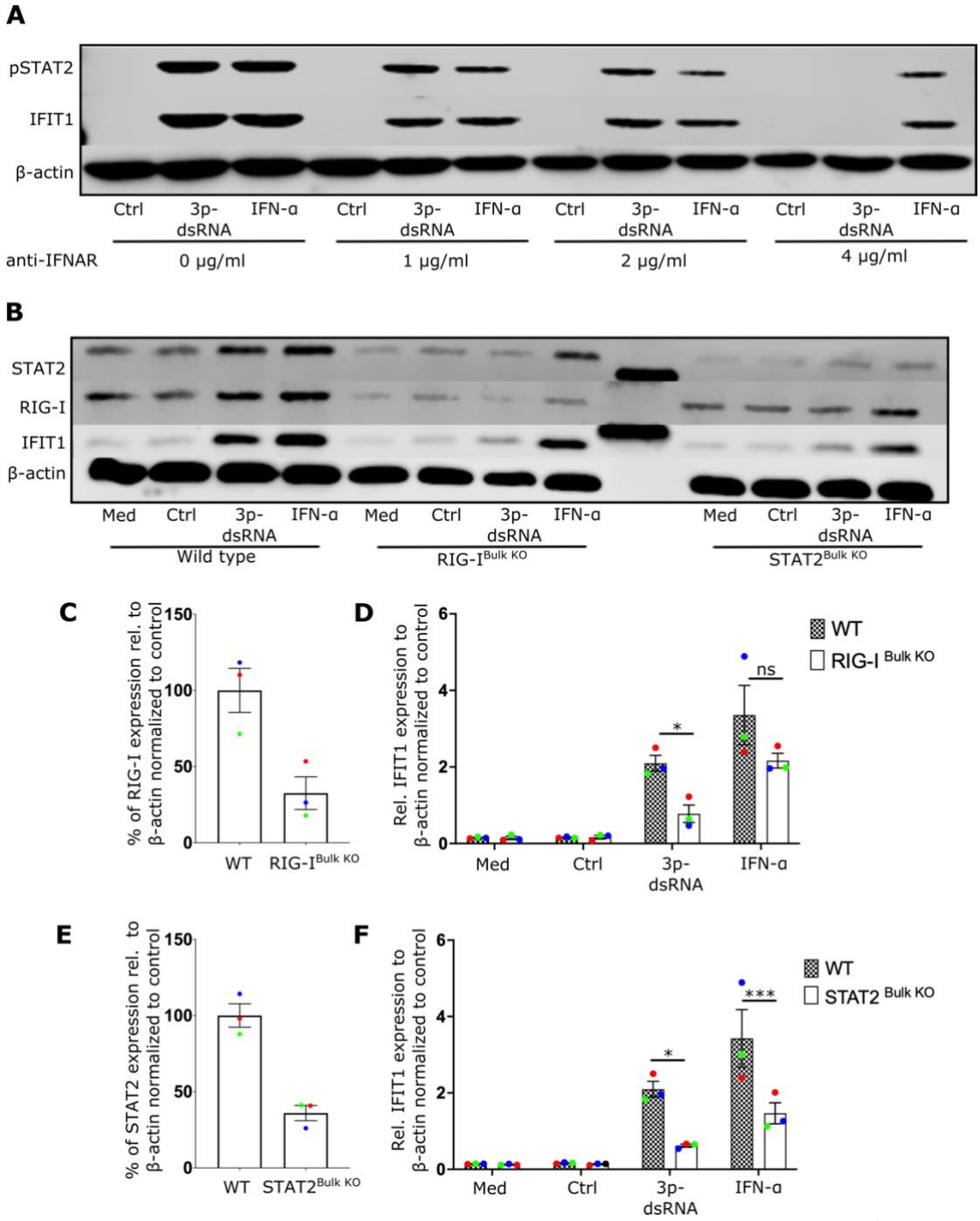

Figure 29. 3p-dsRNA drives type I IFN response in NK cells via RIG-I and IFRAR/STAT2 axis

A) Western blot image for STAT2 and IFIT1 proteins from human primary NK cells treated with different concentrations of IFNAR neutralizing antibodies. **B)** Western blot image for STAT2, RIG-I, and STAT2 proteins from wildtype (WT), RIG-I bulk KO and STAT2 bulk KO human primary NK cells, after overnight culture with media alone (Med), 3p-ssRNA (Control, Ctrl), 3p-dsRNA or IFN-α. Relative RIG-I **(C).** and STAT2 **(E)** expression in % from bulk KO cells. IFIT1 relative expression in RIG-I **(D)** and STAT2 **(D)** bulk KO cell. Each colored dot represents an individual donor (color matching means paired samples), n=3 donors. Bars show mean ± SEM. Statistical analysis was performed using Two-way ANOVA with Bonferroni's correction for multiple comparisons (*$p < 0.05$, and ***$p < 0.001$).

4.2.2.4 RIG-I Stimulation Induces the Transcription of TRAIL and IFN-γ

The *TNFSF10* gene is responsible for the transcription of TRAIL proteins, while *IFNG* produces IFN-γ, both of which play crucial roles in NK cell effector function. TRAIL engagement induces apoptosis in target cells through death receptor activation, while IFN-γ enhances phagocytic activity and promotes CD4 T cell differentiation (Schroder *et al.*, 2004; Kang *et al.*, 2018). Consequently, whether the activation of RIG-I led to changes in the transcription of these genes was assessed. Treatment with non-stimulatory RNA had minimal impact on the mRNA levels of either *IFNG* or *TNFSF10*. In contrast, at 4 hr post treatment, treatment with 3p-RNA resulted in a 16-fold and 7-fold induction of IFN-γ and TRIAL at 4 hr which was markedly reduced by 24 hr post treatment (**Figure 30**). Importantly, the impact of RIG-I stimulation on mRNA levels was greater than that induced by exposure to IFN-α, suggesting a potent effect in promoting the transcription of these two critical effector proteins. Collectively, these findings underscore the dynamic regulation of IFN-γ and TRAIL expression in NK cells in response to RIG-I receptor activation.

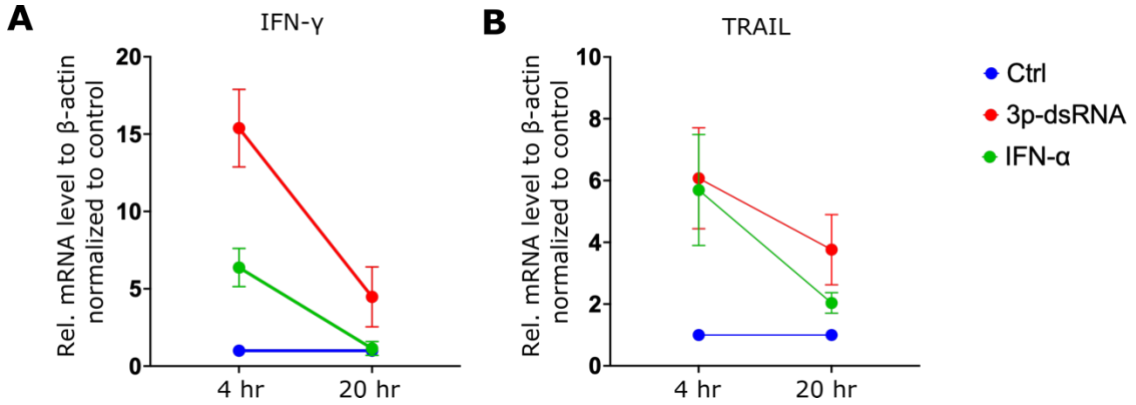

Figure 30. RIG-I stimulation enhances the transcription of IFN-γ and TRAIL independent on target stimulation

Relative fold transcription levels of **(A)** IFN-γ and **(B)** TRAIL normalized to β-Actin housekeeping protein. Human primary NK cells were isolated from buffy coats and stimulated either for 4 hr or 20 hr with negative control (3p-ssRNA), RIG-I ligands (3p-dsRNA), or IFN-α positive control. Data are shown as mean of relative mRNA expression ± SEM, n=3 donors (Delta-delta Ct method was used for normalization).

4.2.2.5 RIG-I Stimulation Enhances NK Cell Effector Function

Since NK cells infected with IAV exhibited an increased effector response towards target cells and targeted RIG-I stimulation also induced the transcription of TRAIL and IFN-γ, the effector response of RIG-I stimulated NK cells was formally evaluated. Purified NK cells were incubated overnight with 3p-dsRNA, IFN-α or control RNA, after which they were incubated with

721.221 target cells. Cells that had been stimulated with 3p-dsRNA showed a significant increase in both degranulation (**Figure 31A,B**) and production of IFN-γ (**Figure 31A,C**) as well as TNF (**Figure 31A,C**) following coculture with 721.221 cells. Again, the magnitude of this enhancement was similar to the enhanced response generated by pre-treatment with IFN-α. To exclude the contribution of other immune cells such as monocytes and B cells that can secrete type I IFN upon RIG-I stimulation to the analyses above, highly purified populations of NK cells were obtained by cell sorting (>99%) (CD56+CD3−CD19−CD14−) and then analysed in a similar manner. Consistent with the initial observations, NK cells stimulated with 3p-dsRNA but not control RNA exhibited elevated levels of CD69 expression, CD107a, IFN- γ , and TNF and therefore confirmed the data obtained from enriched NK cell populations (**Figure 32**). Finally, to exclude the possibility of indirect activation of NK cells through the activation of target cells by residual RIG-I ligand from carry-over, THP-1 dual TBK1$^{-/-}$ IKKα$^{-/-}$ IKKβ$^{-/-}$ IKKε$^{-/-}$ cells were also utilized as target cells. These cells are characterized by the absence of RIG-I downstream signalling and therefore do not induce expression of antiviral proteins or cytokines. No evidence of enhanced NK cell activation was observed following co-culture of NK cells with these cells (**Figure 38**). Indeed, coculture of RIG-I-stimulated NK cells with these cells resulted in a similar enhancement in NK cell effector function observed in the coculture with 721.221 target cells (**Figure 32C-G**). These results confirm that the enhanced activation of NK cells by RIG-I ligands resulted from the intrinsic activation of the RIG-I receptors within NK cells themselves.

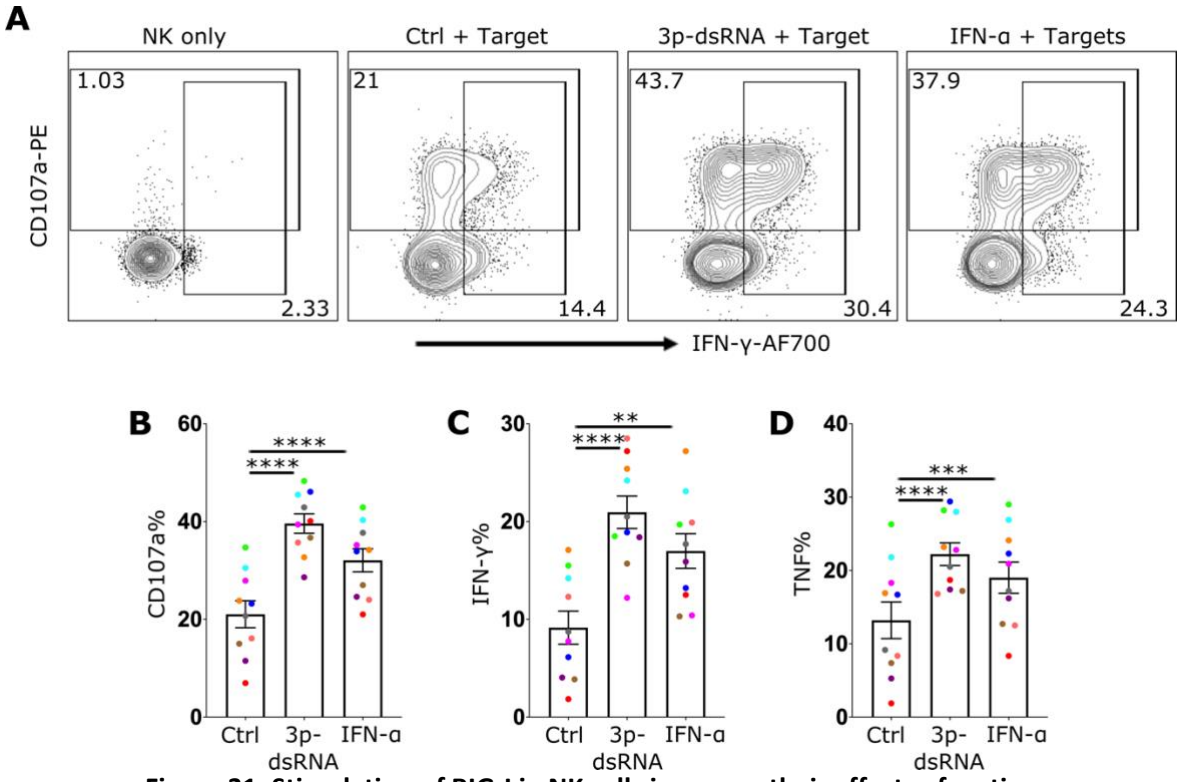

Figure 31. Stimulation of RIG-I in NK cells increases their effector function

A) Flow cytometry plots representing CD107a and IFN-γ of 721.221-activated NK cells after overnight incubation with either media alone (NK only), 3p-ssRNA (Ctrl), 3p-dsRNA or IFN-α. Proportion (%) of NK cells positive for **B)** CD107a, **C)** IFN-γ and **D)** TNF is shown respectively. Each dot represents an individual donor (color matching means paired samples, n=10 donors). Bars show mean ± SEM. Statistical analysis was performed using repeated measures one-way ANOVA with Dunnett's correction (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

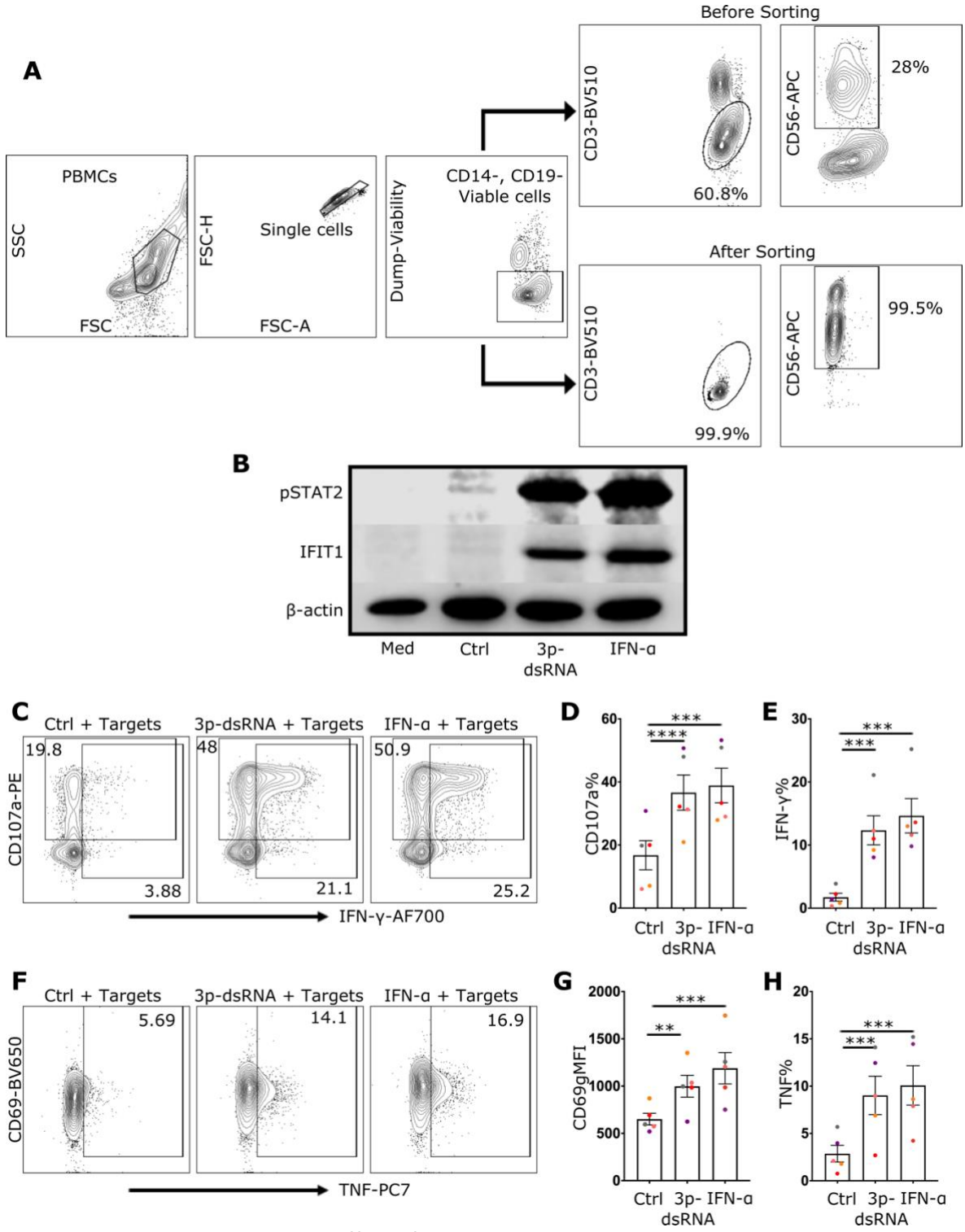

Figure 32. The effect of RIG-I stimulation on sorted NK cells.

A) Flow cytometry plots representing the gating strategy used for NK cell sorting and the purity before and after sorting. **B)** Western blot image for sorted NK cells treated with media, 3p-ssRNA (Ctrl), 3p-dsRNA or IFN-α. Flow cytometry plots representing CD107a vs IFN-γ **(C)** and CD69 vs TNF **(F)** for sorted NK cells cocultured with 721.221 cells for 4 hr at 1:1 ratio. Quantification of CD107a+% **(D),** IFN-γ+% **(C),** CD69 gMFI **(G)** and TNF+% **(G)** is shown. Each colored dot represents one donor (color matching reflects data from NK cells from the same donor), n=5. Bars show mean ± SEM. Statistical analysis was performed using repeated measures one-way ANOVA with Dunnett's correction (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

4.2.2.6 3'Sequencing Indicates Predominant Interferon Signature By both IAV and 3p-dsRNA

To better define the global impacts of RIG-I stimulation, IAV infection and IFN-α treatment, NK cells were exposed to each of these stimuli for 4 hr and then gene expression analysis performed on RNA isolated from both resting and treated NK cells. IAV-exposed, RIG-I stimulated, and IFN-α treated cells each revealed strong induction of the antiviral interferon-stimulated genes (ISGs). Specifically, the transcription of 2'-5'-Oligoadenylate Synthetase 1 (*OAS1*), 2'-5'-Oligoadenylate Synthetase 2 (*OAS2*), Interferon Induced Protein With Tetratricopeptide Repeats 1 (*IFIT1*), *IFIT2*, *IFIT3*, Interferon-induced Transmembrane Protein 3 (*IFITM3*), Interferon Induced Protein 44 Like (*IFI44L*), MX Dynamin Like GTPase 1 and 2 (*MX1* & *MX2*), and Interferon Stimulated Exonuclease Gene 20 (*ISG20*) were all significantly elevated relative to control cells (**Figure 33**). Furthermore, genes associated with the activation of NK cells, including Lymphocyte activating 3 (*LAG3*), *IFNG* (IFN-γ), and *TNFSF10* (TRAIL), also exhibited increased expression (**Figure 33**), suggesting that in NK cells these genes are modulated by exposure to type I IFN.

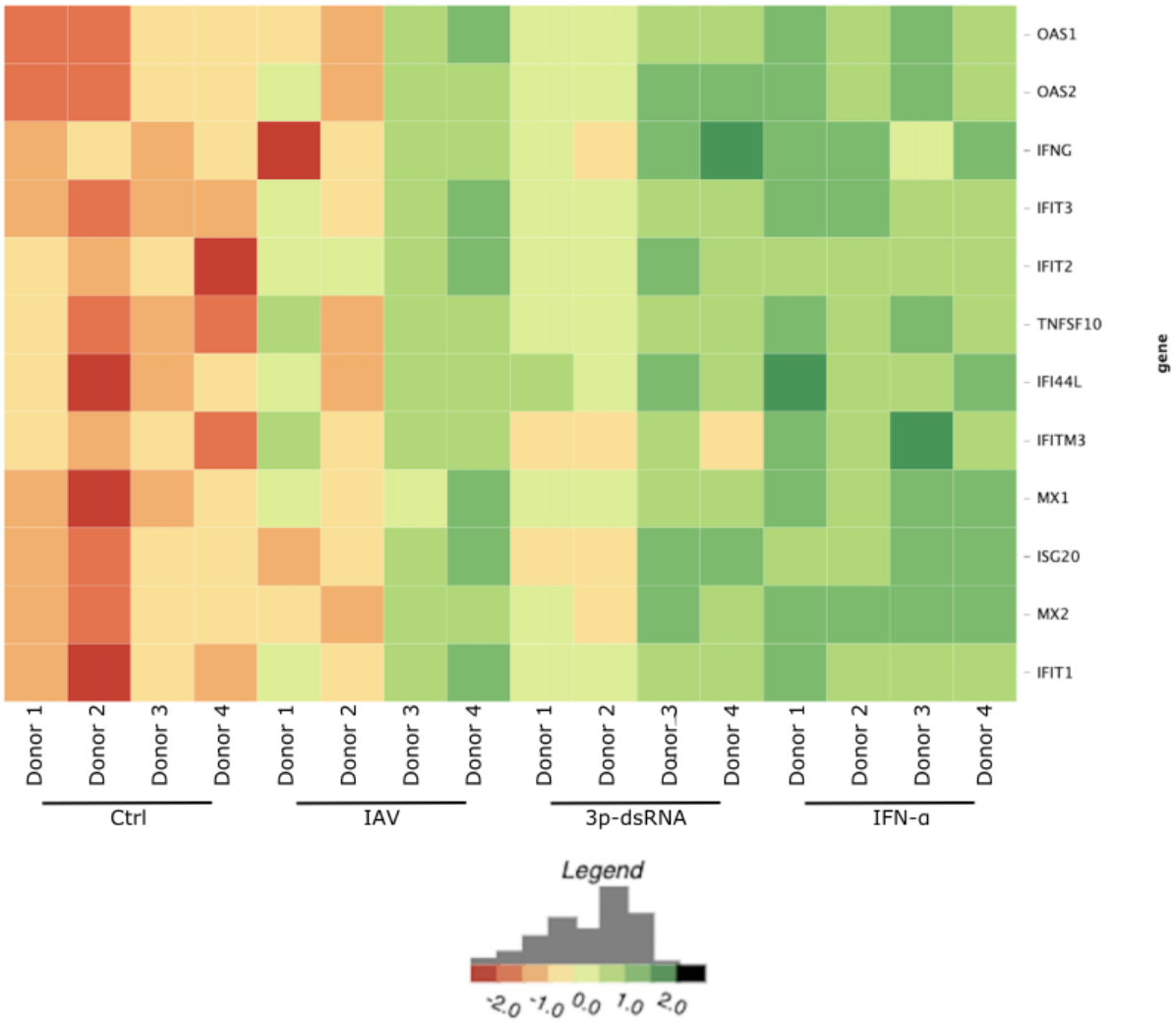

Figure 33. 3' Sequencing Confirms Similarities Between IAV Infection and RIG-I Stimulation in NK Cells

Purified NK cells were infected with IAV or transfected with 3p-dsRNA complexed with lipofectamine, or treated with IFN-α and RNA was purified using the RLT buffer. The heatmap illustrates substantial elevation in various type I interferon stimulated genes, including 2'-5'-Oligoadenylate Synthetase 1 (*OAS1*), 2'-5'-Oligoadenylate Synthetase 2 (*OAS2*), Interferon Induced Protein With Tetratricopeptide Repeats 1 (*IFIT1*), *IFIT2*, *IFIT3*, Interferon-induced Transmembrane Protein 3 (*IFITM3*), Interferon Induced Protein 44 Like (*IFI44L*), MX Dynamin Like GTPase 1 and 2 (*MX1* & *MX2*), and Interferon Stimulated Exonuclease Gene 20 (*ISG20*) as well as genes associated with the activation of NK cells, including Lymphocyte activating 3 (*LAG3*), *IFNG* (IFN-γ), and *TNFSF10* (TRAIL). Extracted RNA was subjected to the aforementioned treatments for a duration of 4 hr (n=4 donors). Total RNA was used to generate libraries of sequences close to the 3’ end of polyadenylated RNA. The libraries were sequenced on an Illumina HiSeq1500 device. The reads were aligned to the human reference genome using STAR (Dobin *et al.*, 2013). The transcripts were quantified using HTSeq (S Anders, T P Pyl, W Huber: HTSeq — A Python framework to work with high-throughput sequencing data. bioRxiv 2014). Differential expression analysis was performed using EdgeR (McCarthy *et al.*, 2012). The colour scale represents the transcription levels, ranging from lowest (-2.5, shown in red) to highest (+2.5, shown in black).

4.2.2.7 CGAS and TLR8 Stimulation Do not Lead to a Significant Function Enhancement

Finally, it was assessed whether the activation of various nucleic acid receptors also affects NK cell function. cGAS was stimulated by transfecting plasmid DNA while TLR8 was activated by co-culture with the TLR8 agonist (TL8-506). The modulation of NK cell activation following exposure to cGAS was compared with that following treatment with 3p-ssRNA (used as a negative control) and IFN-α. cGAS stimulation consistently resulted in a modest enhancement of activation and effector phenotypes, notably CD69 upregulation and slightly increased degranulation, and cytokine production which likely would have reached statistical significance with an increased number of donors. (**Figure 34A-D**) Similarly, TLR8 (**Figure 34E-H**) stimulation also elevated NK cell responses relative to cells treated with controls, the TLR8 stimulation reaching statistical significance. These results further underscore the capacity of nucleic acid receptors, in a general context, to positively influence the effector function of NK cells targeted towards other cells.

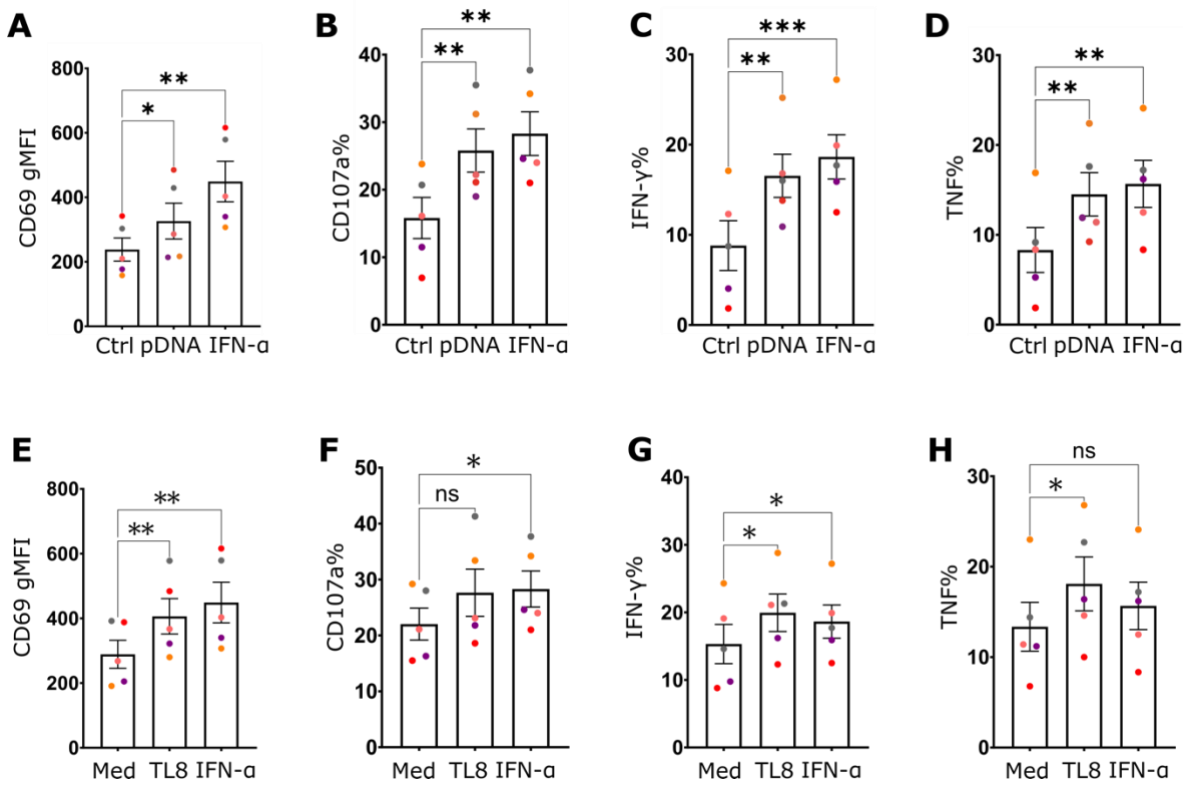

Figure 34. Stimulation of cGAS and TLR8 in NK cells enhances their effector function

NK cells cocultured with 721.221 cells, after the overnight incubation with cGAS ligand (pDNA) and TLR8 ligand (TL8). Demonstrated bar charts show analysis of **A,E**) CD69 gMFI, **B,F)** CD107a%, **C,G)** IFN-γ% and **D,H)** TNF% of NK cells in response to pDNA (A-D) and TL8 (E-H). Each data point is an individual donor (n=5). The bars show mean ± SEM. The numbers are cell frequencies. Repeated measures one-way ANOVA with Dunnett's correction was used for comparison (ns= not significant, *p<.05; ** p<.01).

4.3 Discussion

While RIG-I plays a crucial role in detecting RNA viruses across various tissues and cell types, its potential to regulate NK cell responses is unclear (Bartok and Hartmann, 2020). Prior studies have shown that activating RIG-I with synthetic RNA can boost NK cell-mediated killing of melanoma cells (Poeck *et al.*, 2008; Daßler-Plenker *et al.*, 2019). However exposure of NK cells to IAV itself is known to stimulate RIG-I and been reported to diminish NK cell cytotoxicity (Mao *et al.*, 2009; Lin *et al.*, 2012). Nevertheless, the specific role of RIG-I signaling in how NK cells respond to IAV infection remains relatively uncharted. Through a systematic examination of RIG-I activation and downstream signaling, coupled with CRISPR/Cas9-mediated genome editing in primary NK cells, this study focused on the impacts of RIG-I activation during IAV infection. The findings revealed that activated NK cells were more susceptible to IAV infection compared to resting cells. This underscores the significance of understanding the role of nucleic acid sensing within NK cells during direct infection, especially considering their exposure to a high local concentration of infectious virions when interacting with infected cells.

Both IAV infection and stimulation with 3p-dsRNA, a specific synthetic RNA ligand for RIG-I resulted in elevated CD69 expression, TBK1 phosphorylation, and the secretion of type-I IFN, all of which were dependent on RIG-I. Moreover, they also stimulated RIG-I downstream pathways including the activation of NF-κB that is reported to be required for NK cell cytotoxic function (Vicioso *et al.*, 2021). Critically, this could potentially enhance NK cell activation without the requirement for signaling through IFNAR. Confirmation of cellular activation and the subsequent interferon response was obtained through transcriptomic profile analysis using 3' sequencing. As suggested, upon encountering target cells, IAV infection and RIG-I stimulation increased NK cell degranulation (indicated by CD107a surface expression) and the production of IFN-γ, and TNF. Exposure of NK cells to IFN-α also enhanced their effector functions, however the effect was less pronounced than when stimulated by RIG-I ligands. The deletion of STAT2 from NK cells and the antibody-mediated neutralization of IFNAR prevented the induction of the type I IFN response. Taken together, the data indicate that many of the effects of IAV/RIG-I on NK cell function are contingent on RIG-I stimulation as well as the type-I IFN signaling pathway. Nevertheless, the flow cytometric analyses showed that infected cells exhibited greater activation levels than non-infected bystander

cells, suggesting that direct IAV infection of NK cells contributed to enhancing responses to HLA-I-deficient target cells above and beyond in the impact of extrinsic exposure to type I IFN. This effectively implicates a contribution of intrinsic signalling to the elevated response to target cells.

Previous work had observed enhanced melanoma cell killing by NK cells following their transfection with a synthetic RIG-I ligand and as found here, this effect was not strictly replicated by pre-incubation of NK cells with IFN-β (Daßler-Plenker *et al.*, 2019) and it was concluded that RIG-I stimulation of NK cell enhanced target cytotoxicity via the upregulation of TRAIL rather than the secretion of type I IFN or increased IFN-γ or TNF responses and that RIG-I activation in NK cells may trigger additional genes beyond those induced by IFN- β. Similar to that study, we observed the upregulation of CD69 and TRAIL in RIG-I stimulated NK cells independent of co-culture with target cells. Additionally, the work herein shows for the that following exposure to ligands, RIG-I receptors in NK cells are fully competent to direct type I IFN secretion which can additionally enhance the function of bystander NK cells via extrinsic signalling through IFNAR. Furthermore, when using myeloid tumor cells (THP1) or HLA-I-deficient B-lymphoblastoid cells (721.221) as target cells, augmented IFN-γ, TNF, or CD107a expression induced by IAV, 3p-dsRNA, or pre-incubation with IFN-α was observed which contrasted somewhat with the earlier study by Daßler-Plenker et al., (Daßler-Plenker *et al.*, 2019). Intriguingly, unlike 3p-dsRNA and IFN-α, IAV exposure even without the presence of target cells was able to induce some IFN-γ production. This difference may be attributable to the capacity of viral proteins such as the HA present on IAV virions to directly engage activating receptors like NKp46 (Mandelboim *et al.*, 2001; Arnon *et al.*, 2004), indicating that IAV not only boosts NK effector function through RIG-I/type I IFN but also through additional stimulation of NK cell receptors (reviewed in Luczo et al. (Luczo *et al.*, 2021)).

While the findings contrast with prior studies that reported impairments in NK cell cytotoxicity due to IAV infection or exposure (Lin *et al.*, 2012; Mao *et al.*, 2017), in line with the results presented herein, Lin et al. (Mao *et al.*, 2009) demonstrated that exposure to IAV increased CD69 expression and enhanced degranulation in human NK cells. However, although they observed heightened activation and degranulation, they noted reduced target cell killing, possibly due to the initiation of infection-induced apoptosis of prior to their use in

activation assays. In contrast, in the studies herein, there was limited NK cell death in the first 24 hours post-virus infection, which could be attributed to the use of different virus strains (RG-PR8-Brazil78 HA, NA (H1N1) compared with WSN virus/33 (H1N1)). Indeed, strain to strain variation across the influenza genome and in particular NS1 may be associated with differences in their ability to suppress or limit RIG-I activation. However, it was noted that NK cell viability was markedly lower in some donors compared to others 24 hours post-culture, independent of IAV infection. The experimental design aimed to observe differences between conditions under a single stimulation factor; therefore, only a very low concentration of IL-2 was used. This could have caused the drop in viability, especially in the absence of other supportive cytokines such as IL-15. It could also have occurred due to varying responsiveness to the low concentration of IL-2, which might be linked to lower expression levels of IL-2 receptors, leading to reduced cellular survival.

Nonetheless, the data here unequivocally demonstrate RIG-I-dependent activation within virus-infected NK cells enhances NK cell effector function at least over the time immediately post-infection. Such activation might both enhance the capacity of NK cells to target virus-infected cells that cannot produce type I IFN as well as confer a degree of protection on NK cells themselves from potential longer-term adverse effects of viral infection. Understanding the *in vivo* significance of these observations will require further investigation possibly making use of a wider range of influenza strains which differ both in their HA-specificity as well as in genes which directly antagonize RIG-I activation such as NS1.

5. Aim 3: Studying The Effect of Nucleic Acid Receptor Stimulation in Target Cells on NK Effector Function

5.1 Introduction

It is well-established that the activation of nucleic acid receptors, whether initiated by natural infection or synthetic ligands, serves as the trigger for the production of pivotal proinflammatory cytokines and type I interferons (Mohamed *et al.*, 2023). These molecular signals, in turn, orchestrate the complex interactions of innate immune responses, shaping adaptive immune responses. As a result, there is significant interest in understanding the extent to which the administration of nucleic acid receptor ligands for both TLR and RIG-I-like receptors can have immunomodulatory and adjuvant-like effects (Gosu *et al.*, 2012; Y. Jiang *et al.*, 2023) with view to improving immunotherapies and vaccine regimens (Y. Jiang *et al.*, 2023). While it is well-documented that these ligands can indeed bolster the overall immune response in the context of tumor therapy and vaccination (Bishani and Chernolovskaya, 2021), a critical question arises: what precisely is the impact of these ligands when they stimulate their corresponding receptors within target cells and how is this manifest in the crosstalk with other cell types, and in particular of cytotoxic cells, such as NK cells?.

5.2 Results

5.2.1 Nucleic Acid Receptors Play a Crucial Role in IAV Infection

To better understand the role of nucleic acid receptors during IAV infection of target cells, wild type THP-1 cells along with RIG-I or cGAS-deficient variants either stimulated with 3-dsRNA, pDNA or IFN-α and their response assessed by Western blot (**Figure 35**). In wild-type cells, stimulation with 3p-dsRNA, pDNA, and IFN-α led to the upregulation of RIG-I receptors as well as IFIT1, indicating a robust type I IFN response. As expected, RIG-I$^{-/-}$ and cGAS$^{-/-}$ THP1 cells failed to respond to their respective ligands, 3p-dsRNA or pDNA respectively, confirming the specificity of the ligands and the efficiency of the knockout. Importantly, all three cell lines remained responsive to IFN-α stimulation, as evidenced by the upregulation of RIG-I and/or IFIT1 following its addition (**Figure 35**).

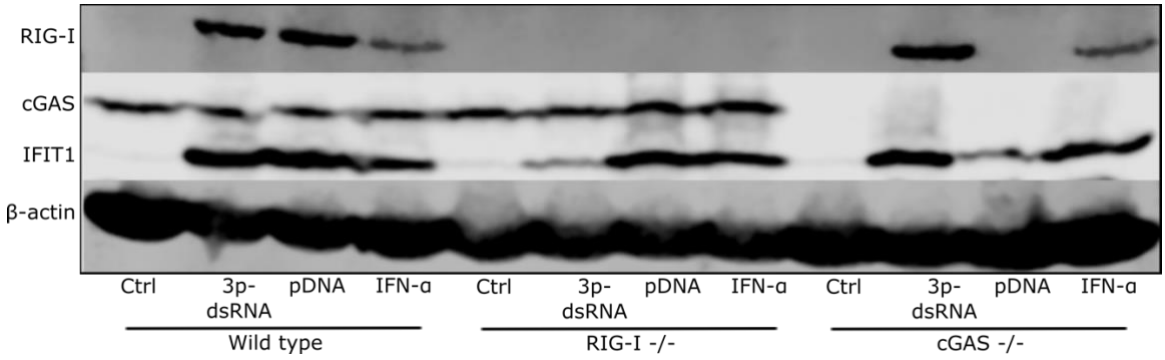

Figure 35. Confirmation of THP1 Knockout Cells

Representative Western blot of RIG-I, cGAS, IFIT1 and β-actin from wildtype, RIG-I$^{-/-}$ and cGAS$^{-/-}$ THP1 cells. The cells were treated overnight with negative control (3p-ssRNA), RIG-I ligand (3p-dsRNA), cGAS ligand (pDNA) and 1000 U/ml IFN-α as a positive control, (n=3).

To evaluate the importance of different nucleic acid receptors in cell susceptibility to IAV viral infection, we then made use of THP1cells that were either RIG-I$^{-/-}$ or cGAS$^{-/-}$, or lacking TBK1$^{-/-}$ IKKα$^{-/-}$ IKKβ$^{-/-}$ IKKε$^{-/-}$ and hence could not signal downstream of nucleic acid receptors, These were infected with IAV and their susceptibility to infection determined by flow cytometry by assessing the proportion of NP+ cells. Interestingly, while approximately 10% of wild type THP-1 cells were NP+ at an MOI of 1, the proportion of NP+ cells was higher in the cell lines that were deficient in nucleic acid sensing, with the THP-1 dual TBK1$^{-/-}$ IKKα$^{-/-}$ IKKβ$^{-/-}$ IKKε$^{-/-}$ cells being more susceptible to IAV infection than either cGAS$^{-/-}$ and RIG-I$^{-/-}$ cells (**Figure 36**). Since IAV is an RNA virus, the lack of RIG-I RNA sensors was associated with higher infection than cGAS DNA sensors. The relatively highest infection in the cells that lack the four kinase enzymes downstream of nucleic acid suggests that other nucleic acid receptors such as TLRs, including TLR7 and 8, might be involved in sensing and inhibiting viral replication. This underscores the complementary importance of different nucleic acid receptors in the antiviral immune response against IAV infection, which may limit viral infection.

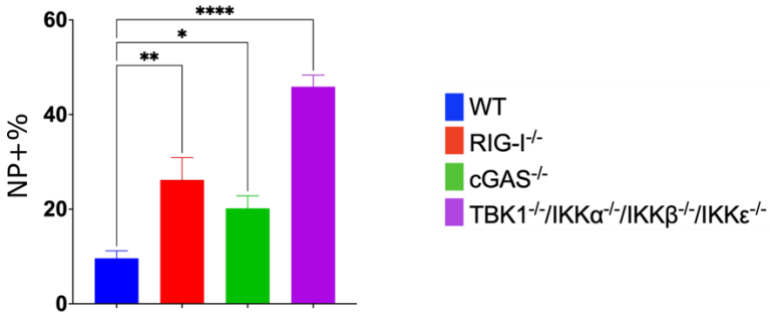

Figure 36. IAV Infection of different THP1 cell lines

Proportion of NP+ cells of different THP1 dual cell lines 8 hr post-IAV exposure. At least three independent experiments were performed (n=3-5). The bars show mean ± SEM. The numbers are cell frequencies. Statistical significance was tested using ordinary one-way ANOVA (ns= not significant, *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$).

5.2.2 IAV Infection of THP1 Cells Enhances NK cell Degranulation and Cytokine Production

To assess how nucleic acid receptor signalling within target cells modified NK cell responses, THP1 dual cells were treated with IAV, media or IFN-α for 4 hr. THP1 cells were then extensively washed to remove free stimulants and were co-cultured with purified NK cells for another 4 hr after which their functional responses were assessed by flow cytometry. In all cases, IAV Infection of WT, RIG-I$^{-/-}$ and cGAS$^{-/-}$ cell lines was associated with increased degranulation and IFN-γ production relative to mock-infected cells (**Figure 37A,B**). While this was statistically significant for wild type, RIG-I$^{-/-}$ and cGAS$^{-/-}$ cells, it did not reach statistical significance in the quadruple kinase KO cell line for CD107a response. Incubation of target cells with IFN-α did not result in enhancement of either degranulation or the production of IFN-γ (**Figure 37A,B**). Altogether, the data suggests that nucleic acid sensing during IAV infection may play an important role in the infection susceptibility. However, the enhancement of the effector function of NK cells was if at all only slightly influenced by nucleic acid receptor signalling in or priming by IFN-α of target cells.

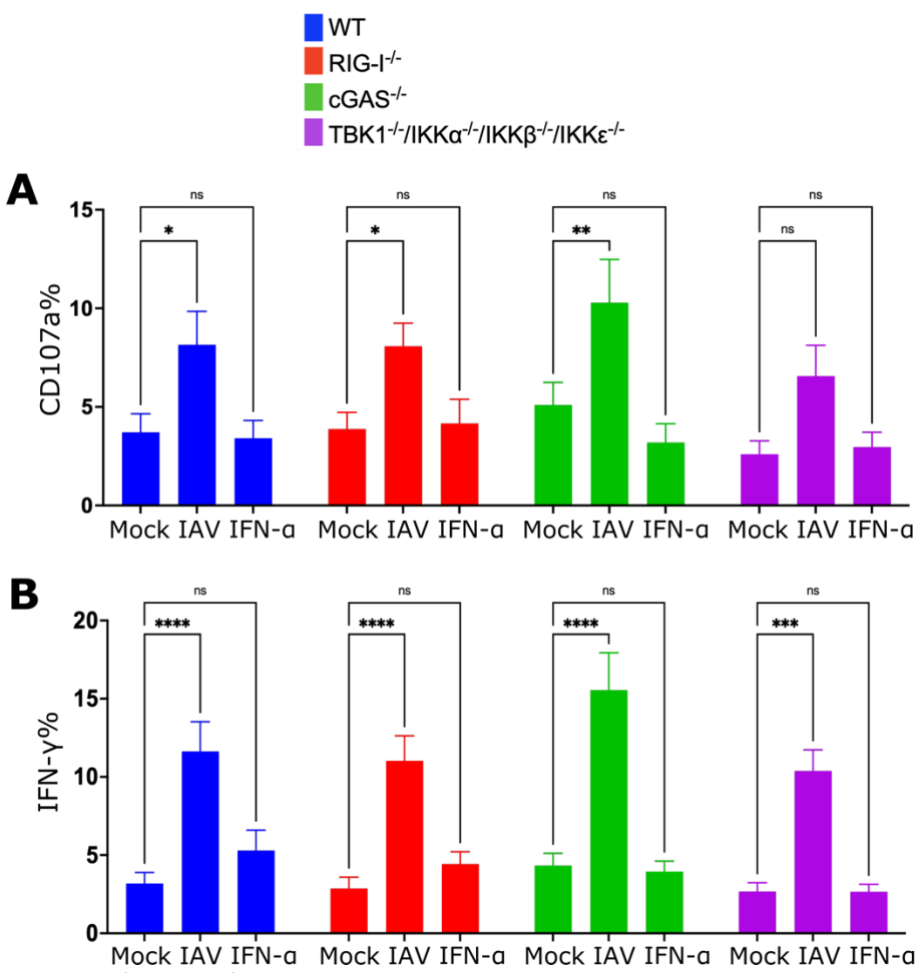

Figure 37. IAV infection of target cells enhances NK response independent on nucleic acid receptors

A) Quantification of CD107a+ NK cells cocultured with media, IAV, and IFN-α treated WT, RIG-I$^{-/-}$, cGAS$^{-/-}$ and TBK1$^{-/-}$ IKKα$^{-/-}$ IKKβ$^{-/-}$ IKKε$^{-/-}$ THP1 cells. **B)** Quantification of IFN-γ+ of the same conditions as in **A**. Five independent experiments were performed (n=5). The bars show mean ± SEM. The numbers are cell frequencies. Statistical significance was tested using two-way ANOVA followed by Bonferroni's correction for multiple group comparisons (ns= not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

5.2.3 Activation of Nucleic Acid Receptors Enhances NK Cell Response

While IAV infection can result in the activation through multiple nucleic acid receptors, the broader impact of IAV infection itself on the target cell is still relatively undefined. Consequently, the impact of targeted activation of RIG-I and cGAS on NK cell activation was next assessed. Following ligand-induced stimulation of both RIG-I and cGAS, THP1 dual cells secreted luciferase activity in the supernatant confirming the capacity to activate cells via each receptor (**Figure 38A**). As anticipated, the RIG-I and cGAS knockout cells showed no response to their respective ligands, further confirming their ligand specificity (**Figure 38A**). As expected, the THP-1 dual TBK1$^{-/-}$ IKKα$^{-/-}$ IKKβ$^{-/-}$ IKKε$^{-/-}$ cells exhibited no response to either

RIG-I and cGAS ligands, reinforcing the crucial role of these downstream components (**Figure 38A**). When examining the effect of these nucleic acid receptor ligands on the degranulation capability of NK cells, there was no appreciable effect of stimulation of wild type THP-1 cells with RIG-I or cGAS agonists (**Figure 38B**). Consistent with this, genetic deletion of RIG-I or cGAS also had little impact on the degranulation response. In contrast, stimulation of wild type THP1 cells with either 3p-dsRNA or pDNA but not IFN-α markedly augmented the IFN response of NK cells (**Figure 38C**). Moreover, this enhanced NK cell IFN-γ response was abrogated by RIG-I deletion when THP-1 cells were stimulated with 3p-dsRNA or by cGAS-deletion when stimulated with pDNA further confirming the specificity of these responses. Similarly, these was no augmented IFN-γ response observed following exposure of $TBK1^{-/-}$ $IKK\alpha^{-/-}$ $IKK\beta^{-/-}$ $IKK\varepsilon^{-/-}$ cells to either 3p-dsRNA, pDNA or IFN-α. Taken together the data indicate that that stimulation of RIG-I and cGAS in THP1 cells resulted in changes within THP cells that drive enhanced IFN-γ responses by NK cells but which are essentially independent of type I, while leaving their degranulation capacity unchanged.

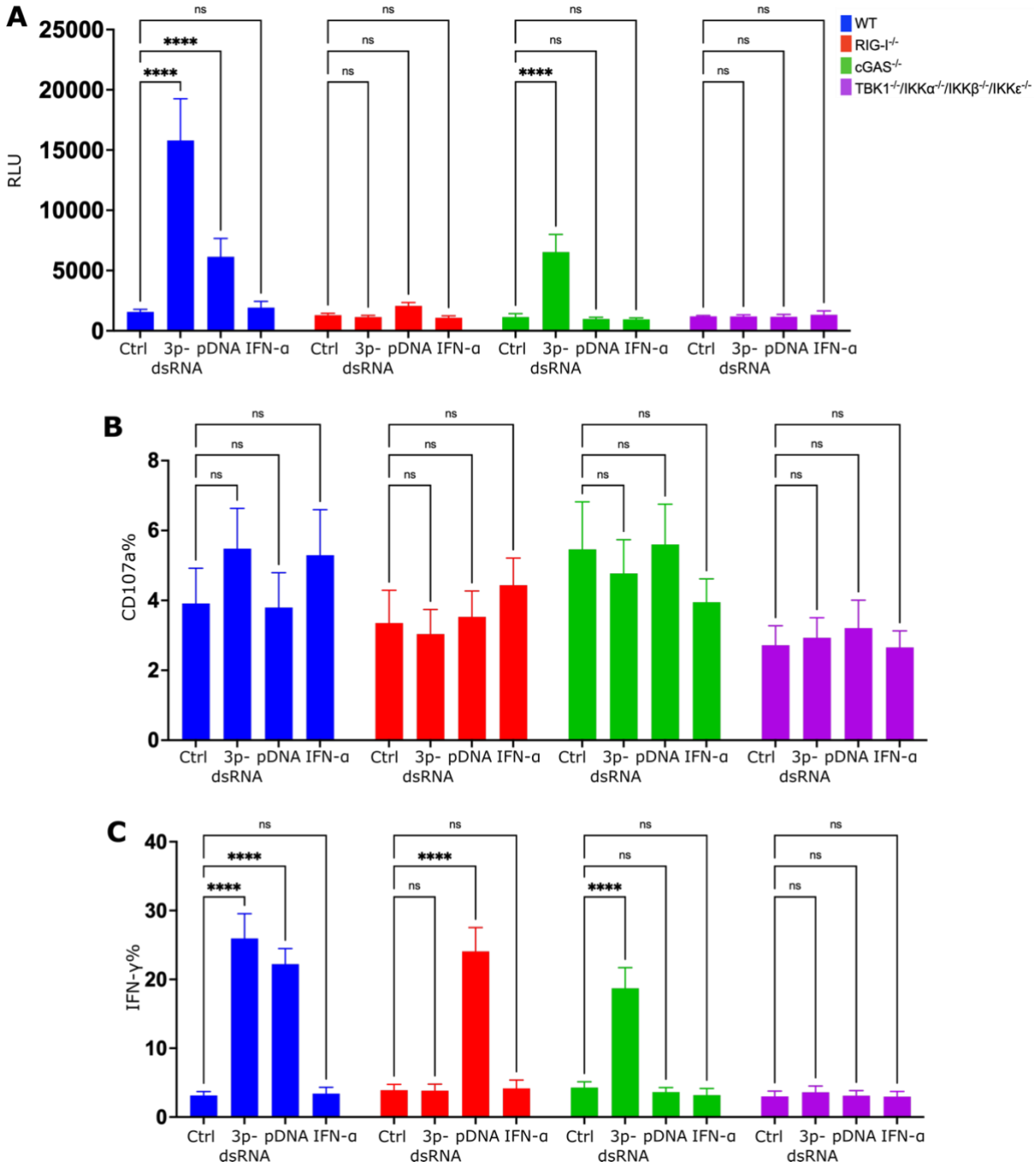

Figure 38. RIG-I and cGAS Stimulation in THP1 cells only increase cytokine production by NK cells

A) Luciferase activity in the supernatant of different THP1 dual cell lines treated with control RNA, RIG-I ligands (3p-dsRNA), cGAS ligands (plasmid DNA) and IFN-α positive control. **B)** CD107a+ NK cells cocultured with the THP1 dual cell stimulated as in A. **C)** IFN-γ+ NK cells treated as in B. Three independent experiments were performed (n=3). The bars show mean ± SEM. The numbers are relative light units or cell frequencies. Statistical significance was tested using two-way ANOVA followed by Bonferroni's correction for multiple group comparisons (ns= not significant, ****$p <$ 0.0001).

5.3 Discussion

Previous studies had identified links between nucleic acid receptor signalling and NK cell activation, with reduced NK cell activation observed in TLR3 deficient mice following infection with Friend retrovirus (Gibbert *et al.*, 2014). Additionally, RIG-I stimulation in mice enhances NK cell recruitment, activation, and proliferation within papillomavirus-associated cancer tissue, indicating the potential of nucleic acid receptors to regulate NK cell activation through intrinsic and/or extrinsic mechanisms (Gibbert *et al.*, 2014). To better understand the potential of extrinsic mechanisms to impact NK cell activation, the capacity of primary NK cells to respond to THP1 cells following their exposure to either viral infection or specific nucleic acid receptor ligands was evaluated.

These investigations into IAV infection of THP1 cells, which results in stimulation of nucleic acid receptors in immune cells such as RIG-I (Mohamed *et al.*, 2023), showed that it enhanced their capacity to elicit degranulation and IFN-γ production by uninfected cocultured NK cells. This functional enhancement of NK cells aligns with recent studies demonstrating elevated antibody-mediated activation of NK cells in response to IAV infection (Jegaskanda *et al.*, 2019). Strikingly, the NK cell response to IAV-infected THP1 cells lacking RIG-I, cGAS and IKKs was not significantly different to that elicited by IAV-infected wildtype cells. This suggests that the enhanced NK cell effector responses were largely independent of individual nucleic acid sensing receptors. This may reflect a degree of redundancy in the mechanisms driving the enhanced responses. Alternatively, it may also reflect the capacity of IAV to manipulate nucleic acid sensing pathways as exemplified by the inhibition of RIG-I's CARD ubiquitination by the NS1 protein (Mibayashi *et al.*, 2007; Guo *et al.*, 2007; Opitz *et al.*, 2007).

Priming of target cells with IFN-α before coculture did not enhance NK cell responses. This implies that IAV infection induces intrinsic changes in infected target cells that IFN-α treatment alone fails to replicate. These changes likely involve alterations in surface ligands, such as MHC-I downregulation (Koutsakos *et al.*, 2019) or upregulation of activating ligands (Esteso *et al.*, 2017; Gunasekaran *et al.*, 2022). Given that target cells were washed to remove supernatants containing unbound viral particles from the assay before adding NK cells, the accumulation of secreted cytokines would be largely limited that which occurred during the period of coculture with the NK cells themselves. Nevertheless, it is still possible that some

potent cytokines such as IL-12 and IL-18, which may be secreted in high local concentrations such as through synaptic contacts between target cells and NK cells were still sufficient to enhance NK responses (Hyodo *et al.*, 1999; Nguyen *et al.*, 2002).

Surprisingly, nucleic acid receptor stimulation in THP1 cells led to significantly increased IFN-γ production but not degranulation responses. This selectivity, favouring elevated IFN-γ responses over degranulation, suggested that activation of nucleic acid receptors in target cells might preferentially target factors directing this NK cell response, such as IL-12 and IL-18 production and secretion. The secretion of these cytokines occurs through various pathways, including activation via TLRs (Hamza *et al.*, 2010) and inflammasomes (Zheng *et al.*, 2023), both of which are associated with the activation of NF-κB (Ma *et al.*, 2015; Liu *et al.*, 2017). NF-κB is also activated by nucleic acid receptors such as cGAS and RIG-I (Liu *et al.*, 2017). Consequently, stimulation of RIG-I and cGAS in target cells induces downstream pathways including NF-κB, leading to secretion of proinflammatory cytokine such as IL-12 and IL-18. This selective activation of IFN-γ response in NK cells is a result.

IAV infection of target cells induced notable increases in both NK cell degranulation and cytokine production. However, the increased degranulation response was not observed when RIG-I or cGAS were directly stimulated in target cells. Although both viral infection and nucleic acid ligands will lead to type I IFN secretion. (Besch *et al.*, 2009; Liu *et al.*, 2015), infection might be associated with wider immune responses that are not induced by selective activation of cGAS or RIG-I. Particularly, IAV infection stimulates RIG-I, TLR3, TLR7, and NLRP3 (Iwasaki and Pillai, 2014). These different pathways might contribute to a stronger and multifaceted immunological response by NK cells when compared with single receptor stimulation. Additionally, viral infection can induce the upregulation of ligands for activating NK cell receptors such as DNAM-1 ligands (Cifaldi *et al.*, 2019).

In experiments involving RIG-I and cGAS agonists, which are known to induce IFN-I secretion (Hornung *et al.*, 2006; Sun *et al.*, 2013; Wang *et al.*, 2013), NK cells did not show increased degranulation. Despite their ability to induce IFN-I secretion, the co-culturing process of NK cells with target cells for a short period might have masked the effect of IFN-I enhancing NK cell degranulation as shown in this thesis. This observation was consistent with the fact that

IFN-α stimulation of target cells also did not lead to enhanced degranulation by NK cells. The nuanced interplay between nucleic acid receptors, type I IFNs, and NK cell behaviour highlights the complexity of NK cell regulation and opens exciting avenues for future exploration.

6. GENERAL DISCUSSION

Recognition of foreign genetic material in the form of DNA and RNA has emerged as a key component of the host's strategic defence against both viral and bacterial infection (Schlee and Hartmann, 2016). The enhancement of cytotoxic lymphocyte effector functions through nucleic acid receptor ligands represents a growing field of study. TLRs have exhibited the ability to augment cytotoxic lymphocyte function through both extrinsic and intrinsic mechanisms. For instance, exposure of human dendritic cells (DCs) to TLR7/8 ligands was shown to elevate antigen presentation and induce the production of IL-12p70 , ultimately bolstering CD8 T cell activation (Sköld *et al.*, 2018). Intravenous administration of RIG-I ligands in mice synergistically complemented checkpoint inhibition, leading to the expansion and activation of antigen-specific CD8 T cells *ex vivo* and enhancing their antitumor response *in vivo* (Heidegger *et al.*, 2019). The pivotal role of RIG-I receptors has been underscored by investigations involving IAV infection and downstream immune responses, with RIG-I knockout mice displaying compromised granzyme release and cytokine production by CD8 T cells following IAV infection, further emphasizing the impact of RIG-I signalling (Kandasamy *et al.*, 2016). On the other hand, intrinsic stimulation of TLR3 in murine CD8 T cells has resulted in increased IFN-γ production, all without impinging on their cytolytic or proliferative capacity (Tabiasco *et al.*, 2006). Similarly, *in vitro* studies have also indicated that TLR7 ligands can directly amplify the activation and cytokine production of human CD8 T cells (Li *et al.*, 2019). Nucleic acid receptor ligands have also garnered attention for their capacity to augment effector functions of NK cells. For example, exposure to the STING agonist cGAMP increased the cytolytic activity of CAR-NK-92 cells and lead to reduced tumour burdens in a murine model of pancreatic cancer (Da *et al.*, 2022). Transfection of TLR7/8 ligands into PBMCs resulted in enhanced antibody-dependent NK cytotoxicity against A549 lung cancer cell line (Khanna *et al.*, 2021). Moreover, intrinsic RIG-I stimulation of purified human NK cells enhanced their cytotoxicity against melanoma cells via TRAIL upregulation, yet no observed effects on the level of degranulation and cytokine production (Daßler-Plenker *et al.*, 2019).

Despite the critical role of these molecular recognition mechanisms in initiating coordinated immune responses, the direct consequences of RIG-I activation within both NK and CD8 T cells remain poorly understood. In this thesis, the primary objective was to investigate the function of nucleic acid receptors in cytotoxic lymphocytes, specifically NK cells and CD8 T cells focussing primarily on RIG-I.

Activation of RIG-I. within NK cells and CD8 T cells resulted in augmented degranulation, as evidenced by increased degranulation and was accompanied by the elevated production of key effector cytokines, including type I IFNs, IFN-γ, and TNF. This also enhanced the function of uninfected NK cells most probably as a result of the paracrine effect of secreted type I IFN from infected cells. Additionally, somewhat surprisingly nucleic acid receptor stimulation of target cells enhanced NK cell cytokine secretion but not degranulation. This highlights the complexity in regulating the function of cytotoxic lymphocytes through nucleic acid receptor stimulation, intrinsically or extrinsically, and sheds light on the potential use of RIG-I and type I IFN to shape immune outcomes.

Understanding Viral Susceptibility

In the process of assessing the impact of RIG-I activation on functional CD8 T cell and NK cell responses, it was by definition essential to stimulate these cells either by crosslinking with antibodies or by co-culture with target cells. Somewhat inadvertently however it was evident that both forms of stimulation gave rise to increased proportions of infected cells when compared with resting cells. Indeed IAV as well as HTLV-I virus has previously been shown to preferentially infect activated human lymphocytes (Mock *et al.*, 1987; Lo *et al.*, 1992). This "increased permissiveness" may be due to different glycosylation profiles, where activated cells express higher levels of sialyl glycans that may allow increased IAV entry (De Bousser *et al.*, 2020). Alternatively the boosted metabolic pathways in activated cells might also promote viral infection and replication, leading to increased expression of viral proteins (Hollenbaugh *et al.*, 2011). On the other hand, resting cells might exhibit a more efficient endogenous anti-viral response, similar to resting CD4 T cells that are less susceptible to HIV infection than their activated counterparts (Stevenson *et al.*, 1990). Alternatively, naïve cells may undergo rapid cell death upon infection, resulting in reduced proportions of infected cells in our flow cytometry data. Further work will be required to better define the reasons for the increased proportions of NP+ cells that were observed upon activation of either NK cells or CD8 T cells.

RIG-I Activation and IFN Signalling in NK and CD8 T Cells

The data herein showed that whether prompted by IAV infection or the introduction of synthetic ligands like 3p-dsRNA, RIG-I receptor engagement can augment effector function of CD8 T cells and NK cells. Both experimental approaches lead to the activation of the NF-κB

and TBK1 pathways in each cell type. This subsequently triggered the secretion of type I interferons (IFN-I), establishing a paracrine activation loop via IFNAR. Signalling through IFNAR resulted in the phosphorylation of STAT2 proteins and was accompanied by the induction of transcription of an array of interferon-stimulated genes (ISGs), prominently IFIT1. The findings from this study align with existing literature and previous research on RIG-I stimulation in numerous cell types, including monocytes, macrophages, dendritic cells, and lung epithelial cells (Wang *et al.*, 2013; Fekete *et al.*, 2018; Yap *et al.*, 2020). While IFIT1 is thought to be a direct target gene for RIG-I receptor downstream signalling via IRF3 (Rusinova *et al.*, 2013), the data here shows that STAT2 deletion as well as antibody-blockade of IFNAR markedly impaired the induction of IFIT1 expression in both CD8 and NK cells. This suggests that expression of ISGs, represented by IFIT1, was primarily a response to secreted type I IFN. Consequently, this observation reveals a potential mechanism for inducing ISGs within both NK and CD8 T cells through the delivery of ligands for nucleic acid receptors.

The altered activity of cytotoxic cells extended beyond the increased transcription of ISGs with a range of effector mechanisms including degranulation, the production of IFN-γ and TNF along with the transcription of TRAIL, all of which were enhanced following stimulation via RIG-I. While previous evidence pointed to the induction of TRAIL expression alone as a response to RIG-I stimulation in NK cells (Daßler-Plenker *et al.*, 2019), the data here revealed a multifaceted enhancement of NK cell function. The specific mechanism behind this enhanced function of both NK and CD8 T cells and the extent to which it is absolutely dependent on type I IFN awaits further study.

The findings have provided insight into the mechanistic underpinnings of RIG-I-induced IFN-I responses in cytotoxic lymphocytes. The dependency of this response on STAT2, a downstream mediator of IFNAR signalling, underscores the coordinated interplay between nucleic acid receptor activation and the ensuing IFN cascade, particularly following IAV infection of NK cells. This aligns with existing literature (Yoneyama *et al.*, 2004), which shows that RIG-I stimulation induces IFN-I response and cellular activation in a range of other human immune cells such as monocytes and reinforces the notion that RIG-I can more broadly orchestrate immune activation.

IAV-Induced NK Activation: Unravelling Novel Responses in Direct Infection

Beyond their conventional role in targeting virus-infected cells, NK and CD8 T cells exhibited an intrinsic effector function enhancement in response to direct viral infection *in vitro*. Perhaps most significantly in this respect, small but measurable responses in NK cells were observed even by IAV infection even in the absence of co-culture with target cells. This effect was not observed following stimulation with purified RIG-I agonists, indicating that IAV infection might be inducing additional activation pathways beyond that stimulated by RIG-I ligands. In line with that, NK cell exposure to purified hemagglutinin and neuraminidase proteins has been to increase their cytolytic activity (Arora *et al.*, 1984). Moreover, the influenza hemagglutinin protein can directly interact with the activating receptor NKp46 and as such might directly contribute to NK cell activation (Mandelboim *et al.*, 2001; Arnon *et al.*, 2004). Therefore, in settings involving exposure to IAV, it is tempting to suggest that this NK cell activation observed following infection with IAV is the result of some crosstalk between RIG-I signalling and activating receptor engagement that may be crosslinked by IAV HA. Further analyses of these effects using a broader range of IAV strains or indeed purified recombinant HA in conjunction with stimulation with 3p-dsRNA may be informative.

NK Cell Responses to Intrinsic and Extrinsic Nucleic Acid Receptor Stimulation

NK cells primarily recognize abnormal cells, such as virally infected cells that downregulate MHC-I molecules or upregulate stress-related molecules, leading to their activation (Topham and Hewitt, 2009). Since foreign nucleic acids are among the most important signatures of viral infection (Schlee and Hartmann, 2016), synthetic ligands that can stimulate different nucleic acid receptors in target cells, mimicking viral infections, were employed to compare extrinsic and intrinsic role in enhancing NK cells. Stimulation of RIG-I and cGAS within target cells significantly increased cytokine production, although not degranulation, in NK cells. This effect differs from that observed following NK cell intrinsic activation of RIG-I or their exposure to type I IFN. Intrinsic RIG-I stimulation increased both cytokine production and degranulation within NK cells. Unlike extrinsic stimulation, which is dominated by secreted cytokines, including type I IFN (Rehwinkel and Gack, 2020), intrinsic RIG-I stimulation can activate several immune pathways within NK cells, such as NF-κB, as demonstrated in this thesis. This activation enhances their function (Mohamed *et al.*, 2023). Moreover, intrinsic stimulation of nucleic acid receptors is likely associated with increased spatial availability of

secreted cytokines, predominantly IFN-I and pro-inflammatory cytokines, acting as an immediate autocrine stimulant (Vacchelli *et al.*, 2015) to boost NK cell effector function including degranulation (Barnes et al. 2022). On the other hand, in experiments where NK cells were stimulated with RIG-I ligands, the cells were cultured overnight with the stimulant, resulting in prolonged exposure to the secreted type I IFN before target coculture. This extended exposure, compared to shorter exposure to target-driven IFNs during coculture with RIG-I-stimulated target cells, might have influenced the NK cell response to the added targets.

This suggests that the intrinsic role of RIG-I stimulation in NK cells is multifaceted and advantageous for driving NK cell effector function compared to the extrinsic approach. Consequently, there is potential to enhance cytolytic lymphocyte function through nucleic acid receptor stimulation. However, it is crucial to note that this enhancement might vary based on whether the stimulation occurs intrinsically or extrinsically. Therefore, it is worth investigating these differences in *in vivo*/*ex vivo* models to evaluate the efficiency of each approach.

Challenges and Complexities

Several limitations were encountered during the execution of this study, which warrant careful consideration in interpreting the findings. One notable challenge arose in the required increase of the viral MOI to achieve infection of cytotoxic lymphocytes. The MOI of 10 used for NK and CD8 T cell infection contrasted starkly with the MOI of 1 needed for cell lines like THP1, posing difficulties accurately quantifying the extent of *de novo* viral replication compared to the input virus that had remained attached to the cell surface of either NK or CD8 T cells. Moreover, robust quantification of the induction of type I interferons also proved challenging due to the presence of numerous subtypes, encompassing 13 alpha and 2 beta subtypes. Consequently, a broader approach which focussed on IFNAR activity via secreted type I IFN was used but nevertheless limited the capacity to specifically identify which subtypes of type I interferons were driving the enhanced lymphocyte function.

Additionally, the utilization of knockout cells in degranulation assays introduced complexities, as they required longer term in vitro culture to generate which in turn resulted in less robust functional responses (Data not shown). The interpretation of data from such cultures also is

impacted by the fact that the CRISPR gene editing approach while clearly effective and measurable in primary cells, did not result in all of the cells in a given culture being deficient in the edited gene. As such there was the potential of residual activity in bulk pool of knockout cells to limit the interpretation of the data.

7. APPLICATIONS AND FUTURE EXPERIMENTS

The activation of nucleic acid receptors, such as Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs), holds promise for enhancing the immune response and improving the effectiveness of cell-based therapies (Y. Jiang *et al.*, 2023). These receptors are integral to the innate immune system's ability to detect viral pathogens through their recognition of specific nucleic acid patterns. Stimulating these receptors can activate numerous cells of the immune system both directly and indirectly. Combining cell-based therapies such as adoptive NK or T cell therapy or chimeric antigen receptor (CAR) T cell therapy with nucleic acid receptor agonists may lead to a more robust immune response against cancer cells (Napoleon *et al.*, 2022). This approach aims to harness the immune system's ability to recognize and respond to foreign or abnormal nucleic acids, thus reinforcing both innate and adaptive immune responses mediated by engineered or adoptively transferred cytotoxic cells. Early preclinical studies to manipulate anti-cancer immune response using ligands for nucleic acid receptors have shown promising results in animal models, indicating that their stimulation can enhance the efficacy of cell-based therapies by promoting immune cell activation, cytokine production, and tumor cell killing (Medler *et al.*, 2019). Based on the work conducted in this thesis along with current clinical trials (Gosu *et al.*, 2012; Medler *et al.*, 2019; Mcwhirter and Jefferies, 2020) employing nucleic acid receptor ligands, stimulation of nucleic acid receptors, such as RIG-I receptors, in CD8 T cells or NK cells or alternatively the systemic injection of the ligands of these receptors has the potential to augment NK or CD8 T cell-based therapies, albeit some there is significant risk of systemic RIG-I activation driving adverse events.

Several novel findings have come to light, which hold significant promise for further investigation. For instance, while it is established that type I IFNs stimulate IFNAR, resulting in the induction of interferon-stimulated genes, it was found that NF-κB and TBK1 were activated following IFN-α stimulation. This prompts two primary questions: 1) Could there be endogenous nucleic acid sequences within NK cells or CD8 T cells that, when interferon-stimulated genes are upregulated, increase the expression of nucleic acid receptors, thus lowering the threshold for receptor stimulation and activating downstream pathways, including NF-κB and TBK1? 2) Do these cells employ different pathways than those documented in the literature? For instance, could there be shared kinases between IFNAR signalling and nucleic acid receptor signalling, or is there a unique self-amplification loop involving these two pathways? Addressing these questions may involve using genetic editing

approaches. Nucleic acid receptors such as RIG-I, MDA5, cGAS, and TLRs could be knocked out in different combinations to assess their roles in inducing NF-κB and TBK1 pathways in response to IFN-α stimulation. Additionally, deletion of genes encoding key kinases such as IKKs and TBK1 may help elucidate the connection between these pathways and the interferon response. Another intriguing finding is the upregulation of *IFNG* gene mRNA in NK cells as a response to RIG-I stimulation and IFN-α exposure, even in the absence of target cell-induced activation. However, there is a lack of corresponding protein expression during this time frame. This suggests the involvement of RNA regulatory processes that are exclusively triggered by target signals. One theory proposes that PKR, the interferon-inducible protein kinase activated by ds-RNAs, inhibits mRNA translation through eIF2α phosphorylation (Kaempfer, 2006). Therefore, utilizing a PKR knockout model to compare the levels of expressed IFN-γ in the absence and presence of target stimulation could be a valuable approach to unravel the mechanism behind this intriguing discovery.

8. CONCLUSION

This study explored how Influenza A Virus affects NK and CD8 T cells, revealing complex connections between viral infections and lymphocyte responses. The infection triggered a cascade of events, activating nucleic acid receptors, most notably RIG-I, in cytotoxic lymphocytes and in target cells. Intriguingly, this viral encounter was associated with a noticeable surge in the effector functions of both NK and CD8 T cells. Building on these observations, the contribution of RIG-I receptors to this enhanced activation was assessed more comprehensively. To this end, RIG-I ligands were employed to simulate the effects observed in IAV infection experiments. Remarkably, the stimulation of RIG-I in either cell type, as well as in the target cells, resulted in enhanced cytokine production, degranulation, and the upregulation of activation markers. Among these markers, CD69, CD107a, IFN- γ, TNF, and TRAIL also experienced a boost, adding a layer of complexity to the activation process. Furthermore, this coordinated response was accompanied by an upsurge in the secretion of type I interferons, not only potentially influencing the responding cells but also potentially shaping the broader immune milieu. These findings illustrate the interconnected dynamics between viral infections, activation of cytotoxic lymphocytes, and potential amplifying roles of nucleic acid receptors. Collectively, these insights not only deepen our understanding of immune responses to viral threats but also lay the groundwork for potential avenues to modulate immune reactions for therapeutic benefit utilizing nucleic acid receptors, particularly RIG-I.

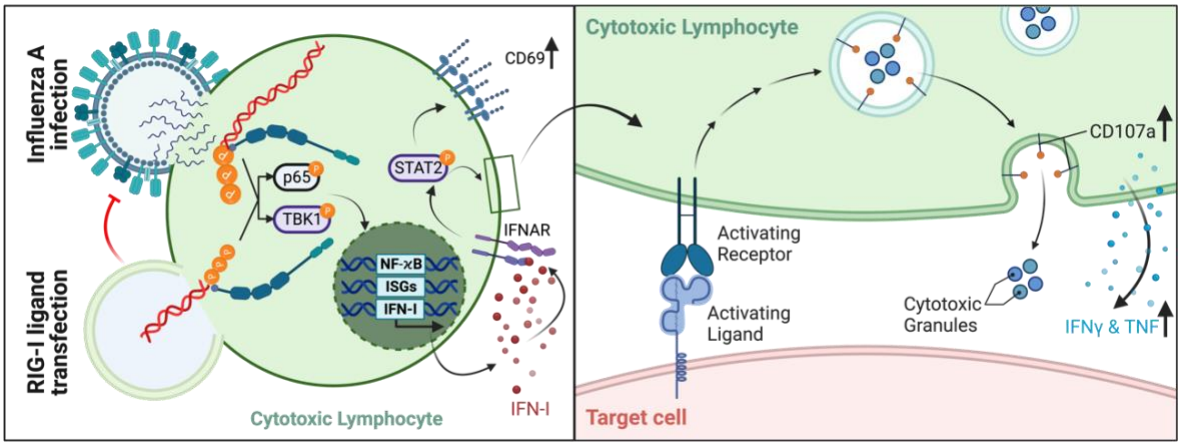

Figure 39. Activation of Cytotoxic Lymphocytes Through RIG-I Receptors: Enhancing Degranulation and Effector Cytokines

Left panel: The stimulation of RIG-I receptors in cytotoxic lymphocytes, either through RNA viruses like influenza A infection or by 3p-dsRNA synthetic ligands, initiates downstream signaling pathways involving TBK1 and NF-κB. Consequently, this leads to the secretion of type I IFN (IFN-I), which acts in an autocrine manner to trigger IFNAR signaling and induce the expression of interferon-stimulated genes (ISGs). These events culminate in the activation of CD8 T cells, as evidenced by the upregulation of the CD69 activation marker. Right Panel: Once activated and encountering target cells, cytotoxic cells exhibit enhanced degranulation, enabling direct killing of the target cells. The enhanced degranulation of cytotoxic lymphocytes can be detected by the presence of CD107a, a lysosomal protein associated with the degranulation process. Additionally, the activated CD8 T cells secrete IFN-γ and TNF cytokines, which possess the ability to modulate other immune responses.

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