Functional and mechanistic studies on engineered antibodies that engage TRIM21

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**NANYANG
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SINGAPORE

**Functional and mechanistic studies on Engineered Antibodies
that engage TRIM21**

Chin Wen Jie

SCHOOL OF BIOLOGICAL SCIENCES

**Joint Doctoral program of Singapore Immunology Network,
A*STAR and Nanyang Technological University**

2020

**Functional and mechanistic studies on Engineered Antibodies
that engage TRIM21**

Chin Wen Jie

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal/from papers accepted at conferences in which I am listed as an author.

Results for Chapter 3.2 and 3.3.1 were either from re-validation experiments done by me or published as: Ng, P.M.L., Kaliaperumal, N., Lee, C.Y., Chin, W.J., Tan, H.C., Au, V.B., Goh, A.X.-H., Tan, Q.W., Yeo, D.S.G., Connolly, J.E., Wang, C.-I., 2019. Enhancing Antigen Cross-Presentation in Human Monocyte-Derived Dendritic Cells by Recruiting the Intracellular Fc Receptor TRIM21. The Journal of Immunology, ji1800462.

The contributions of the co-authors are as follows:

- Dr Wang Cheng-I, Dr John E Connolly and Dr Patricia Ng provided the project direction and edited the manuscript drafts.
- I prepared sections of the manuscript drafts pertaining the Western Blot experiment. The manuscript was written by Dr Patricia Ng and revised by Dr Wang Cheng-I.
- I co-designed the co-immunoprecipitation experiment with Dr Patricia Ng and performed all the laboratory work at the Singapore Immunology Network. I also repeated most of the published experiments for validation and analyzed the data.
- Nivashini Kaliaperumal co-designed the staining panel and ran the flow cytometry for the experiments involving T cells.
- Tan Hwee Ching, Angeline Goh, Tan Qiao Wen and Darren Yeo assisted in the generation of Fc phage library and library panning.
- Veonice helped ran the Luminex assay and assisted in the analysis of its data.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	II
TABLE OF CONTENTS.....	IV
List of Figures	VIII
List of Supplementary Figures.....	X
List of Tables	XI
List of Supplementary Tables	XI
ABBREVIATION.....	XII
ABSTRACT.....	XV
1. INTRODUCTION	1
1.1. Dendritic cells	1
1.1.1. The link to adaptive immunity	1
1.1.2. MHC-I-restricted cross-presentation by DCs.....	1
1.1.3. DC maturation state determines a tolerogenic or inflammatory response	3
1.1.4. <i>Ex vivo</i> DC-based and <i>in vivo</i> DC-targeting therapeutics	5
1.1.5. DC-targeting vaccines: Which DC subset and receptor to target?.....	8
1.1.6. Adjuvant and its essential role of stimulating DC in vaccines.....	11
1.1.7. Co-delivery of antigen and adjuvant to DCs.....	13
1.2. TRIM21.....	17
1.2.1. Tripartite motif (TRIM) family proteins and their role as immune regulators	17
1.2.2. The use of ubiquitin in TRIM-mediated protein modification pathway	18
1.2.3. TRIM21/Ro52: the autoantigen in autoimmune disorders.....	19
1.2.4. Cytosolic TRIM21 interacts with immune complex to mediate antigen clearance and immune signaling pathways.....	22
1.2.5. Weaker binding between antibody Fc and TRIM21 compromises the strength of immune signaling but does not affect ADIN.....	22
1.3. Engineering antibodies to improve their affinity to TRIM21	24
1.3.1. Antibody phage display library	24
1.3.2. Generation of Fc phage display library	25
1.3.3. Isolation of Fc clone with high affinity for TRIM21 from Fc phage display library	28
1.4. The application of modified antibody on dendritic cells	31
1.4.1. Antibody-mediated TRIM21 effector response on DCs	31
1.4.2. Increase affinity for TRIM21 to improve antibody vaccines	32
1.4.3. Inherent limitation of modified antibody vaccines	32
1.4.4. Using adenovirus infection model to test the effect of modified antibodies..	33

1.4.5.	Application of the modified antibodies in enterovirus 71 (EV71) model.....	34
1.4.6.	Application of tumor antigen-coupled anti-DEC205 antibodies with modified Fc against cancer.....	35
1.5.	Cancer antigen to be coupled on anti-DEC205 antibodies	37
1.5.1.	Ovalbumin (OVA).....	37
1.5.2.	Glypican-3 (GPC3)	38
1.6.	Objective of this study	40
2.	MATERIALS AND METHODS.....	42
2.1.	Viruses, Cells, mice and related cell-processing methods.....	42
2.1.1.	Human and mouse cell lines.....	42
2.1.2.	Mice.....	42
2.1.3.	Viruses.....	43
2.1.4.	Peripheral blood mononuclear cell (PBMC) preparation.....	44
2.1.5.	Generation of immature monocyte-derived dendritic cells (moDCs).....	44
2.1.6.	Generation of mouse bone marrow-derived macrophages (BMDMs).....	44
2.2.	Preparation of antibodies and other materials.....	46
2.2.1.	Engineered antibodies	46
2.2.2.	Expression of mouse and human TRIM21 PRYSPRY domain	50
2.2.3.	Peptide-MHC production and tetramerization	50
2.2.4.	Conjugation of ovalbumin (OVA) to chimeric anti-mouse DEC205 antibody	51
2.3.	General methods	52
2.3.1.	Enzyme-linked immunosorbent assay (ELISA).....	52
2.3.2.	Western blot	53
2.3.3.	Quantitative PCR (qPCR)	53
2.3.4.	Surface Plasmon Resonance (SPR) Experiment	54
2.3.5.	Flow Cytometry.....	56
2.3.6.	TRIM21 small interfering RNA (siRNA) knockdown and luciferase plasmid transfection.....	57
2.3.7.	NF- κ B reporter assay	57
2.4.	<i>In vitro</i> cellular assays	59
2.4.1.	Antibody-dependent intracellular neutralization (ADIN) assay	59
2.4.2.	moDC maturation assay	59
2.4.3.	Cytokine/chemokine detection with Multiplex assay	60
2.4.4.	Immunofluorescence	60
2.4.5.	Internalization and Degradation Assay with activatable fluorescence-quencher probe.....	61
2.4.6.	moDC: autologous CD14- PBMC co-culture assay (Adenovirus)	61

2.4.7.	Peptide re-stimulation assays	62
2.4.8.	Co-immunoprecipitation (co-IP)	63
2.4.9.	Quantitative mass spectrometry and gene ontology analysis	63
2.4.10.	Illumina RNA sequencing and calculation of Molecular Distance to Medium (MDTM)	64
2.4.11.	moDC: autologous CD14- PBMC co-culture assay (Enterovirus 71)	65
2.5.	<i>In vivo</i> mouse experiments	66
2.5.1.	Ovalbumin antigen model	66
2.5.2.	GPC3 antigen model	66
2.5.3.	Isolation of tumor cells from mouse	67
3.	RESULTS	69
3.1.	Biophysical properties of antibodies used in experiment	69
3.1.1.	Modified anti-adenovirus antibodies possess improved affinity for human TRIM21, but abolished binding for mouse Trim21	69
3.1.2.	Affinity of modified antibodies on Human and Mouse Fc receptors (FcRs)	71
3.2.	Immunological effect of modified antibodies in an adenovirus model	74
3.2.1.	WT and Modified antibodies demonstrated similar adenovirus neutralization efficacy in Hela cells and moDCs	74
3.2.2.	Modified immune complex (IC) significantly induced moDC maturation	78
3.2.3.	Modified IC stimulates moDC to secrete T _H 1-associated cytokines and chemokines	83
3.2.4.	Modified IC-treated moDCs promoted autologous T cell activation and expansion	86
3.2.5.	Antigen-specific CD8 T cells were promoted by modified antibody	90
3.3.	Mechanistic study of the activity of modified Abs on DCs	95
3.3.1.	Co-immunoprecipitation of antibodies confirmed intracellular interaction between TRIM21 and antibodies	95
3.3.2.	Internalized modified antibody interacted with ribosomal proteins and ISG15 ligase TRIM25	97
3.3.3.	Modified antibody was strongly retained in moDCs after IC treatment	101
3.3.4.	TRIM21 is imperative in NF- κ B signaling by internalized antibodies and modified antibodies induced higher levels of NF- κ B signaling than WT antibodies	105
3.3.5.	Modified immune complex induced drastic transcriptional changes in human moDCs	107
3.3.6.	Modified immune complex induced upregulation of proinflammatory transcriptional response while promoting asynchronous metabolic pathway relative to PBS control	111
3.3.7.	Transcriptional profile of modified IC-treated moDCs is analogous to PAMP-stimulated moDCs	115

3.3.8.	CD8 T cell proliferation stimulated by modified IC-treated moDCs is dependent on proteasomal activity.....	117
3.3.9.	Modified anti-human DEC205 (hDEC205) antibodies do not induce moDC maturation	121
3.3.10.	Modified anti-mouse DEC205 antibodies do not induce TRIM21-KI BMDC maturation	125
3.4.	Vaccine test of antigen-conjugated modified antibody in <i>in vivo</i> mouse model	130
3.4.1.	Ovalbumin-targeting to DCs by modified anti-mDEC205 induces OT-I CD8 T cell expansion in the lymph nodes.....	130
3.4.2.	Modified anti-mDEC205-GPC3 immunization before tumor challenge delayed the onset of tumor growth.....	134
4.	DISCUSSION	139
4.1.	Biophysical effects of Fc engineering on modified antibody	139
4.1.1.	Modified antibody displayed a strong affinity for human TRIM21 but not mouse TRIM21	139
4.1.2.	Fc modification did not drastically affect the binding to classical human Fc receptors.....	140
4.2.	Immunological effects of modified antibody in adenovirus infection model..	142
4.2.1.	Increase in Fc affinity to TRIM21 improved immune signaling but not ADIN	142
4.2.2.	Activation of moDCs by modified IC enabled efficient cross presentation to CD8 T cells	143
4.3.	Mechanism of modified antibody in spurring DC cross presentation	145
4.3.1.	Modified antibody requires adenovirus to increase its rate of internalization, which is critical for efficient DC maturation	145
4.3.2.	Possible involvement of TRIM25 in immune response by modified antibody	147
4.3.3.	Signaling pathways stimulated by modified antibody	148
4.4.	The application of modified DEC205-targeting antibody as cancer vaccine ..	152
4.4.1.	Inability of modified anti-DEC205 to stimulate DC maturation <i>in vitro</i>	152
4.4.2.	Prophylactic use of antigen-conjugated modified anti-DEC205 in mouse model	153
5.	CONCLUSION.....	155
	References.....	156
	Supplementary Figures	176
	Author's publications.....	189
	Posters and Conference talks	189

List of Figures

Figure 1.1. The three signals necessary for an antigen-specific T cell activation	3
Figure 1.2. DC-based cancer vaccination strategies	6
Figure 1.3. A modified Fc that exhibits increased affinity for TRIM21.....	28
Figure 2.1. Structure of all engineered antibodies used in the study.	48
Figure 2.2. Timeline for anti-mDEC205 vaccine conjugated to mGPC3 antigen.	67
Figure 3.1. Surface Plasmon Resonance measurements of the binding between recombinant PRYSPRY and anti-adenovirus WT/Modified antibodies.	70
Figure 3.2. Fc modification does not disrupt antibodies binding to human Fc receptors.....	73
Figure 3.3. Improving Fc affinity to TRIM21 did not affect adenovirus neutralization in Hela cells and moDCs.	77
Figure 3.4. Improving Fc affinity to TRIM21 stimulated the expression of moDC maturation markers.....	81
Figure 3.5. Improving Fc affinity for TRIM21 stimulates moDC to secrete Th1-associated cytokines and chemokines.	84
Figure 3.6. Immune complex with modified antibody promoted CD8 T cell expansion.	89
Figure 3.7. Immune complex with modified antibody promoted adenovirus-specific CD8 T cell expansion.....	91
Figure 3.8. Immune complex with modified antibody did not promote adenovirus-specific CD4 T cell expansion.....	93
Figure 3.9. Co-immunoprecipitation of antibodies in moDCs 4 hours after immune complex treatment	96
Figure 3.10. Proteomics and gene ontology analysis of co-immunoprecipitates with internalized antibodies.	100
Figure 3.11. Modified antibodies improved immune complex internalization rate into moDCs.	103
Figure 3.12. TRIM21 is essential for NF- κ B upregulation by modified antibody in HEK293T cells.	106
Figure 3.13. Modified immune complex induced drastic transcriptional changes in human moDCs.	110
Figure 3.14. Modified immune complex induced an inflammatory transcriptional response but staggered the oscillation of metabolism-related gene expression relative to control.	114

Figure 3.15. Modified IC stimulated moDCs to adopt a transcriptional profile resembling that of immunogenic maturation.	116
Figure 3.16. Proteasome inhibitor MG132 suppress CD8 T cell expansion by immune complex with modified antibody	119
Figure 3.17. Anti-human DEC205 antibodies did not stimulate moDC maturation.	123
Figure 3.18. Anti-mouse DEC205 antibodies did not stimulate mouse BMDC maturation.	127
Figure 3.19. Intracellular staining of antibodies in BMDCs.....	129
Figure 3.20. Modified anti-mDEC205-Ova expanded OT-I CD8 T cells in lymph nodes, but not in blood and spleen.	132
Figure 3.21. Prophylactic treatment of modified vaccine moderately delayed the onset of tumor growth.....	136
Figure 3.22. Tetramer for GPC3-specific CTLs indiscriminately stains both CD8 and non-CD8 T cells.	138
Figure 4.1. Modified antibody mediates immune response through both TRIM21 and TRIM25.....	148
Figure 4.2. Changes in dendritic cell metabolism through development, quiescence and activation.....	151

List of Supplementary Figures

Supplementary Figure 1. Enterovirus 71/ Coxsackie Virus A16 Binding ELISA of chimeric 5H7 and 1C6 antibodies.....	176
Supplementary Figure 2. Surface Plasmon Resonance measurements of the binding between recombinant PRYSPRY and anti-EV71 WT/Modified antibodies.....	177
Supplementary Figure 3. Effect of modified antibodies + virus in Enterovirus 71 model....	178
Supplementary Figure 4. Treatment with modified IC stimulates moDC to upregulate NF- κ B and AP-1 signaling downstream transcripts (Adenovirus model).	180
Supplementary Figure 5. moDCs from Donor 118, 119, 120, 123 and 124 undergo maturation upon modified IC treatment (Samples sent for RNA-sequencing).....	181
Supplementary Figure 6. Expression levels of circadian genes in RNA-seq data.....	182
Supplementary Figure 7. Gating strategy for proliferated OT-I CD8 T cells.....	183
Supplementary Figure 8. Gating strategy for tetramer+ CD8 T cells.....	184
Supplementary Figure 9. Comparison of human WT and modified Fc interaction with Human/Mouse TRIM21.....	185
Supplementary Figure 10. Internalization of pre-labeled anti-human DEC205 antibody into human moDCs.	186

List of Tables

Table 1.1. DC subsets and their properties are grouped by the most prominently associated induced T-cell response	9
Table 1.2. List of amino acids on human Fc that contacts TRIM21	27

List of Supplementary Tables

Supplementary Table 1. List of ‘Core Up’ and ‘Core Down’ genes during dendritic cell maturation according to Dalod et al.	188
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ABBREVIATION

A

Ad5	Adenovirus type-5
ADCC	Antibody-dependent cell cytotoxicity
ADIN	Antibody-dependent intracellular neutralization
APCs	Antigen-presenting cells

B

BMDMs	Bone marrow-derived macrophages
-------	---------------------------------

C

CAR	Coxsackie and Adenovirus Receptor
CC	Coiled-coil
CCC	Covalently closed circular
cDCs	Conventional DCs
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CHO	Chinese hamster ovary
CLR	C-type lectin receptor
co-IP	Co-immunoprecipitation
CpG ODNs	CpG oligonucleotides
CTLs	Cytotoxic T lymphocytes
CVA16	Coxsackievirus A16

D

DAMP	Damage-associated molecular pattern
DCs	Dendritic cells
DEGs	Differentially expressed genes
dLNs	Draining lymph nodes
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl Sulfoxide
dsDNA	Double-stranded DNA

E

EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
EV71	Enterovirus 71

F

Fc	Fragment crystallizable
Fab	Fragment antigen binding

G

GPC3	Glypican-3
GMP	Good Manufacturing Practices
GM-CSF	Granulocyte-macrophage colony stimulating factor
GO	Gene ontology

H

HA1	Hemagglutinin 1
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HCV	Hepatitis C virus
HLA-DR	Human Leukocyte Antigen – DR isotype

HRP	Horseradish peroxidase
HPV	Human papillomavirus
I	
IC	Immune complex
IgG	Immunoglobulin G
IFN	Interferon
IFN- γ	Interferon-gamma
IL-4	Interleukin-4
IPTG	Isopropyl β -d-1-thiogalactopyranoside
IRF	Interferon regulatory factor
K	
K _D	Dissociation constant
KO	Knockout
L	
LPS	Lipopolysaccharide
M	
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signaling protein
MDTM	Molecular distance to medium
MEF	Mouse embryonic fibroblasts
MHC-I	Major histocompatibility complex class I molecule
MHC-II	Major histocompatibility complex class II molecule
moDCs	Monocyte-derived DCs
MOI	Multiplicity of infection
MPLA	Monophosphoryl lipid A
N	
NEM	N-ethylmaleimide
NGS	Next-Generation-Sequencing
NK cells	Natural killer cells
O	
OVA	Ovalbumin
P	
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDCs	Plasmacytoid DCs
Pen/Strep	Penicillin Streptomycin
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PRRs	Pattern recognition receptors
pVI	Membrane lytic protein VI
Q	
qPCR	Quantitative PCR
R	
RD cell	Rhabdomyosarcoma cell
RIG-I	Retinoic acid-inducible gene I
RING	Really Interesting New Gene
RLU	Relative luminometer units
RSV	Respiratory syncytial virus

RU	Response units
S	
SA-PE	Streptavidin R-Phycoerythrin
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
SMCC	Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate
SPF	Specific Pathogen Free
SPR	Surface Plasmon Resonance
SS	Sjogren's syndrome
ssDNA	Single-stranded DNA
T	
TBST	Tris buffered saline Tween-20
TCEP	Tris (2-carboxyethyl) phosphine
TCR	T cell receptors
Th cells	T helper cells
TMB	Tetramethyl benzidine dihydrochloride
TNF- α	Tumor necrosis factor-alpha
TPM	Transcripts per million
TLR4	Toll-like receptor-4
Tregs	Regulatory T cell
TRIM21	Tripartite motif protein 21
V	
V _H	Variable heavy
V _L	Variable light
W	
WT	Wild type

ABSTRACT

Depending on their state of maturation, dendritic cells (DCs) might initiate immune suppression or immune activation in DC-targeted therapies. In cases where immune activation is desired, adjuvants are often co-administered to augment the immune response. But adjuvants that rely on the use of pathogen-associated molecular patterns (PAMPS) pose the potential threat of toxicity. Thus, we explored antibody receptors in the body and found that we can utilize the Tripartite Motif-containing 21 (TRIM21) pathway. TRIM21 is a cytosolic receptor of the Fragment *Cystallizable* (Fc) region of antibodies. Antibodies bound to cell-invading pathogens can in some cases enter the cytosol as an immune complex (IC) and recruit TRIM21. Following the binding to IC, TRIM21 mediates intracellular neutralization of the pathogen and immune signaling pathways. We postulated that improving the affinity of antibody Fc to TRIM21 should enhance TRIM21-mediated activity and possibly improve the activation status of DCs. In this study, we have designed and generated modified antibodies carrying an Fc region that was engineered for enhanced binding to human TRIM21. As an investigative model, adenovirus was used as an infection model that carries anti-adenovirus antibody into the cytosolic compartment of human monocyte-derived DC (moDC). Adenoviruses complexed with modified antibodies promoted heightened moDC maturation, which was independent of FcγR binding. The matured moDCs led to an increase in IFN-γ production and adenovirus-specific T cell proliferation in a co-culture containing T cells. Co-immunoprecipitation of antibodies revealed intracellular interaction between antibody and TRIM21, but only antibody bearing the engineered Fc was massively retained within the cell. This likely augmented moDC activation. Transcriptomic data suggests that modified antibody boosted immune-related signaling in human moDCs, which was supported by the upregulation of TRIM21-dependent NF-κB signaling response observed in HEK293T cells. Further work is required to elucidate the involvement of TRIM21 in antibody retainment. Preliminary results on hTRIM21-

transgenic mouse model indicated that antigen-conjugated anti-DEC205 with engineered Fc might lead to the expansion of antigen-specific CD8 T cells and modulate anti-tumor immune response. Our study presents a novel alternative to conventional adjuvants, and can be translated for application with DC-targeted therapies.

1. INTRODUCTION

1.1. Dendritic cells

1.1.1. The link to adaptive immunity

Dendritic cells (DCs) belong to a specialized group of mononuclear phagocytes known as antigen-presenting cells (APCs) that bridge the innate immunity to adaptive immunity. Unlike other leukocytes such as natural killer cells (NK cells) and neutrophils, DC's main role is not to directly kill the pathogen, instead they make use of a roundabout approach to prime and stimulate long-lasting immunity. Steinman's lab first discovered DC [1-5] as a cell that "constantly extend and retract many small cytoplasmic dendrites". Heterogeneous populations of DCs reside in peripheral non-lymphoid tissues (e.g. Langerhans cells in the skin), where they make use of their dendritic morphology and expression of various cell surface receptors to help interrogate the environment and capture both self- and non-self-antigens [6]. As professional APCs (which also include macrophages and B cells), DCs have the ability to ingest foreign antigens, process them via the endocytic pathway into peptides, and load the digested peptides onto major histocompatibility complex class II molecules (MHC-II). The MHC-II complex is then trafficked to the cell surface for presentation to CD4+ T helper cells (Th cells) [7], which release cytokines to facilitate and further boost immune responses against the antigen. What separates DCs from other APCs though, lies in their additional capacity to initiate cellular immunity against invading pathogens. This initiation of cellular immunity in naive CD8 T cell is mediated by the MHC-I complex of dendritic cells.

1.1.2. MHC-I-restricted cross-presentation by DCs

In the past, it was generally accepted that antigen presentation via major histocompatibility complex class I molecules (MHC-I) to CD8+ cytotoxic T lymphocytes (CTLs) is exclusively limited to endogenous antigens, whereas exogenous antigens can only be presented via MHC-

II molecules to CD4⁺ Th cells. This paradigm stems from the assumption that exogenous proteins are bound by the endosomal membrane and cannot trespass into the cytosol for processing by proteasomes, which is a pre-requisite for its subsequent loading onto MHC-I. However, this theory could not explain how CTL responses can be activated against pathogens which do not readily infect APCs. It was only until the discovery of ‘cross presentation’ that finally resolved the contradiction [8, 9]. In general, there are two main pathways of antigen cross-presentation that had been described: ‘vacuolar’ and ‘cytosolic’. For ‘vacuolar pathway’, exogenous antigens remain within their endosomal compartment after internalization, undergo lysosomal degradation into peptides and are loaded onto MHC-I molecules in the Golgi apparatus [10]. This mechanism goes against conventional wisdom, bypassing the need for proteasomal degradation to generate peptides that can be loaded on MHC-I. On the other hand, the ‘cytosolic pathway’ abides to the ordinance of MHC-I peptides being processed by proteasomes, but entails a membrane transport pathway unique to DCs. Out of all the APCs, DC demonstrates the most efficient channeling of exogenous antigens from endocytic compartments to cytosol [11]. This exposes exogenous antigens to proteasomal degradation, so their peptides can be loaded onto MHC-I and eventually presented as MHC-peptide complexes on the cell surface to stimulate antigen-specific CTLs [12-14]. As a result, when compared to other APCs such as macrophage, DC displays an exceptional ability to cross-present antigens and prime CTL responses.

The ability to cross-present allows DCs to actively present tumor and viral antigens without being infected themselves. Cognate T cell receptors (TCR) are then able to recognize the antigenic peptides presented by MHC-I molecules, giving rise to TCR-MHC ligation (also known as ‘signal 1’) that is essential in triggering antigen-specific cellular immunity (Figure 1.1) [15, 16]. Priming of T cell responses by antigen-loaded DCs had been demonstrated both

in vitro [17, 18] and *in vivo* [19, 20]. However, the influence of DCs on subsequent T cell response can work in both ways, as the lack of additional signals toward T cells can steer the response southward, into a tolerogenic, or immune-dampening response [21, 22]. This largely depends on the maturation status of the DCs involved.

Figure 1.1. The three signals necessary for an antigen-specific T cell activation [23]. Signal 1 comprises recognition between MHC-peptide complex and the cognate TCR. Signal 2 involves the binding of costimulatory molecules (e.g. CD80/CD86) to their receptors (e.g. CD28) to stabilize the immunological synapse and generate activation signals. Signal 3 is produced by the cytokine secretion from APCs, which is received by cytokine receptors on T cells.

1.1.3. DC maturation state determines a tolerogenic or inflammatory response

One important feature of DC biology is its maturation state, which plays a key role in regulating the balance between tolerance and immunity [24]. The process of immunogenic DC maturation is characterized by the acquisition of three abilities: antigen presentation, T cell co-stimulation and migration to lymphoid tissues [25].

Under steady state conditions, DCs are typically in an immature/resting state, distinguished by suboptimal phenotypic display of MHC and T cell co-stimulatory molecules, which includes CD80, CD86 and CD40. Immature DC constantly surveys its environment for external perturbations in order to respond immediately to any situation. The ability of DCs to sense the change in its surrounding is realized through the expression of a variety of receptors (e.g. pattern recognition receptors (PRRs), cytokine receptors) capable of detecting a plethora of biological cues (e.g. pathogen-associated molecular patterns (PAMPs), cytokines) [26]. Notably, the engagement of PRRs to its corresponding PAMP indicates the presence of an infecting pathogen in the vicinity, which sets off a cascade of signaling responses that leads to the immunogenic maturation of DCs. Prominent examples include cell surface toll-like receptor-4 (TLR4) which detects lipopolysaccharides (LPS) found in the outer membrane of gram-negative bacteria [27], and cytosolic retinoic acid-inducible gene I (RIG-I) which identifies viral double-stranded RNA (dsRNA) in the cytosol during a viral infection [28]. DCs which mature from such immunogenic response downregulates its capacity to internalize antigens, upregulates cell surface expression of MHC and co-stimulatory molecules, migrates to draining lymph nodes (dLNs) via CCR7 expression and releases pro-inflammatory cytokines to activate other immune cells [29-32]. Depending on the stimulus, the secreted cytokines and chemokines recruit specific immune cell types and skew T cell differentiation towards the subtype geared towards the stimulus in question [33-36]. To sum it up, with the phenotypic changes, immunogenically matured DCs has a higher capacity to provide ‘signal 2’ (co-stimulatory molecules) and ‘signal 3’ (cytokines) to incite the proliferation and activation of antigen-specific effector T cells (Figure 1.1) [25].

On the other end of the spectrum, DCs can also undergo maturation in the absence of immunogenic stimulus. In this case, it is referred to as a ‘homeostatic maturation’ [37]. Similar to immunogenic maturation, homeostatic maturation upregulates cell surface expression of MHC molecules and promotes DC migration to dLN via CCR7 [38], which encourages these DCs to engage with lymphoid naïve T cells. However, homeostatic maturation does not confer DCs the upregulation of co-stimulatory molecules and cytokine secretion. As a result, these DCs cannot provide ‘signal 2’ and ‘signal 3’ to T cells, promoting immunological tolerance which limit the development of naïve T cells into effector T cells, pushing them down the development route into regulatory T cell (Tregs) [39-42]. Antigens loaded on tolerogenic DCs are generally regarded as self-peptides, and this strategy has the tremendous benefit of weeding out self-reactive T cells that escaped central tolerance and preventing unwarranted autoimmune responses.

The pivotal role of DCs in promoting cellular immunity, both immunogenic and tolerogenic, has garnered much attention in the field of immunotherapy and led to the development of a number of DC-based therapeutics.

1.1.4. *Ex vivo* DC-based and *in vivo* DC-targeting therapeutics

In the fight against both cancer and infectious diseases, the goal is to stimulate robust and enduring T cell responses in the patient. In that sense, DC serves as an interesting and promising therapeutic toolbox which regulates cellular immunity. There are two distinct ways DCs can be used to formulate therapies: 1) *ex vivo* generation of antigen-loaded DCs to be injected into patient’s body or 2) *in vivo* activation and loading of antigens on DCs in patient’s body (Figure 1.2;[43]). For the *ex vivo* DC-based method, it involves extracting the patient’s own cells, loading the antigens *ex vivo* and re-injecting them back to the patients. Other than Sipuleucel-

T which was approved by the US FDA (Food and Drug Administration), there are twenty other DC-based vaccines in clinical trials as of 2019 [44]. *Ex vivo* DC-based vaccine therapy have the luxury of working directly on the DCs to be injected, which ensures quality control of the cells to be delivered to the patient. However, the extraction and maintenance of DCs in GMP (Good Manufacturing Practices)-grade facilities have the downsides of high costs and long processing time in contrast to *in vivo* targeting of DCs [45, 46]. The laborious cell-culturing procedures pose a major hurdle that prevents universal implementation of this therapy.

Figure 1.2. DC-based cancer vaccination strategies [43]. DC-based cancer vaccines stimulate anti-cancer immune responses stimulating DCs to activate tumor-specific T cells. This is done by either infusing patients with *ex vivo* antigen loaded DCs (left) or targeting antigens directly to DCs *in vivo* (right).

As an alternative, antibody-guided vaccines can be directly injected into patients to target DCs *in situ*. This simplifies the entire treatment and makes the medical process easier for both

patients and doctors. Moreover, facilitating the direct homing of antigens to DCs in spleen and lymph nodes overcomes the need for cell migration. In this method, the antigens from tumors or pathogens are bound to the antibodies targeting DC-restricted receptors. Such vaccines come in the form of antibody-antigen conjugate or functionalized particle (Figure 1.2).

Many preclinical studies have established that administration of DC-targeting vaccines can help elicit potent antigen-specific T cell responses in various cancer and infectious diseases [47-51]. Yet, with all the successes in the laboratory, most *in vivo* DC-targeting vaccination clinical trials have failed to show substantial improvement in disease remission and patient survival rates. One major hurdle faced by cancer vaccines, as with most immunotherapy treatments, is the immunosuppressive nature of the tumor microenvironment. This is often exacerbated by the absence of PAMPs to stimulate immune responses in cancer. Immunosuppression can stem from the presence of immune-dampening cell populations like Tregs [52-54] and the immune escape mechanisms of the disease agents [55, 56]. In addition, progress is further hampered by the challenging task of identifying targetable tumor antigens. For DC-targeting vaccines against infectious pathogens, the targetable antigens are usually defined, and innate immune responses are expected to be initiated against PAMPs. However, many important infectious diseases without an existing protective vaccines, such as AIDS and tuberculosis, are caused by intracellular pathogens [57], which requires CD8 T cell activation to control its progression. To overcome the problems of vaccines against both cancer and infectious pathogens, there is a need to enhance T cell-mediated immunity. To do so, we will delve into the current approaches of DC-targeting vaccine and find out if there are any avenues for us to improve on.

1.1.5. DC-targeting vaccines: Which DC subset and receptor to target?

There is a myriad of DC receptor options for the antigens to be delivered. Depending on the receptor's expression on different DC subsets, the choice will also determine the DC subset to be targeted. Figdor's group has compiled a comprehensive list of endocytic receptors, along with the corresponding immune response and DC subsets they stimulate (Table 1.1; [58]). Endocytic receptors have differential potential to stimulate T-cell immunity and despite the vast amount of research so far, there is still much debate over which receptor is superior amongst them. To best navigate the array of DC target receptors, we first require an overview of the human and mouse DC subsets and the respective role they play in immunity.

Immune response	DC subset	Uptake receptor	TLR	Cross-presentation	Comments
Th1	BDCA3 ⁺ H CD8α ⁺ M	Clec9A, Langerin ^M , DEC-205, Clec12A, DCAR1 ^M	1, 2, 3, 4 ^M , 6, 8, 9 ^M	++	Treg ^[59]
	Langerhans	Langerin, DEC-205, Dectin1, Dectin2, DCIR ^H	1 ^H , 2, 3, 4 ^M , 6 ^{H,M?} , 9 ^M	+	CTL induction or licensing ^[60] Th2, Th17, Th22
	Dermal CD1a ⁺ H Dermal CD103 ⁺ M	Langerin, DEC-205, MGL ^H , DCIR ^H	3 ^M , 4 ^H	++	Th17 ^[61]
Th2	BDCA1 ⁺ H CD8α ⁻ M	DCIR2 ^M , Clec12A, DCIR, Dectin-1 ^H , DEC-205 ^H , mMGL1 ^M , 2 ^M ,	1, 2, 3 ^H , 4, 5, 6, 7, 8, 9 ^M	+	
Th1/Th2	moDC ^H	DC-SIGN, DEC-205, MR, DCIR	1, 2, 3, 4, 5, 6, 8, 10	++	Th17
IFN-I	pDC	Siglec-H, BST-2, BDCA-2 ^H , Clec9A ^M , Clec12A, Dectin-2, DEC-205 ^H , DCIR ^H , Dectin-1 ^M , mMGL1 ^M	1 ^{H,M?} , 6 ^{H,M?} , 7, 9	+	Activation of myeloid DC and NK, B and T cells

Table 1.1. DC subsets and their properties are grouped by the most prominently associated induced T-cell response [58]. Expression of uptake receptors and TLRs as well as cross-presentation potential is of further importance in the choice of suitable targets. M, mouse; H, human.

There are three major subsets of DCs, namely the conventional DCs (cDCs), which comprises of cDC1 (mouse CD8+XCR1+ or human BDCA3+XCR1+) and cDC2 (mouse CD8-CD11b+ or human BDCA1+CD11b+), the plasmacytoid DCs (pDCs; mouse CD11c-B220+Ly6C+ or human CD11c-CD123+BDCA4+) and inflammatory DCs generally derived from circulating CD14+ classical monocytes (human monocyte-derived DCs; moDCs; CD14-CD11c+) [62, 63]. Importantly, equivalents between mouse and human DC subsets were identified, for which they share similarities in terms of phenotypic profile, transcriptional program and function.

To put things into perspective, cDC1s are recognized to be strong inducers of Th1 and CTL responses. This is because of their remarkable ability to take up antigen [64-67], release chemokines such as CXCL9/10 for the recruitment of CD8 T cells [68], and also secrete IL-12 for the activation of effector CTLs [69, 70]. cDC2 preferentially initiates TH2 and CD4 T cell responses through the release of IL-10 and IL-33 [71]. pDCs are known as a poor antigen-presenting DC subset, but an exemplary producer of type I IFN (IFN) in response to TLR-7 and TLR-9 stimulation during viral infection [72, 73].

Lastly, human moDC is the DC subset used in most *in vivo* DC-based vaccine clinical trials. Before going into the advantages of using moDCs, it is important to take note that there are researchers who refute the validity of moDCs as natural DCs, since they are ‘artificially induced’ by high concentrations of GM-CSF and IL-4 cytokines not commonly observed in

normal physiology. In addition, cDCs can be ontogenically traced back to a common CD34+ progenitor cells (unlike moDCs which are derived from monocytes) and are believed to be the bona fide DC subtype orchestrating immune responses *in vivo* [74-76]. In spite of the controversy surrounding moDCs' legitimacy as a DC, it still manages to dominate adoptive transfer therapies as the go-to cell type, due in part to the ease in large-scale *in vitro* culturing under clinical-grade conditions [77, 78]. On top of that, moDCs are responsive to adjuvant stimulation and readily presents antigens on MHC-I and MHC-II molecules. These qualities make moDC not only an excellent experimental cell type for vaccine development, but also a promising tool for clinical DC-based vaccination. Although reports suggesting the use of natural DCs (cDC1, cDC2 and even pDCs) for DC-based vaccines are gaining traction [79-82], we have decided to use moDCs as the proxy DC subset for most of our experiments in human model due to the reasons stated above.

As for the choice of DC receptor, we will focus mainly on DEC-205/CD205, which belongs to the C-type lectin receptor (CLR) group that facilitate uptake of carbohydrate ligands [83]. DEC-205 is a 205 kDa endocytic receptor highly expressed on DCs, which has been reported to bind to ligands such as necrotic cell debris, keratin and CpG oligonucleotides [84-86]. Vaccines coupling viral or cancer antigens to monoclonal antibody against DEC-205 hold a strong track record in potentiating an efficient cellular immunity in preclinical trials with the combination of TLR ligands as adjuvants [87-97]. Clinical trials involving the DEC-205-targeting vaccine developed by Celldex Therapeutics, CDX-1401 (Anti-DEC205 monoclonal antibody fused to full-length cancer antigen NY-ESO-1) had consistently showed an acceptable safety profile and increase in antigen-specific cellular and humoral responses [98]. However, it struggled to demonstrate significant improvement in response rate and patient survival despite the assortment of adjuvants (e.g. poly-ICLC, resiquimod) and immune checkpoint

inhibitors (e.g. pembrolizumab, ipilimumab, nivolumab) it has been tested in combination with (ClinicalTrials.gov Identifier: NCT02413827, NCT01834248, NCT02661100, NCT02129075, NCT00948961).

We suspect the reason for the lack of patient response lies in the less-than-ideal administration of adjuvants. In view of this, we would like to reinspect the current adjuvants for DC-targeting vaccines and identify the fault within the system.

1.1.6. Adjuvant and its essential role of stimulating DC in vaccines

The main component of vaccine is the antigen derived from the disease agent, such as viruses, bacteria or tumor cells. In the case of live attenuated virus or bacterium, the weakened pathogen functions as both the antigen and adjuvant due to the endogenous PAMPs (e.g. viral nucleic acids or bacterial LPS) present within the microbe. However, the use of live attenuated viruses has drawn backlash with the inherent risk of reversion to wild type virulence. With ever-increasing scrutiny on vaccine safety, subunit vaccines, which are purified recombinant proteins that are clinically safe but non-immunogenic, have become the focal point of vaccine development [99]. When administered alone without an immunostimulant, subunit vaccines are typically inadequate initiators of a protective immunity due to their low immunogenicity [100]. To overcome this issue, adjuvants are co-administered to enhance the quality, magnitude and duration of the immune response [101, 102]. Adjuvants can serve at least one of the two following purposes: the first is to prolong antigen accessibility, allowing sufficient antigen exposure for APCs to garner an efficient T cell-priming activity; the second is to stimulate immunogenic maturation of DCs [103]. Both functions encourage DCs to optimally stimulate naïve antigen-specific T cells into effector T cells. For instance, oil-in-water emulsion formulations such as incomplete Freund's adjuvant or microcapsules are used for fulfilling the

first purpose by forming antigen depots that delay clearance and stretch out the bioavailability of the vaccine. The second purpose of adjuvants: stimulation of DC maturation, is fulfilled through the use of PAMPs or DAMPs-inducing adjuvants (e.g. alum) [104-106] which serve as agonists of PRR (e.g. TLR). To date, only few adjuvants were approved by FDA for human vaccines. These include the commonly used aluminum salts, which is known to instigate Th2 responses through the induction of uric acid and inflammasomes [107-109], emulsion preparations such as MF59 and AS03 to generate antigen depots, and MPLA, which is an altered form of lipid A from *Salmonella minnesota* R595 LPS with much improved safety profile [110].

For DC-targeting vaccines, adjuvants promoting Th1-polarization and cellular immunity is paramount to the success of treatment against cancer and viral diseases [111-113]. Of note, the emphasis should be placed more on provoking DC maturation and not on forming antigen depot. This is because DCs have the intrinsic ability to store antigens in lysosome-like organelles for extended presentation, and depot formation might on the contrary disrupt the delivery of the DC-targeted cargo [114]. With that in mind, TLR ligands like poly ICLC and CpG oligonucleotides (CpG ODNs) were preferred, which serve only to promote DC maturation in clinical trials involving DC-targeting vaccines.

Nonetheless, current vaccine formulations failed to bring about clinical success [115-117] and sustained adaptive immune response [118, 119]. We believe this is largely due to the difficulty in striking a balance between achieving drug efficacy and lowering drug toxicity. Conventional adjuvants are non-targeting in nature and are injected as separate entities from the antigens, so they do not home towards their intended recipient (APCs such as DC) within the body. Instead, delivery of adjuvants from the injection site to DCs relies on passive biodistribution. If adjuvants were to approach the potency of live infections to stimulate an effective immune

response while distributing itself systemically, it will introduce major safety concerns like the risk of a cytokine storm (a phenomenon describing the uncontrollable release of proinflammatory cytokines). It was demonstrated that targeting adjuvants to DCs can reduce the required effective adjuvant dosage by 100-fold as compared to a non-targeting adjuvant, lowering the risks of serum cytokine storm and related toxicity [120].

Although it remains to be an understudied area in vaccine therapeutics, the timing which DC receives the antigen and inflammatory cue (adjuvant) is crucial in the successful induction of immune response. DCs that are inadvertently activated by the adjuvant but do not present the desired antigen can instead prime autoimmune responses against self-antigens. Likewise, when DCs are exposed to the antigen but not the adjuvant, it will conversely promote a tolerogenic immune response that can discourage the intended activation of cellular immunity [58]

To minimize toxicity and increase potency, the solution we have come up with is to ensure codelivery of the antigen and adjuvant to the same DC at a synchronized timing.

1.1.7. Co-delivery of antigen and adjuvant to DCs

During an infection, DCs often encounter both the antigens and PAMPs (similar to adjuvant) together as a particulate package (e.g., bacteria or virus). It is rational to theorize that concerted delivery of antigens and adjuvants will simulate an actual infection, reduce immune tolerance and help enhance subsequent antigen-specific CD8⁺ T cell priming. Reduction in immune tolerance is difficult to verify, since most vaccine trials assess their efficacy based on antibody production and T cell response, while readouts to measure tolerance are typically overlooked. On the other hand for antigen-specific responses, Ruslan Medzhitov's group reported that the co-presence of antigen and adjuvant within the same phagocytosed cargo by DCs ensures optimal antigen processing and presentation [102].

Codelivery of adjuvants and antigens can be assured by two common approaches: packaging the two components using a delivery particle or covalently coupling them together. A number of studies chose to package both components into vehicles such as nanoparticles and managed to show that vaccines delivered this way enhanced immune efficacy and reduced toxicity in various mouse model studies [121-124]. For example, mice injected with liposome-encapsulated CpG + ovalbumin (OVA) or HER2/neu antigen showed higher levels of antigen-specific antibodies and/or interferon-gamma (IFN- γ) cytokine in serum in comparison to a simple mix of the two components [125, 126]. Other groups have demonstrated that cross-linking antigens and adjuvants exhibited a similar profile as vaccines packaged in a vehicle: stimulating stronger humoral and cellular immunity whilst reducing toxicity in comparison to unlinked antigens and adjuvants [127-129]. Cross-linking is performed via chemically conjugation or gene fusion. For example, Martin Kreutz and colleagues chemically coupled CpG ODN to the fusion protein of monoclonal anti-DEC205 antibody and OVA antigen. The antibody-antigen-adjuvant conjugate was significantly superior in CTL priming than control conjugate co-injected with an equivalent dose of soluble CpG ODN in a C57BL/6 mouse model [130]. Similarly, when the TLR5 ligand flagellin is genetically fused to the hemagglutinin 1 (HA1) fragment from the H1N1 influenza virus to form the fusion vaccine VAX128C, a phase I clinical trial also managed to show a strong immunogenicity and better safety profile than administering the antigen alone [131]. Further results suggested that conjugated products can redirect immune response away from Th2 associated with hypersensitivity as compared to co-administered antigen and adjuvant. Tighe et al., observed that when CpG ODN is bound to the major allergen Amb a 1 from short ragweed pollen, it directed Th1 cell polarization, higher immunoglobulin G (IgG) antibody titers in rabbit and monkey and significantly lower histamine production from human patient basophils [132].

Herein, we have presented reports that codelivery of adjuvants and antigens can: 1) stimulate a powerful and more precise immune response in the form of higher specific antibody titers and antigen-specific CTL activation, 2) prevent off-target toxicities and 3) reduce the adjuvant dosage required for effective immune stimulation.

That being said, several clinical trials using antigen-adjuvant linked vaccines have yielded less-than-satisfactory results. These vaccines either failed to induce an acceptable patient response or instigated adverse toxicity issues. One example is the virus-like nanoparticle cancer vaccine loaded with CpG-ODN and conjugated to tumor antigen Mart-1, which is tested on melanoma patients in a phase IIa clinical trial. Although results showed detectable tumor-specific memory CD8 T cell response and well tolerated toxicity level, only 2 out of 15 patients with detectable metastases showed positive response to the treatment while 11 patient displayed disease progression [133]. In another case, the vaccine STF2.4xM2e (VAX102), which is a recombinant fusion protein that covalently connects matrix protein 2 (M2e) antigen from influenza A virus and the TLR5 ligand flagellin, was tested in a phase I clinical trial. Subjects showed dose-dependent local and systemic reactions, with up to 40% of the subjects in the highest dosage group (10ug) experiencing severe symptoms such as headache, nausea and fever, suggesting the onset of a cytokine storm. In terms of immune response, the vaccine prompted a 4-fold or more increase in M2e antibody levels in all vaccinated subjects, showing the vaccine's strong immunostimulatory properties.

The two examples above illustrate the difficulty in designing an antigen-adjuvant conjugate. Even with the improved safety profile shown in preclinical models, designing a vaccine that in human subjects display minimal safety concerns, while generating immunological response

great enough to overcome the disease, still proved to be tremendously difficult especially in the case of cancer. We surmised that homing the antigen-adjuvant conjugate to their intended targets, the antigen-presenting cells such as dendritic cells, will bring about more potent immunological effects while reducing the toxicity. Notably, to our knowledge, we have not come across an antibody-guided vaccine conjugated with adjuvants that have enter clinical trials. In view of this, we would like to propose a novel vaccine-adjuvant design based on antibodies, and explore if this new vaccine format can bring about the much-needed improvement in clinical efficacy. To do that, we will draw inspiration from an unconventional intracellular antibody receptor TRIM21 (tripartite motif protein 21), which has been reported to trigger a series of immune signaling events.

1.2. TRIM21

1.2.1. Tripartite motif (TRIM) family proteins and their role as immune regulators

TRIM21 belongs to the TRIM family, which comprises over 70 individual protein members. True to its name, members from the family share three conserved protein motifs: a Really Interesting New Gene (RING) domain, followed by one or two B-Box domains and a coiled-coil (CC) domain [134-137]. The RING domain is a 40 to 60-residues long cysteine-rich zinc finger motif which mediates the E3 ubiquitin ligase activity. The B-box and coiled-coil domains both play a part in facilitating self-assembly of the TRIM proteins and substrate interaction [138]. Other than the RBCC (RING, B-box and coiled coil) domain group that form the basis of its structure, each TRIM protein may also display a supplemental domain at their carboxyl terminus. The additional domain contributes to the protein's function via purposes such as target recognition and enzymatic regulation [139-141].

Members of the TRIM family are involved in many cellular processes, including autophagy, carcinogenesis and most importantly in our context, immunity [142-144]. In immune responses, many of the TRIM proteins help to regulate innate immunity, ward off viral infection and control autoimmunity [140, 145-148].

TRIM proteins can directly or indirectly meddle with the process of virus infection [139]. For instance, TRIM5 α prevents lentiviral and retroviral infection by directly binding to the viral capsid protein. This interferes with the uncoating process and directs the virus for proteasomal degradation [149-151]. Similarly, TRIM14 directly binds to hepatitis C virus (HCV) non-structural protein NS5A to induce viral degradation [152]. TRIM proteins that indirectly prevents viral infections include TRIM25 which delay the onset of virus infection by priming immune cells for defense. It activates viral RNA-bound RIG-I during viral infection, allowing RIG-I binding to mitochondrial antiviral signaling protein (MAVS) for downstream signaling.

This leads to the production of cytokines such as type I IFN by leukocytes, which alert neighboring cells to resist the infection [153]. Likewise, TRIM31 induces the conformational switch of MAVS into prion-like aggregates, which initiates the cellular antiviral signaling pathway [154].

1.2.2. The use of ubiquitin in TRIM-mediated protein modification pathway

Most of the TRIM proteins rely heavily on RING domain for their function, which is to enzymatically catalyzes ubiquitin conjugation. Ubiquitin is a 76-residues long protein highly conserved in eukaryotes. It can be attached to a target protein via the process of ubiquitination. This is a post-translational modification of proteins that modulates biological processes such as protein degradation and signal transduction pathways. Ubiquitin conjugation requires the cooperation of three kinds of enzymes, namely the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. To start off, E1 ubiquitin-activating enzyme triggers the formation of thioester bond between ubiquitin molecule and itself in an ATP-dependent manner. Next, the activated ubiquitin is transferred to an E2 ubiquitin-conjugating enzyme. E3 ubiquitin ligase then recognizes both the ubiquitin-charged E2 enzyme and the target substrate protein, forming a transient E2-E3-substrate complex [155, 156]. As this happens, the E3 ligase will transfer the ubiquitin molecule from E2 enzyme to the substrate protein, during which the C-terminus of ubiquitin will bind to the α -amino group of a methionine (M) or ϵ -amino of lysine (K) of the substrate protein via an isopeptide bond. For TRIM proteins, the RING domain functions as the E3 ligase and TRIM proteins associate with several E2 enzymes such as UBE2D and UBE2E. In many cases, multiple ubiquitin molecules are successively linked together, forming a polyubiquitin chain. In total, ubiquitin has its N-terminus and 7 internal residues (M1, K6, K11, K27, K29, K33, K48, and K63) available for polyubiquitination [157]. Depending on which residue undergoes conjugation, distinct chain

conformations are formed. For example, the K48-linked chains adopt a “compact” conformation which allows adjacent ubiquitin moieties to interact with each other. K63-linked chains have an “open” conformation akin to beads strung on a string, granting it a large degree of structural flexibility [158]. Binding partners are able to perceive the distinct orientation and geometric structure of each polyubiquitin chain to coordinate interaction and carry out its intended purpose [159]. Each type of polyubiquitin chains can mediate different protein modification functions, which encompasses proteasome-dependent degradation of proteins, intracellular signaling, chromatin repair, membrane trafficking, and transcriptional regulation [160]. By the code of polyubiquitin chains, proteins covalently labeled with the canonical K48-linked polyubiquitin chain are usually directed for proteolytic degradation while those with K63-linked polyubiquitin chain are involved in activation of cell signaling pathways [161]. Furthermore, unanchored K63-linked polyubiquitin chains detached from substrate proteins by deubiquitinating (DUB) enzymes have also been suggested to activate protein kinases leading to downstream signal transduction pathways [162, 163].

In particular, the formation of K63-linked polyubiquitin chains is known to be central in executing the function of TRIM21.

1.2.3. TRIM21/Ro52: the autoantigen in autoimmune disorders

TRIM21 is a cytosolic protein expressed ubiquitously in all mammalian cells. Other than the RBCC module found in all TRIM proteins, TRIM21 contains an additional PRYSPRY domain at its C-terminus [164, 165]. PRYSPRY is also referred to as the SPRY/B30.2 domain, consisting of a ~160-residues long SPRY domain (derived from a sequence repeat found in amoeba dual-specificity kinase splA and mammalian ryanodine receptor), preceded by a ~40-residues long PRY extension [166, 167]. There are more than 500 eukaryotic proteins

containing the PRYSPRY domain, which all display a bent β -sandwich fold structure consisting of 15 β -strands [168]. Despite the resemblance in structure, sequence homology between PRYSPRY domains from different protein can be as low as 20%, which explains its ability to arbitrate protein-protein interaction with many different targets [169]. TRIM21 has been associated with the regulation of several members of the interferon regulatory factor (IRF) family [170], including IRF3, IRF5, IRF7 and IRF8 [170-172].

Due partly to its heavy influence on IFN responses, dysfunction of TRIM21 is closely linked to the pathogenesis of autoimmune diseases [173]. This is exemplified by TRIM21 knockout (KO) mice developing severe autoimmune symptoms similar to that of lupus disease. TRIM21 KO mouse embryonic fibroblasts displayed an upregulation of TLR-mediated signaling pathways relative to wild type (WT) cells. This includes IRF and NF- κ B activation that led to the induction of proinflammatory cytokines such as IL-1 β , IL-6, TNF α , CXCL10 and IFNs [174, 175]. These findings suggest that TRIM21 plays a role in the negative regulation of proinflammatory cytokine responses.

Indeed, although TRIM21 is a cytosolic protein, it has always been known as a target of autoantibodies in systemic lupus erythematosus (SLE) and Sjogren's syndrome (SS) [176, 177]. Autoantibodies to Ro52 (another name for TRIM21 before it was grouped under the TRIM family; From here on Ro52 will be referred to as TRIM21) and a non-homologous protein Ro60 were historically designated the common name anti-Ro/SSA antibodies [178]. However, later reports have shown that clinical manifestation appears to be related solely to anti-TRIM21 autoantibodies [179, 180]. Anti-TRIM21 antibodies are detected in around half of the patients with SS, and one-third of the patients with SLE, systemic sclerosis and autoimmune myositis [181, 182].

Although anti-TRIM21 antibodies have been repeatedly linked to autoimmune diseases, its pathogenic role is not entirely clear since antibodies should not be able to access TRIM21 localized in the cytosol of cells. One hypothesis is based on the data that UV irradiation induces the translocation of TRIM21 to the cell surface of keratinocytes [183, 184]. This exposes TRIM21 antigen to autoantibodies in the circulation, which triggers antibody-dependent cell cytotoxicity (ADCC) leading to cell apoptosis [185]. This theory is supported by another study, which shows that anti-TRIM21 antibody titer in circulation was correlated with cell surface TRIM21 expression in skin biopsy samples from SLE patients, but only in those suffering from photosensitive forms of Lupus. [186].

In patients who do not display photosensitivity, TRIM21 predominantly remain within the cell even after exposure to UV light. For anti-TRIM21 antibodies to interact with the cytosolic TRIM21 antigen, the antibodies would have to traverse the cell membrane and enter the cytosol. Leo James and colleagues have found that this can happen in the event of an adenovirus infection, when non-entry-blocking anti-adenovirus antibodies bound on virus particles were carried into non-immune cells during the course of infection. In this case however, the antibody binds to TRIM21 via its Fc (Fragment crystallizable) domain [187]. This came in conflict with the prior belief that binding to TRIM21 was through the Fab (Fragment antigen-binding) region. In fact, TRIM21 shows a remarkably broad specificity to Fc of different antibody isotypes, with its PRYSPRY domain binding to human IgG ($K_D \sim 150$ nM), IgM ($K_D \sim 17$ μ M), as well as IgA ($K_D = 50 \pm 24$ μ M) [165, 188]. This disproportionality in Fc-TRIM21 binding by different antibody isotypes might have given rise to the likelihood of inaccurate serum antibodies measured as anti-TRIM21 in an ELISA test for previous studies. As a result, the number of TRIM21 autoantibodies-positive SLE patients could have been misleadingly skewed.

Nonetheless, interaction between antibody Fc and TRIM21 mediates a series of immune activities that are particularly interesting, which we can take advantage of to improve future vaccine therapeutics.

1.2.4. Cytosolic TRIM21 interacts with immune complex to mediate antigen clearance and immune signaling pathways

Since the discovery that the PRYSPRY domain of TRIM21 binds avidly to the CH2–CH3 domain interface of an antibody Fc region [189], several reports had went on to illustrate the importance of TRIM21 as a cytosolic antibody receptor that mediates antigen clearance intracellularly [190-193]. Antibodies typically survey the extracellular environment, where they bind to specific targets such as pathogens to limit disease progression. However, during infection, antibodies that attached to cell-invading pathogens can be piggybacked into the cytosol and interact with TRIM21. TRIM21 activation upon binding with antibody Fc directs the entire antibody-pathogen complex (from here on will be referred to as immune complex (IC)) for proteasomal degradation and the process is termed antibody-dependent intracellular neutralization (ADIN). The clearance of antigens has been reported in the case of non-enveloped viruses [191, 194], bacteria [192] and tau protein assemblies [190] in non-immune cells. In conjunction, signaling pathways like NF- κ B and AP-1 are activated by TRIM21 to help fight against the infection [195-197]. One condition that determines the strength of TRIM21-mediated immune response is the affinity of antibody Fc to TRIM21.

1.2.5. Weaker binding between antibody Fc and TRIM21 compromises the strength of immune signaling but does not affect ADIN

To elucidate the importance of Fc-TRIM21 affinity in TRIM21-mediated immune response, Stian Foss and colleagues generated anti-adenovirus IgG clones with attenuated affinity to TRIM21, up to a K_D difference of 100-fold. Using these antibody clones, they revealed that induction of NF- κ B signaling from IgG-TRIM21 interaction can be heavily suppressed in an adenoviral infection model when binding is compromised. However, ADIN did not seem to be affected as IgG-opsonized adenovirus particles continued to be degraded by proteasomes under suboptimal binding [197]. Conversely, we hypothesized that antibodies with higher affinity to TRIM21 might enhance TRIM21-mediated immune responses, which includes NF- κ B signaling that is known to be instrumental in regulating multiple aspects of innate and adaptive immunity [198]. In other words, improving Fc affinity to TRIM21 has the potential to promote a more pronounced immune response in scenarios where antibodies can access cytosolic TRIM21. In order to improve Fc affinity to TRIM21, our lab came up with an engineering approach involving the generation of an Fc phage display library.

1.3. Engineering antibodies to improve their affinity to TRIM21

1.3.1. Antibody phage display library

The development in genetic engineering and phage display technologies has fueled the progress of biological drugs in the past few decades [199]. Phage display was first described by George P. Smith in 1985, when he displayed exogenous antigens on filamentous phages by fusing the antigen's DNA sequence with the phage gene III [200, 201]. This idea of phage display has been employed to establish the antibody phage display technique and earned George P. Smith the Nobel Prize in Chemistry in 2018.

The antibody phage display technology is based on the genetic engineering of bacteriophages to prepare a phage library, for the selection of monoclonal antibody clones that bind to a bait antigen. For construction of an antibody phage display library, RNA from peripheral blood mononuclear cells (PBMCs) is isolated and reverse-transcribed into complementary DNA (cDNA). From there, polymerase chain reaction (PCR) products of the variable heavy (V_H) and variable light (V_L) regions are fused to the phage display vector. The resulting phage display library reflects the entire antibody repertoire of the B cells present in the PBMC source. The library will go through repeated rounds of antigen-guided selection and eluted phage propagation. Ultimately, the phages displaying antibody clones that bind to the target antigen will be enriched and selected [202]. DNA from the selected phages will be sequenced and used for expression of said antibodies.

For Fc phage display library, site-directed mutagenesis will have to be conducted since variation in natural Fc repertoire exists only between different antibody isotypes.

1.3.2. Generation of Fc phage display library

Site-directed mutagenesis is frequently used for protein engineering experiments. In particular, Kunkel mutagenesis is a popular technique for generating protein variants in M13 bacteriophages [203-207]. For this method, an uracil-incorporated, circular, single-stranded DNA (ssDNA) template is used to synthesize a heteroduplex double-stranded DNA (dsDNA) using mutagenic oligonucleotide primers. The dsDNA will then be inserted into bacteria, where the mutated strand will remain and replicate, while the original template strand with uracil residues will be cleaved. Following that, the bacteria will be propagated to produce the bacteriophages with the mutated plasmids.

As the processes of generating the Fc library and mutant Fc panning were not performed by me, the results of both experiments are placed in the Introduction (section 1.3.2 and 1.3.3), to distinguish them from the data in the Results section which I have produced.

Before construction of the Fc phage display library to select for clones with strong affinity for TRIM21, our lab first studied the crystal structure of a human WT IgG Fc-TRIM21 complex to identify contact residues between the two proteins (PDB ID: 2IWG [164]). Although some studies suggest otherwise [208], it is more likely than not that amino acid mutations at the contact interface will strongly impact the resulting binding affinity. Jones et al., defines contact residues between two proteins as those within the distance of 7Å [209]. To further zero in on more important residues, we further increased the stringency of selection by using a cut-off distance of 5Å. As a result, we identified 21 residues on WT IgG Fc which were within this distance (Table 1.2; Supplemental Table I from [210]). After excluding residues which are part of an alpha helix to avoid destabilizing the antibody structure during mutagenesis, we decided

to finally pick seven amino acids in the CH3 domain whose side groups are facing towards and in the closest proximity to TRIM21 (Figure 1.3.A and 1.3.B; Figure 1 from [210]).

Using degenerate oligonucleotide primers to introduce randomized amino acids in the seven selected positions, our lab was able to generate a library of 2 billion bacteriophages by Kunkel mutagenesis as described by Tonikian et al [204]. Briefly, the human IgG1 Fc gene fused to an amber stop codon (TAG), followed by a truncated phage gene III, was inserted into the in-house phagemid vector PN04-44AD. Oligonucleotide-directed mutagenesis was carried out to generate covalently closed circular (CCC) heteroduplex dsDNA mismatched at the seven selected positions. The CCC dsDNA was electroporated into *E. coli* TG-1 cells that were pre-infected with helper phage and uracil-containing template ssDNA strand was resolved by the bacterial host. The resulting library was packaged into phage particles and used to isolate Fc clones binding to recombinant TRIM21 PRYSPRY domain.

List of amino acids on human Fc that contacts TRIM21

Amino acid	CH domains	secondary structure
252(MET)	CH2	Loop
253(ILE)		Loop
254(SER)		Loop
309(LEU)		Helix
310(HIS)		Helix
311(GLN)		Helix
314(LEU)		Helix
315(ASN)		Helix
345(GLU)	CH3	Loop
428(MET)		sheet
430(GLU)		Loop
431(ALA)		Loop
432(LEU)		Loop
433(HIS)		Loop
434(ASN)		Loop
435(HIS)		Loop
436(TYR)		sheet
437(THR)		sheet
438(GLN)		sheet
439(LYS)		sheet
440(SER)		sheet

Table 1.2. List of amino acids on human Fc that contacts TRIM21 [210]. A total of 21 amino acids are found to be in contact with TRIM21 PRYSPRY domain based on the criteria that they are at ≤ 5 angstrom distance apart from TRIM21. The seven amino acids that were selected (pink) for randomization have side groups nearest and facing towards TRIM21.

Figure 1.3. A modified Fc that exhibits increased affinity for TRIM21 [210]. a, Crystal structure from Protein Data Base (PDB; 2IWG) showing TRIM21 PRYSPRY domain (blue) in complex with Fc (green). The amino acids selected for randomization are in red and shown labeled in the zoom-in box. **b,** the protein sequence of Fc is shown. The amino acids selected for randomization are in red. **c,** Frequency of the different clones identified after Round 2 and Round 3 of biopanning.

1.3.3. Isolation of Fc clone with high affinity for TRIM21 from Fc phage display library

For biopanning, recombinant human TRIM21 PRYSPRY domain was used as the bait protein (see Methods and Materials section). We introduced harsher washing conditions after each round of biopanning to enrich clones with exceptionally strong affinity for TRIM21. In the end, ~50 clones from the output of Rounds 2 and 3 were sequenced and one clone was found to dominate the Round 3 output (Fig. 3C, Clone no. 1; [210]). This variant bears five residue mutations (All sequences follow the EU numbering system [211, 212]: H433T, N434R, Y436F, S440I and a spurious mutation T256P in the CH2 domain. Via DNA recombination, the mutant/engineered Fc can be grafted onto any Fab region to form an IgG antibody. From here

on we refer to antibodies grafted with the mutant Fc as modified antibody. This thesis will focus on using these modified antibodies, which the affinity to TRIM21 is revealed to be ~100 fold stronger than WT Fc in our preliminary results, for most of our experiments.

1.3.4. Potential effect of Fc modification on affinity to Fc receptors

Mutations in the Fc region may inadvertently alter antibody's binding to other Fc receptors, which will affect downstream cellular processes.

For instance, Fc region of immunoglobulin engages cognate Fc receptors expressed on leukocytes to mediate immune-modulatory effector functions. Fc gamma receptors (FcγRI/CD64, FcγRII/CD32, and FcγRIII/CD16) found on immune cell surface interacts with extracellular IgG Fc to induce downstream cellular processes such as antibody-dependent cell cytotoxicity (ADCC) for cytotoxic T cells [213] and antibody-dependent cell phagocytosis (ADCP) for phagocytic cells [214]. More importantly in our context, FcγR mediates antibody penetration into cells [215] and may subsequently lead to the interaction between modified antibody and its intended cytosolic target TRIM21.

Numerous studies have concurred that the lower hinge and CH2 regions of IgG are critical for binding to FcγRs [216, 217]. The key motifs in close proximity to the FcγR:Fc interface were identified to be P232-S239, D265-D270, Y296-T299 and N325-I332 [218-221]. Despite the fact that the mutated residues in our modified antibody (T256P, H433T, N434R, Y436F, S440I) do not coincide with the aforementioned motifs and are therefore expected not to affect its interaction with the FcγRs, we proceeded to confirm this as interference to FcγRs binding will affect FcγR-mediated dendritic cell maturation and MHC-I-restricted antigen presentation [222].

On the other hand, neonatal Fc receptor (FcRn) has a predominant intracellular localisation and binds endocytosed IgG at acidic pH within the endosome/lysosome [223]. Binding to FcRn rescues the antibody from intracellular degradation by recycling them back to surface and extends its half-life in circulation [224]. Any improvement in affinity to FcRn, which can further facilitate antibody exit out of the cell, may be a concern for the modified antibody, as its intended mode of action was to enter the cytosol and interact with TRIM21. The key motifs for FcRn:Fc interactions are L251-S254, L309-Q311 and N434-H435 [225]. In contrast to Fc γ R, FcRn binds to Fc at the amino acid positions where many of our modifications (T256P, H433T, N434R, Y436F, S440I) lies, or to which they are close. This increases the likelihood for modified Fc:FcRn binding to be affected.

To address our concerns, we compared the affinity of both WT and modified antibodies to Fc γ Rs and FcRn by immunosorbent and surface plasmon resonance assays. To elucidate if modified antibody requires Fc γ R binding for its downstream effects, a LALA variant of the modified antibody (i.e. a modified antibody that possesses L234A, L235A mutations which abolishes binding to Fc γ R) [226] was included in our experiments.

1.4. The application of modified antibody on dendritic cells

1.4.1. Antibody-mediated TRIM21 effector response on DCs

TRIM21-mediated ADIN and immune signaling pathways are reported to be initiated by antibodies within non-immune cell [190-192, 194]. At the time when we started this study, there were no studies that investigated the effect of intracellular antibodies interacting with TRIM21 in the immune cells. Interestingly, we found that DCs express high levels of TRIM21 amongst immune cells [174] and TRIM21 is involved in immune-related response. This included the regulation of type I interferon pathway during virus infection [227] and the enhancement of proinflammatory cytokine secretion in Behçet's disease [228]. Given the role of TRIM21 in regulating immune responses in DCs, we and others [229, 230] began to hypothesize that TRIM21 can play a major role in aiding a DC's immune function if given the chance.

For example, in the case of a virus infection on DCs, antibodies opsonized on the virus can initiate ADIN which theoretically generates viral peptides for loading on MHC-I, supporting its antigen presentation activity. Besides, TRIM21-mediated immune signaling pathways such as NF- κ B and IRF, are critical in driving DC maturation [231-233]. Concurrently, the boost in NF- κ B and AP-1 signaling can also confer protection by prompting innate anti-viral and pro-survival responses [234-236]. Putting them altogether, TRIM21-mediated response has the potential to elicit DC maturation and antigen presentation, stimulating a robust adaptive immune response in the form of antigen-specific T cell activation, which is what we desire in anti-viral and anti-cancer vaccines.

1.4.2. Increase affinity for TRIM21 to improve antibody vaccines

By increasing the affinity between antibody and Fc receptor TRIM21, we hypothesized that it could stabilize the complex and create a more secure interaction. With higher likelihood of complex formation, this might further improve TRIM21-mediated immune function and encourage DC cross presentation. If our hypothesis is true, it can give rise to a new antibody vaccine format which would not have to rely on additional adjuvants to provoke DC maturation. Instead, slight genetic modification in the form of five amino acid mutation can imbue antibody with additional immunostimulatory property like an adjuvant. In the case of a DC-targeting antibody vaccine, application of antigen-conjugated modified antibodies would mimic the co-delivery of adjuvants with antigen. This can potentially promote a more precise and potent immune response, reduce the drug dosage required, off-target toxicity and tolerogenic immune response, and save drug manufacturers the hassle of chemically tagging an adjuvant onto the vaccine.

1.4.3. Inherent limitation of modified antibody vaccines

It is known that DC-restricted receptors such as DEC-205 and Clec9A facilitate uptake and internalization of binding ligands into endocytic compartments of DCs [89, 237, 238]. As a result, these receptors were often the target of antigen-coupled antibody vaccines to deliver antigens into DCs for processing and presentation to both CD4 and CD8 T cells [237, 239, 240]. However, what remains enigmatic is the event of antigen egress from endosomes after cell entry. Studies have shown that soluble and complexed antigens internalized through Fc gamma receptors can be exported into the cytosol in mouse DCs [11, 13]. Specifically, horseradish peroxidase (HRP), which is ~44 kDa in molecular size, remained enzymatically active in the cytosolic compartment, suggesting that antigens delivered as IC remained fully intact and functional. But antigen export to cytosol appears to be size-selective, as small

molecular mass dextrans of ~30 kDa are rapidly transported to the cytosol while higher molecular mass dextrans of ~500 to 2000 kDa remained in vacuoles [11]. To our knowledge, it has not been clarified if DC-targeting antibodies of ~150 kDa are eligible for relocation to the cytosol.

Although still widely debated, one popular hypothesis has garnered the most experimental support – the presence of transporter proteins which export proteins from the endosomes into the cytosol. [241-244]. Given the narrow pore size of known transporter proteins, tremendous conformational stress would be imposed on translocating proteins in the native state. Therefore, proteins to be translocated were expected to undergo protein unfolding [245, 246]. For an antibody, this step can severely compromise its structure and its affinity for TRIM21. It is unknown if DC-targeting antibody can refold into its native conformation after translocation to interact with the cytosolic TRIM21 molecules. To investigate the effect of modified antibody, it is extremely vital for us to use an alternative internalization model which ensures intact antibody trafficking.

1.4.4. Using adenovirus infection model to test the effect of modified antibodies

As a proof-of-concept model, we decided to use adenovirus to transport anti-adenovirus antibodies into the cytosol, as what Leo James' group has done in a number of their publications [165, 187, 195, 197, 247]. Adenovirus is a non-enveloped virus which infects the cell by first going through endocytosis via receptors such as Coxsackie and Adenovirus Receptor (CAR) and DC-SIGN [248, 249]. Upon encountering low pH conditions in the endosome, adenovirus releases membrane lytic protein VI (pVI) that ruptures the endosome, allowing it to gain entry into the cell cytosol [250]. In this way, antibodies that attach to its capsid protein can be carried into the cytosol to interact with TRIM21. One such example is the non-entry blocking

hybridoma clone 9c12 [251] which functions via intracellular neutralization. Binding of 9c12 to adenoviruses allow the antibodies to reach the cytosol when the viruses infect the cell, and from there recruit the intracellular machinery for viral neutralization. For our work, the variable domain of 9c12 will be used with a WT or Fc-modified human constant region to test the effect of modified antibodies in the adenovirus infection model.

Our preliminary results have shown that moDCs incubated with the modified antibodies-adenovirus IC (from here on referred as modified IC) exhibited an increase in maturation markers, which was not observed in moDCs incubated with WT antibodies-adenovirus IC (from here on referred as WT IC) [252]. In other words, dendritic cells displayed a higher degree of maturation when IC that enter the cytosol bind better to TRIM21. Our study will aim to further unravel the function and mechanism of modified antibodies in this model.

1.4.5. Application of the modified antibodies in enterovirus 71 (EV71) model

In the initial phase of the project, enterovirus 71 (EV71) was selected to be the alternative target pathogen aside from adenovirus. EV71 is a non-enveloped positive sense single-stranded RNA virus that causes Hand, Foot Mouth Disease (HFMD), which is highly contagious and mainly manifests in young children below the age of 5. The virus is known to bring about severe neurological complications like encephalitis and aseptic meningitis, which can ultimately result in death of patients. In the recent years, EV71 outbreaks had plagued Asia-Pacific countries such as China, Singapore, Malaysia, Taiwan, Thailand and Australia [253-256].

One challenge which we anticipated for EV71 model is that the difference in mode of infection to that of adenovirus, which may hamper the delivery of antibody to TRIM21. During infection, EV71 punctures the endosomal membrane and releases its nucleic acid into the cytosol, leaving

its viral proteins that antibodies bind to in the endosome [257]. The fate of the remaining viral proteins has not been reported. Since DCs are known to translocate antigens from endosomes to cytosol [258-261], we postulated that antibodies attached to the EV71 viral proteins may still end up in the cytosol to interact with TRIM21. However, after a number of functional assays, we realized the main dilemma faced in this model is the constitutive immune-activation by the EV71 alone. To simply put it, the immunostimulatory effect by modified antibodies was overshadowed by the immune response to EV71. The results for EV71 model will be placed in the supplementary figures and further explained in the discussion section.

As a result, for the alternative disease model, we decided to re-explore the prospect of applying the modified Fc in DC-targeting cancer vaccine despite its intrinsic limitation. We will focus particularly on the use of tumor antigen-coupled DEC205-targeting antibodies.

1.4.6. Application of tumor antigen-coupled anti-DEC205 antibodies with modified Fc against cancer

As previously mentioned, Anti-DEC205 antibodies carrying an antigen load were demonstrated to orchestrate endocytic entry into dendritic cells for loading onto MHC molecules and eventual stimulation of T-cell responses [97, 262-264]. The treatment usually entails the combination of adjuvants such as TLR agonists to promote DC activation. Since modified antibody showed promises to activate moDC via TRIM21-mediated response, we speculated that anti-DEC205 modified antibodies fused with a desired antigen can be developed into a therapeutic antibody vaccine. The model will be mainly tested *in vivo*, but one issue in the translation to mouse model is the fact that engineered Fc displayed abolished binding to mouse TRIM21 (Figure 2.1). To overcome this problem, immunocompetent

transgenic C57BL/6 mouse bearing a human TRIM21 PRYSPRY domain sequence will be used.

1.5. Cancer antigen to be coupled on anti-DEC205 antibodies

To assess the immunological function of modified anti-DEC205 antibodies in the context of both human and mouse, we would first have to select the antigen to be conjugated on the molecule. The choice of antigen is a crucial aspect in cancer vaccine design. We have decided on the use of two antigens, ovalbumin and glypican-3, for their high immunogenicity and research tools available for in-depth studies on cancer vaccine.

1.5.1. Ovalbumin (OVA)

OVA is the main protein found in egg white. It is one of the best characterized and most frequently used model antigens in vaccine research. Its immunogenic T cell epitopes have been mapped and many tools (e.g. transgenic mouse models such as OT-I, MHC-peptide multimers) are commercially available to be used in studies on antigen delivery and measuring T cell responses of a treatment [265-267]. Laura Bonifaz and colleagues have established a protocol that uses OVA-specific CD8+ T cells from OT-I mice as a reporter cell to track and measure OVA-specific CD8 T cell activation *in vivo* [268]. Using the protocol, it would allow us to assess the function of OVA-conjugated modified anti-DEC205 antibodies in mice.

Although OVA is useful as a proof-of-concept antigen model, it is not derived from disease agents such as tumor and viruses, and thus will not serve as a favorable affirmation for proving the worth of modified antibody as a vaccine. In order to further understand the value of modified antibody in modulating a real disease agent, we intend to test it with the natural tumor antigen Glypican-3.

1.5.2. Glypican-3 (GPC3)

GPC3 is a tumor antigen overexpressed in hepatocellular carcinoma (HCC). Other than embryonic kidney and placenta, GPC3 is not found on normal healthy tissues, which makes it an excellent target for anti-cancer therapeutics [269]. GPC3 belongs to the heparan sulfate proteoglycan family and controls cell cycle progress via Wnt and Hedgehog signaling pathways [270-272]. The human and mouse GPC3 gene encode precursor core proteins which are 580- and 579-residues long respectively. They are naturally cleaved by furin to form an N-terminal fragment (334 and 333 amino acid respectively) and a C-terminal fragment (222 amino acid long). Another reason for choosing GPC3 is because for both mouse and human homologues, immunogenic CD8 T cell epitopes have been identified. Interestingly, all the peptides are found on the N-terminal fragment. They are HLA-A*24:02-restricted hGPC3₂₉₈₋₃₀₆ (EYILSLEEL) and HLA-A*02:01-restricted hGPC3₁₄₄₋₁₅₂ (FVGEFFTDV) peptides for human, and H2-K^b/H2-D^b restricted mGPC3₁₂₇₋₁₃₆ (AMFKNNYPSL) peptide for mouse [269, 273, 274]. All three aforementioned peptides are able to efficiently induce GPC3-specific CD8 T cell immune response. For the work on GPC3 antigen, we aimed to focus solely on establishing the mouse model due to time constraint, so the section on human GPC3 is left for future studies.

Deciding the portion of GPC3 antigen to be conjugated on anti-DEC205 is crucial to the success of the experiment. After much discussion, we determined the segment to be included based on the following criteria:

- Include the immunogenic CD8 T cell epitope H2-K^b/H2-D^b restricted mGPC3₁₂₇₋₁₃₆ (AMFKNNYPSL) peptide
- Make the conjugated GPC3 antigen as large as possible to encompass other unidentified epitopes which may synergistically stimulate CTL responses

- Exclude cysteine residues from the sequence to avoid the formation of disulfide bonds

This led us to select the 123-residues long mGPC3₇₃₋₁₉₅ fragment to be conjugated on the anti-DEC205 antibody. We cloned the desired GPC3 sequence to the C-terminus of the antibody heavy chain and expressed this as a fusion protein in HEK293-6E or Chinese hamster ovary (CHO) cell cultures. In this paper, we tested the fusion protein as a prophylactic vaccine, by immunizing TRIM21 transgenic mice before they are challenged with mGPC3-expressing tumors.

1.6. Objective of this study

In this study we will explore the feasibility of using modified antibodies with a stronger affinity to TRIM21 in antiviral and anti-cancer antibody vaccines. To do that, we need to first verify if modified antibodies can induce any immunostimulatory effects directly on DCs and indirectly on T cells. If so, we have to validate if the mechanism involves TRIM21-mediated activity and test the effect of such antibodies in *in vivo* mouse models.

Using the aforementioned adenovirus infection model, we are going to:

- Elucidate the biological function of modified antibodies
 1. Reinforce the preliminary data of modified IC stimulating moDC maturation by increasing the sample size.
 2. Examine the effect of modified antibodies in promoting subsequent T cell activation through moDCs
 3. Validate the antigen specificity of T cell response by peptide restimulation experiments
- Understand the underlying mechanism
 4. Confirm the entry of modified antibodies into moDC and its interaction with cytoplasmic TRIM21.
 5. Investigate the role of TRIM21 in activating the NF- κ B signaling pathway by knocking down TRIM21.
 6. Examine the transcriptomic profile of moDCs treated with modified antibodies and identify key signaling pathways stimulated.

Next, we will test the efficacy of modified DEC205-targeting antibody vaccines in TRIM21 transgenic mice using two different antigens:

- Using ovalbumin as the antigen

7. Elucidate the capability of modified antibodies in activating antigen-specific CTLs
- Using GPC3 as the antigen
8. Evaluate vaccines efficacy in boosting anti-tumor response by monitoring the tumor size and detection of tumor infiltrating CD8 T cell count

2. MATERIALS AND METHODS

2.1. Viruses, Cells, mice and related cell-processing methods

2.1.1. Human and mouse cell lines

Human rhabdomyosarcoma cell line (RD-114 ATCC number VR-805), HeLa cell line (ATCC number CCL-2) and HEK293T cell line (ATCC number CRL-3216) were obtained from American Type Culture Collection (ATCC). Parental murine colon cancer cell line MC-38-null was obtained from Singapore Immunology Network. MC38-GPC3 stably expressing murine GPC3 were generated as described (Addgene: Generating Stable Cell Lines with Lentivirus, 2007). All cell lines were grown in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% Penicillin Streptomycin (Pen/Strep, Gibco, USA) at 37°C with 5% CO₂.

2.1.2. Mice

C57BL/6 wild-type mice and C57BL/6-Tg(TcraTcrb)1100Mjb mice (OT-I; CD8 T cells express transgenic T cell receptor which recognizes chicken ovalbumin peptide residues 257-264 in the context of H2K^b [275]) were maintained and housed in the A*STAR Biological Resource Center (BRC) facilities in Specific Pathogen Free (SPF) conditions. Mouse model possessing mouse/human chimeric TRIM21 (TRIM21-KI mouse) was established in C57BL/6 mice by Applied Stemcell (Applied Stemcell, USA). Briefly, CRISPR/Cas9 technology was used to replace a segment of mouse exon9 coding region with human exon9 sequence in chromosome 7. The gene sequence inserted was the 567bp human TRIM21 PRYSPRY domain

— 5'-
CACATCACTCTGGATCCAGACACAGCCAATCCGTGGCTGATACTTTCAGAAGATC
GGAGACAAGTGAGGCTTGGAGACACCCAGCAGAGCATACCTGGAAATGAAGAG
AGATTTGATAGTTATCCTATGGTCCTGGGTGCCCAGCACTTTCCTCTGGAAAAC

ATTACTGGGAGGTAGATGTGACAGGAAAGGAGGCCTGGGACCTGGGTGTCTGCA
GAGACTCTGTGCGCAGGAAGGGGCACTTTTTGCTTAGTTCCAAGAGTGGCTTCTG
GACAATTTGGTTGTGGAACAAACAAAAATATGAGGCTGGCACCTACCCCCAGAC
TCCCCTCCACCTTCAGGTGCCTCCATGCCAAGTTGGGATTTTCCTGGACTATGAG
GCTGGCATGGTCTCCTTCTACAACATCACTGACCATGGCTCCCTCATCTACTCCTT
CTCTGAATGTGCCTTTACAGGACCTCTGCGGCCCTTCTTCAGTCCTGGTTTCAATG
ATGGAGGAAAAACACAGCCCCTCTAACCCTCTGTCCACTGAATATTGGATCACA
AGGATCCACTGACTATTGA-3'. F1 animals with the correct single strand mutation were
verified by Next-Generation-Sequencing (NGS) and crossed in A*STAR BRC to generate
double knock-in progenies. All experiments involving mice were carried out under Institutional
Animal Care and Use Committee (IACUC) approval. Mice were 6-8 weeks old at the start of
each *in vivo* experiment.

2.1.3. Viruses

Non-replicative adenovirus type-5 (Ad5)-null (VQAd empty-2.6del, an Ad5 vector having no
inserted transgene) and Ad5-eGFP (VQAd eGFP-2.6del, an Ad5 vector having enhanced green
fluorescent protein transgene) were purchased from Viraquest (Iowa, USA). The adenoviruses
were titrated using Adeno-X Rapid Titer kit (Clontech, USA) following the manufacturer's
protocol, aliquoted and stored at -80°C until use. Wild-type EV71-B5 sub-genogroup strain
(GenBank FJ461781.1) and coxsackievirus A16 (CVA16) strain (U05876) were obtained from
the Department of Microbiology and Immunology, Yong Loo Lin School of Medicine,
National University of Singapore. These viruses were propagated as described by Jia et al [276].
Briefly, viruses were added to RD cells grown in DMEM supplemented with 2% FBS at 37°C
with 5% CO₂. Cell culture supernatants were harvested at 4 days post infection (dpi). After
three freeze-thaw cycles, the debris was removed by centrifugation and virus aliquots were

stored at -80°C. Virus titer was measured on RD cells with an end-point dilution assay to determine the 50% tissue culture infective dose (TCID₅₀).

2.1.4. Peripheral blood mononuclear cell (PBMC) preparation

Human blood was obtained from healthy volunteers. The research was approved by National University of Singapore Institutional Review Board (IRB code NUS-IRB 10-250). PBMCs were isolated by Ficoll-Paque gradient centrifugation (GE Healthcare, USA). The cells were incubated with CD14 microbeads (Miltenyi, Germany). After washing with MACS buffer, CD14-positive and CD14-negative cells were separated using a LS column (Miltenyi, Germany). The PBMCs were cryopreserved in 10% Dimethyl Sulfoxide (DMSO, Sigma-Aldrich, USA) with 90% FBS before storing in liquid nitrogen for later experiments. PBMCs were rapidly thawed at 37°C, diluted with RPMI containing 10% FBS and subsequently washed twice with PBS containing 5% FBS before using for the subsequent experiments.

2.1.5. Generation of immature monocyte-derived dendritic cells (moDCs)

The CD14-positive cells were incubated in RPMI 1640 (HyClone, USA) supplemented with 10% FBS, 100µg/ml human interleukin-4 (IL-4) and 100µg/ml human granulocyte-macrophage colony stimulating factor (GM-CSF) (Miltenyi, Germany) for 6 to 7 days, to differentiate the cells into immature moDCs.

2.1.6. Generation of mouse bone marrow-derived macrophages (BMDMs)

Skin of mice on the limb area was cut open to expose the muscles. Muscles surrounding the femur and tibia were cut away to expose the bone near the hip joint. An incision is then made through the hip to release the entire leg. In a Microbiological Safety/Class II cabinet, 70% ethanol-soaked scissors and forceps were used to cut both ends of the bones. Then, using a 5

ml syringe attached with a 27-guage needle, RPMI 1640 medium, supplemented with 10% FBS, 1% Pen/Strep was loaded. The needle was inserted into the bone marrow cavity to flush out bone marrow cells. Erythrocytes were then lysed with ACK buffer (Gibco, USA) following the manufacturer's protocol. The remaining bone marrow cells were incubated in a 5% CO₂ incubator at 37°C for 6 days in RPMI 1640 (HyClone, USA) supplemented with 10% FBS, 1% Pen/Strep, 10ng/ml murine IL-4 and 20ng/ml murine GM-CSF (Miltenyi, Germany) to differentiate them into BMDCs.

2.2. Preparation of antibodies and other materials

2.2.1. Engineered antibodies

Mouse hybridomas that express anti-adenovirus hexon monoclonal antibody 9C12 [251] was obtained from the Developmental Studies Hybridoma bank (NICHD/University of Iowa). Mouse hybridomas that express a neutralizing (5H7) and a non-neutralizing (1C6) anti-EV71 monoclonal antibodies were kind gifts from a collaborator, Jimmy Kwang from Temasek Life Sciences Laboratories (TLL) [276, 277]. Sequences of the mouse immunoglobulin gene variable regions were extracted following the protocols described by Wang et al [278]. Briefly, RNA isolation was done using acid guanidinium thiocyanate-phenol-chloroform extraction (TRIzol, Thermo Fisher Scientific, USA) following the manufacturer's protocol. The variable region sequences were then converted to cDNA and amplified using Qiagen OneStep RT-PCR kit (Qiagen, Germany) and inserted into pCR2.1 TOPO vector (Invitrogen, USA). The variable domain sequence of the human anti-human DEC-205 antibody belongs to the published clone 3G9-2D2 (U.S. Patent 8,236,318 B2), while the rat anti-mouse DEC-205 antibody belongs to the published clone NLDC-145. Both variable domain sequences were synthesized by GenScript Biotech (USA). The variable domains of the above antibodies were connected to the constant region of human IgG1 in the pTT5 vector. For the Fc-modified antibodies with improved affinity to TRIM21, the five amino acid modifications in the constant regions are modified by using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, USA) [210]. For the WT and Fc-modified chimeric rat anti-mouse DEC-205 antibody, the 123-residues long mGPC3₇₃₋₁₉₅ fragment was connected to the C-terminus of the antibody through a G₄SA₃ linker as described [279] and further linked to a 6 x His tag via a GSGS linker. The WT anti-DEC205-mGPC3 sequences were synthesized by GenScript Biotech and the Fc-modified equivalent was again generated by using site-directed mutagenesis of the five amino acids. The antibodies were then expressed in HEK293-6E cells as described

by Durocher et al [280] in our laboratory or in CHO cells by WuXi AppTec. Both the vector and cells were obtained from National Research Council of Canada. Antibodies were purified from the culture supernatant using Protein G resin (Merck Millipore, USA). Schematic illustrations of all the engineered antibodies are represented in Figure 2.1. Each batch of antibodies produced within the lab was subjected to occasional Limulus Amebocyte Lysate (LAL) assay tests and ensured to be low in endotoxin level (<1 endotoxin unit (EU)/mg of antibody), before being used in subsequent experiments.

Figure 2.1. Structure of all engineered antibodies used in the study. The variable domains of five different antibodies were fused to the constant region of either WT or modified human IgG1 sequence. Red variable domain - mouse anti-adenovirus hexon antibody 9C12; Light green variable domain - mouse non-neutralizing anti-EV71 antibody 1C6; Dark green variable domain - mouse neutralizing anti-EV71 antibody 5H7; Orange variable domain - human anti-human DEC-205 antibody 3G9-2D2; Purple variable domain - rat anti-mouse DEC-205 antibody NLDC-145. For anti-mouse DEC-205, two antigens were separately conjugated: mGPC3₇₃₋₁₉₅ sequence (grey rod) was genetically fused to the C-terminal end of the antibody

while full length ovalbumin (red star) was chemically conjugated via succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC). Ovalbumin-conjugated anti-DEC205 was prepared by partial sulfhydryl reduction and is known to have an average drug:antibody ratio (DAR) of ~3 with a species distribution of 0 to 8 drugs [281, 282].

2.2.2. Expression of mouse and human TRIM21 PRYSPRY domain

TAGZyme qQE-2 plasmids were inserted with either human TRIM21 or mouse Trim21 PRYSPRY domain sequence fused with 6 x His tag. The plasmids were transformed into BL21 competent cells (New England Biolabs (NEB), USA) and positive transformants were selected after plating on LB agar containing ampicillin. The clones were inoculated into 2xYT medium broth (Sigma-Aldrich, USA) containing ampicillin, grown to $OD_{600} = 0.6$ and induced with 1mM Isopropyl β -D-1-thiogalactopyranoside (IPTG), before grown shaking at 250 rpm, 25°C for 16 hours. The cells were harvested and lysed with BugBuster Reagent (Sigma-Aldrich, USA) in the presence of Ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor mini tablets (Thermo Fisher Scientific, USA). The supernatant was incubated with Ni-NTA agarose beads (Qiagen, Germany) and the His-tagged PRYSPRY protein was eluted with buffer containing high concentration of imidazole (50mM KH_2PO_4 , 300mM KCl, 250mM imidazole, pH 8). The protein was further purified on the ÄKTA pure chromatography system by running the sample through the Superdex 75 10/300 GL column (GE Healthcare, USA).

2.2.3. Peptide-MHC production and tetramerization

Production and refolding of biotinylated mouse H-2Kb and H-2Db monomers was done by our collaborator, Evan Newell and Olaf Rotzschke from Singapore Immunology Network as described [283]. Briefly, recombinant H-2Kb and H-2Db heavy chains and human $\beta 2$ microglobulin light chains were refolded in the presence of a UV-cleavable linker (J) (H2-Kb SIINFEJL and H2-Db FAPGNYJAL, Mimotopes, Australia) and subsequently biotinylated. For tetramerization, 1:4 molar ratio of premium grade Streptavidin R-Phycoerythrin conjugate (SA-PE) to biotinylated MHC monomers was mixed and incubated on ice in the dark for 40 min. High purity (>98%) H2-Kb/H2-Db restricted mGPC3₁₂₇₋₁₃₆ AMFKNNYPSL peptide was synthesized by GenScript Biotech (USA). After tetramerization, peptide exchange reaction

was set up by mixing a molar ratio of 100 peptides to 1 tetramer and subjected to UV irradiation for 15 minutes on ice in UVP Longwave Ultraviolet Crosslinker (Cole-Parmer, USA). The tetramerized peptide-MHC-complexes were then combined and concentrated by using a 10 kDa (Merck Millipore, USA) cut-off filter. After exchanging the buffer into cytometry buffer (PBS, 2% FCS, 2mM EDTA, 0.05% sodium azide) the tetramer cocktail was filtered through a 1 μ m centrifugal filter (Merck Millipore, USA) and used to stain the cells with each peptide-MHC tetramer at a concentration of ~500nM.

2.2.4. Conjugation of ovalbumin (OVA) to chimeric anti-mouse DEC205 antibody

Monovalent IgG's were conjugated to maleimide-OVA (Imject Maleimide Activated Ovalbumin Cat#77126, Lot#TH274021; 11 moles maleimide/mole Ova; Thermo Fisher Scientific, USA) according to the manufacturer's protocol. In brief, the antibodies were reduced using 5 mM tris (2-carboxyethyl) phosphine (TCEP; Sigma-Aldrich, USA) at 1:150 molar ratio for 30 min at room temperature in conjugation buffer (10 mM sodium phosphate buffer pH 7.0 with 10 mM EDTA) that had been previously removed of oxygen by bubbling with argon gas for 30 mins through 30 ml of buffer. Maleimide-OVA powder was reconstituted just before use in water to a concentration of 10 mg/ml and mixed with the reduced antibodies for 2 hours at room temperature and stored overnight at 4°C. The reduced antibody was separated from the reducing agent using Zeba™ Spin Desalting Columns (Thermo Fisher Scientific, USA), further dialyzed with Slide-A-Lyzer dialysis cassette (Thermo Fisher Scientific, USA) and evaluated by SDS-PAGE. Monovalent IgG:OVA conjugates were characterized by SDS-PAGE. Quantification of the OVA content of the conjugates was achieved by comparison with known quantities of OVA on the same blot detected with an HRP-conjugated polyclonal rabbit anti-OVA antibody (Research Diagnostics, Inc.).

2.3. General methods

2.3.1. Enzyme-linked immunosorbent assay (ELISA)

For EV71 ELISA, 96-well Maxisorp flat-bottom plates (Nunc, Denmark) were coated with bicarbonate/carbonate coating buffer (33 mM Na₂CO₃, 100 mM NaHCO₃, pH9.6) containing 10 µg/ml mouse anti-EV71 MAB979 (Merck Millipore, USA) overnight at 4°C. This is followed by blocking with Blocker Casein in PBS (Thermo Fisher Scientific, USA). The coated plate was then incubated with 5×10^4 TCID₅₀/ml EV71-B5 or CVA16 at room temperature for 1 hour, engineered anti-EV71 monoclonal antibody (WT/Mod 5H7 or 1C6, prepared in our lab) for 1 h, and followed by anti-human IgG HRP-conjugated secondary antibody (Jackson ImmunoResearch, USA) for 1 hour. Lastly, tetramethyl benzidine dihydrochloride (TMB) substrate (SurModics, USA) was added for incubation for 15 min at room temperature and the reaction was stopped using 1M hydrochloric acid (HCl). Finally, the absorbance at 450 nm was detected using EnSpire Multimode Plate Reader (Perkin Elmer, USA).

For ELISA with recombinant Fc gamma receptors, the Maxisorp plates were coated with 10 µg/ml human CD64 protein (FcγRI; Sino Biological, China), human CD32a protein (FcγRIIa; Sino Biological, China), human CD32b protein (FcγRIIb; Sino Biological, China), human CD16a protein (FcγR3a; Sino Biological, China) or human CD16b protein (FcγRIIIb; R&D Systems, USA) overnight at 4°C. The coated plate was then incubated with eight 3-fold serial dilutions of engineered anti-adenovirus monoclonal antibody (WT/Mod 9C12). The starting concentration of the antibodies to be diluted were as follow: FcγRI – 600 ng/ml, FcγRIIa – 3000 ng/ml, FcγRIIb – 10000 ng/ml, FcγRIIIa – 900 ng/ml and FcγRIIIb – 150000 ng/ml. Similar to EV71 ELISA, anti-human IgG HRP-conjugated secondary antibody, TMB substrate and HCl were then added and the results were analyzed using the Enspire Plate Reader.

2.3.2. Western blot

Cell extracts and immunoprecipitates were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane using Trans-Blot SD semi-dry transfer cell (Bio-Ra, USA), according to the manufacturer's protocols. After incubation with 5% nonfat milk in Tris Buffered Saline-Tween (TBST) (25 mM Tris [pH 7.2], 140 mM NaCl, and 3 mM KCl, 0.2% Tween 20) for 1 h, the membrane was incubated with antibodies against IgG Fab (1:5000; Jackson ImmunoResearch, USA), Ro52/TRIM21 (1:500, D-12; Santa Cruz Biotechnology, USA), and GAPDH (1:10000, VPA00187; Bio-Rad Laboratories, USA) overnight at 4°C. Membranes were washed with TBST four times, then incubated with HRP-conjugated anti-mouse (1:10000; DAKO, Denmark) or anti-rabbit (1:20000; Thermo Fisher Scientific, USA) antibodies for 1 h. Blots were washed with TBST four times and developed with ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, USA) according to the manufacturer's protocols. Blot images were acquired using the ChemiDoc imaging system (Bio-Rad Laboratories, USA).

2.3.3. Quantitative PCR (qPCR)

Total RNA from moDCs was isolated at 6 time points after immune complex treatment: 1h, 2h, 4h, 6h, 8h and 24h. RNA integrity and concentration were assessed using Nanodrop 2000 (Thermo Fisher Scientific, USA). RNA was reverse transcribed using Maxima First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA). Expression of six direct gene transcripts of NF- κ B and AP-1: BCL2A1, BIRC3, CCL5, CXCL8, CXCL10 and IFNB were measured and analyzed by qPCR using iTaq Universal SYBR Green Supermix (Bio-Rad, USA) and the following primers: 5' - GTC CGT AGA CAC TGC CAG AAC A - 3' and 5' - AAA GTC ATC CAG CCA GAT TTA GG - 3' for BCL2A1; 5' - GGT AAC AGT GAT GAT GTC AAA TG - 3' and 5' - TTA CTG GCT TGA ACT TGA CG - 3' for BIRC3; 5' - CCC AGC AGT CGT

CTT TGT CA - 3' and 5' - TCC CGA ACC CAT TTC TTC TCT - 3' for CCL5; 5' - GCC AAC ACA GAA ATT ATT GTA AAG CTT - 3' and 5' - CCT CTG CAC CCA GTT TTC CTT - 3' for CXCL8; 5' - GAG CCT ACA GCA GAG GAA CC - 3' and 5' - GAG TCA GAA AGA TAA GGC AGC - 3' for CXCL10; 5' - CTC TCC TGT TGT GCT TCT CC - 3' and 5' - GTC AAA GTT CAT CCT GTC CTT G - 3' for IFNB. For EV71 VP1 qPCR, the following primers were used: 5' - GCT CTA TAG GAG ACA GTG TGA GTA GGG - 3' and 5' - ATC TCA GCA GCT TGG AGT GC - 3'. Gene expression was normalized to the housekeeping gene GAPDH using the primers 5' - ACC ACA GTC CAT GCC ATC A - 3' and 5' - TCC ACC ACC CTG TTG CTG TA - 3'. The amplification was carried out on LightCycler 480 II (Roche, Switzerland) as follows: Initial activation of hot-start iTaq DNA Polymerase at 95°C for 3 min; 45 cycles of three steps: 95°C for 10 s, 55°C for 20 s, and 72°C for 10 s. At the end of the amplification cycles, melting temperature analysis was carried out by a slow increase in temperature (0.11 °C/s) up to 95 °C.

2.3.4. Surface Plasmon Resonance (SPR) Experiment

SPR experiments were performed using a ProteOn XPR36 instrument (Bio-Rad, USA) or Biacore T200 system (GE Healthcare, USA) held at room temperature (25 °C). To measure affinity for TRIM21 PRYSPRY, antibodies were immobilized to a GLC sensor chip (Bio-Rad, USA) using standard amine coupling kit. The chip was activated using a 1:1 mixture of EDAC (133 mM) to sulfo-NHS (33 mM) which was injected over the surface at a flow rate of 30 µL/min for 5 minutes. Following activation, WT/Mod 1C6 antibody (5 µg/mL in 10 mM sodium acetate buffer, pH 5.5) was injected at a flow rate of 30 µL/min for 300 minutes. The pH, protein concentration, flow rate and injection time for each ligand were optimized in order to achieve ligand densities of about 2000 RU. Sodium acetate buffer was injected in Lane 6 as a control to monitor non-specific binding to the chip surface. The surface was deactivated by

injecting 1 M ethanolamine, pH 8.5 over all lanes at a flow rate of 30 μ L/min for 300 seconds. Following deactivation, the surface was washed with 10 mM glycine, pH 2 (flow rate = 100 μ L/min for 18 seconds) in order to remove any non-covalently bound protein on the surface. Finally, the running buffer was switched to phosphate buffered saline (PBS), pH 7.4, 0.05% Tween-20 (PBST) and the surface was allowed to equilibrate before injecting the analytes. To determine the affinity, injections of five concentrations of the human and mouse PRYSPRY protein (100, 50, 25, 12.5, 6.25 nM) over the antibody-immobilized GLC sensor chip (flow rate = 100 μ L/min for 60 seconds), followed by a dissociation time of 10 minutes. To measure affinity for human Fc γ R1 (recombinant CD64 protein; Sino Biological, China), antibodies were immobilized to a CM5 sensor chip (GE Healthcare, USA) by standard amine coupling kit using the Biacore Wizard immobilization function (immobilization target level = 1000 RU). 2-fold serial dilutions of analytes (20, 10, 5, 2.5, 1.25 nM) were injected in triplicate binding cycles over the antibody-immobilized CM5 sensor chip (flow rate = 100 μ L/min for 60 seconds), followed by a dissociation time of 10 minutes and the surface was regenerated with 10 mM glycine buffer, pH 2.7 (flow rate = 100 μ L/min for 18 seconds). To measure affinity for neonatal Fc receptors (FcRn), the experiment was performed on Biacore T200 system as described [284]. Briefly, the interaction analysis was done in running buffer with lower pH – 10 mM sodium phosphate pH 5.8, 150 mM NaCl supplemented with 0.05% Tween-20. The analytes, recombinant human and mouse FcRn (Sino Biological, China), were prepared by buffer-exchanging them into the running buffer using Zeba spin columns (Thermo Fisher Scientific, USA). 3-fold serial dilutions of analytes (hFcRn – 180, 60, 20, 6.6, 2.2 nM; mFcRn – 60, 20, 6.6, 2.2, 0.73 nM) were injected in triplicate binding cycles over the antibody-immobilized CM5 sensor chip (flow rate = 30 μ L/min for 180 seconds) with mild regeneration buffer (100mM Tris pH 8.0; flow rate = 30 μ L/min for 180 seconds).

All sensorgrams were double-referenced against reference flow cell (no antibody) and a buffer injection, and fitted globally to a 1:1 Langmuir binding model from which the dissociation constants (K_D) were calculated.

2.3.5. Flow Cytometry

Flow cytometry was performed on BD FACSCanto II Flow Cytometer (Becton Dickinson Biosciences, USA) using FACSDiva software. 1×10^5 moDCs were stained with anti-human monoclonal antibodies specific for CD80 (2D10, Biolegend, USA), CD86 (2331 (FUN-1), Becton Dickinson Pharmingen, USA), CD206 (15-2, Biolegend, USA), HLA-DR (LN3, Biolegend, USA), CD40 (5C3, Biolegend, USA), human TRIM21 (D12, Santa Cruz, USA) and human-IgG (H+L) (Thermo Fisher Scientific, USA) for 30 minutes in the dark at 4°C in PBS containing 1% FBS (FACS buffer). Co-cultured cells were stained with anti-human monoclonal antibodies specific for CD3 (SK7, Biolegend, USA), CD4 (OKT4, Biolegend, USA), CD8 (SK1, eBioscience, USA), CD56 (MEM-188, Biolegend, USA) and IFN γ (4S.B3, Biolegend, USA). For hTRIM21, hIgG and IFN γ staining, cells were first fixed and permeabilized using Fixation/Permeabilization Solution Kit (Becton Dickinson Biosciences, USA), according to the manufacturer's instructions. To enumerate absolute count, CountBright Absolute Counting Beads (Thermo Fisher Scientific, USA) were added to the samples before acquiring. For mouse BMDC experiments, 1×10^5 BMDCs were stained with anti-mouse monoclonal antibodies specific for CD80 (16-10A1, Biolegend, USA), CD86 (GL1, Biolegend, USA), MHCII (I-A/I-E) (M5/114.15.2, Biolegend, USA), CD40 (3/23, Biolegend, USA) and CD11c (N418, Biolegend, USA). For mouse T cell staining, 1×10^5 cells were stained with anti-mouse monoclonal antibodies specific for CD45 (30-F11, Biolegend, USA), CD3 (GL1, Biolegend, USA), CD8 (53-6.7, Biolegend, USA) and H-2Kb/Ova-specific Pro5® MHC Pentamer (SIINFELK) (Proimmune, England).

2.3.6. TRIM21 small interfering RNA (siRNA) knockdown and luciferase plasmid transfection

The two plasmids pGL4.32[luc2P/NF- κ B-RE/Hygro] and pGL4.74[hRluc/TK] were kind gifts from our collaborator Nivashini Kaliaperumal from Institute of molecular and cell biology (IMCB). 1×10^4 cells/well HEK293T cells were plated in antibiotic-free low glucose DMEM medium (Hyclone, USA) supplemented with 10% heat-inactivated FBS. The cells were co-transfected with 50ng pGL4.32 and 1ng pGL4.74 with or without 150 pmol TRIM21 siRNA (UCAUUGUCAAGCGUGCUGC; Dharmacon, USA), using Lipofectamine 2000 as directed by manufacturer for 24 hours in 37°C, 5% CO₂.

2.3.7. NF- κ B reporter assay

NF- κ B reporter assay is performed as previously described, but with slight modifications [187, 197]. Briefly, HEK293T cells transfected with pGL4.32, pGL4.74 with or without TRIM21 siRNA were plated in black 96 well clear bottom plate at a density of 1×10^4 cells/well prior to infection. Ad5-null was mixed with 0.3 μ g/ml WT or Mod 9C12 antibody to form immune complexes and incubated at room temperature for 1h, then used at a final concentration of 4 million infectious units/well, which translates to multiplicity of infection (MOI; Virus to cell ratio) of 400. Controls were treated with PBS only, virus only or 1ng/ml TNF- α (Miltenyi, Germany) and the treated cells were incubated at 37°C, 5% CO₂ for 7 hours. Dual luciferase assays were performed with the dual luciferase reporter assay system (Promega Corp, USA). All reagents were prepared according to the manufacturer's instructions. The 5 $\times$ passive lysis buffer was supplied by the manufacturer and used for cell lysis. Briefly, after the cells were subjected to seven hours of treatment, the supernatant is removed and the cells were gently washed once with PBS. 20 μ l of 1 $\times$ passive lysis buffer was added to each well of cells and the

culture plate was placed on an orbital shaker with gentle rocking at room temperature. After allowing lysis for 15 mins, the lysate was stored at -20°C until use. Luminescence measurements were done with Hidex Sense microplate reader (Hidex Oy, Finland). The following steps were used for luminescence measurements: 100 µl of luciferase assay reagent II (LARII) was added to the test sample, with a 5 seconds time for luminescence measurement and a 5 seconds interval time, followed by addition of 100 µl of the Stop & Glo reagent, 5 seconds time for luminescence measurement and a 5 seconds interval time before proceeding to the next well. The data are represented as relative luminometer units (RLU), which is the ratio of firefly to *Renilla* luciferase activity.

2.4. In vitro cellular assays

2.4.1. Antibody-dependent intracellular neutralization (ADIN) assay

ADIN assay was performed as described [252]. Briefly, for HeLa cells, 1.25×10^5 cells were seeded into 24-well plates. Immune complexes were formed by pre-incubating 1.5×10^5 Ad5-eGFP (MOI = 1.2) with WT/Mod 9C12 antibodies from 0.3 pM to 35 nM for 1 hour at room temperature. The mixture was added to the HeLa cells, which diluted it 55-fold to give a final concentration from 0.006 to 627 pM. The cells were infected for 48 hours before enumeration of eGFP expression by flow cytometry. For moDCs, 1×10^5 immature moDCs were seeded into 96-well plates. Immune complexes were formed by pre-incubating 4×10^7 Ad5-eGFP (MOI = 400) with WT/Mod 9C12 antibodies from 0.17 nM to 690 nM for 1 hour at room temperature. The mixture was added to the moDCs, which diluted it 12.5-fold to give a final concentration from 0.006 to 627 pM. The cells were infected for 48 hours before enumeration of eGFP expression by flow cytometry

2.4.2. moDC maturation assay

For anti-adenovirus antibodies, 4×10^7 Ad5-null was pre-incubated with 1.6 μ g WT/Mod 9C12 antibody for 1 hour at room temperature in PBS to form immune complexes. For negative controls, 'PBS only', 'virus only' or 'antibody only' were used. For positive control, 1 μ g/ml LPS from E. coli O128:B12 (Sigma-Aldrich, USA) was used. 1×10^5 immature moDCs were then treated with the immune complexes in 200 μ l RPMI 1640 supplemented with 10% FBS at MOI of 400 and incubated at 37°C with 5% CO₂. For anti-DEC205 antibodies, no viruses were used. 1×10^5 immature moDCs were incubated directly with 8 μ g/ml of WT/Mod anti-DEC205 antibodies in RPMI 1640 supplemented with 10% FBS and incubated at 37°C with 5% CO₂. To inspect moDC maturation, the moDCs were harvested 24 hours after immune complex treatment or antibody treatment and stained for the surface markers CD80, CD86, CD206, HLA-DR (Human Leukocyte Antigen – DR isotype) and CD40. To examine the gene

expression, the moDCs were harvested at multiple time points after immune complex treatment and RNA extraction was done with TRIzol following the manufacturer's protocol. Gene transcripts were probed using qPCR and RNA sequencing.

2.4.3. Cytokine/chemokine detection with Multiplex assay

Supernatant from the moDC maturation assay were harvested at 24-hour timepoint analysed using Human Cytokine/Chemokine Bead Panel 1 and 2, which measure a total of 64 targets (Milliplex MAP Kits; Millipore) on a Flexmap 3D System (Luminex Corp, TX). Cytokines/chemokines that showed differences following treatments with the modified IC treatment were identified and ranked by a fold change-to-dose correlation, and a Spearman rho² of 0.6 with $p < 0.05$ is used as a cut-off. For cytokines/chemokines within the cut-off, we tested the effect of the different treatments using ANOVA analysis.

2.4.4. Immunofluorescence

1×10^5 immature moDCs were resuspended in fresh medium and treated in the same way as moDC maturation assay but only for 4 hours. The cells were then washed twice with ice-cold PBS and fixed/permeabilized with ice-cold methanol at -20°C for 10 minutes. The fixed cells were washed twice with FACS buffer and stained with antibody cocktail and 1 µg/ml DAPI in FACS buffer for 1 hour at room temperature in dark. Following that, the cells were washed, resuspended in PBS and loaded onto ibidi µ-Slides (ibidi, Germany). Cells were viewed and images were taken using Olympus FV1000 Confocal microscope (Olympus, Japan). The images were analyzed on the Imaris software (Bitplane, United Kingdom).

2.4.5. Internalization and Degradation Assay with activatable fluorescence-quencher probe

Internalization and degradation of antibodies were measured with activatable fluorescence-quencher probe as described [285]. Briefly, 1.25 μ L of IRDye QC-1 NHS ester (Li-Cor, USA) at 10 mg/mL was added to 100 μ L of goat Fab anti-human IgG Fab-AF647 (Jackson ImmunoResearch, USA) at 1 mg/mL. After 2 h incubation at room temperature in the dark, the dual-labeled antibodies (quenched for AF647 fluorescence) were purified using a Zeba desalting spin column. 15×10^6 Ad5-null was pre-incubated with 0.8 μ g WT/Mod 9C12 antibody for 30 mins at room temperature in PBS to form immune complexes. To measure internalization, a 6-fold molar excess of AF647 single-labeled anti-human IgG Fab was incubated with the immune complex on ice for 30 mins. The antibody-dye mixture was then added to 1×10^5 immature moDCs and treated for 15 mins, 30 mins, 1 hour, 2 hours, 4 hours or 24 hours. At the stipulated time points, the cells were washed three times with medium to remove unbound antibody and fixed with ice-cold methanol for 15 minutes at -20°C . Fluorescence was enumerated by flow cytometry at the end. To measure degradation, 6-fold molar excess of AF647 and QC-1 dual-labeled anti-human IgG Fab and QC-1 was incubated with the immune complex prior to the experiment instead. Each experiment was done in duplicate

2.4.6. moDC: autologous CD14- PBMC co-culture assay (Adenovirus)

Treatment of moDC with MG132 is performed by incubating moDC with 10 mM MG132 in complete RPMI medium for 1 hour at 37°C , 5% CO_2 . After 1 h, the cells were washed with fresh medium to remove MG132. MG132-treated or -untreated moDCs were resuspended in fresh medium and treated in the same way as moDC maturation assay but only for 4 hours. During incubation, autologous CD14- PBMC were labeled with 10 μ M carboxyfluorescein

succinimidyl (CFSE) using the Vybrant CFDA SE Cell Tracer kit (Life technologies, USA): briefly, 1×10^7 cells were labeled in 1 ml of 10 μ M CFSE in FBS-supplemented medium for 5 mins at room temperature. Excess dye was then removed by centrifugation at 10,000 x g for 1 min and the cells were washed three times with fresh medium. After 4 h, the cells were co-cultured in fresh medium at a moDC:CD14- PBMC ratio of 1:5. At this juncture, one addition positive control CD3/CD28 agonist T Cell TransACT (Miltenyi, Germany) was introduced. The co-culture was maintained for up to 12 days with replacement of 1/3 old medium with fresh medium on days 4 and 8. Supernatant from the co-culture was collected on day 4, day 8 and day 12 to measure Interferon-gamma (IFN- γ) release, using Biolegend IFN- γ ELISA kit as per the manufacturer's instructions (Biolegend, USA). To measure T cell expansion, the co-cultured cells were harvested on day 12 and stained for the T cell markers CD3, CD4 and CD8. CD56 marker was included in the staining as well to exclude NKT cells.

2.4.7. Peptide re-stimulation assays

To prepare moDC for pulsing of peptides, autologous moDCs were generated using the same method as above from frozen monocyte stock. After 6 days, moDC were suspended in fresh medium containing 100 ng/ml GM-CSF (Miltenyi, Germany), 100 ng/ml IL-4 (Miltenyi, Germany) and 50 ng/ml tumor necrosis factor alpha (TNF- α) (Miltenyi, Germany) and pulsed with 200 μ g/ml Peptide libraries (PepTivator AdV5 Hexon or PepTivator NY-ESO-1, Miltenyi, Germany). Positive controls are a mixture of 20ng/ml phorbol 12-myristate 13-acetate (PMA) plus 1 μ g/ml ionomycin (Sigma-Aldrich, USA), or CD3/CD28 agonist T Cell TransACT. moDCs were peptide-pulsed 1 h before re-stimulation. Thereafter, the peptide-pulsed moDCs were used to re-stimulate an autologous 12-day old moDC: CD14- PBMC co-culture. Similar to the co-culture assay, the peptide-pulsed moDCs were added to the co-culture cells at a

moDC:co-culture cell ratio of 1:5, in fresh medium supplemented with 1.5 µg/ml Brefeldin A. Cells were re-stimulated for 16 hours and labeled for flow cytometry analysis.

2.4.8. Co-immunoprecipitation (co-IP)

Ad5 was preincubated with antibodies for 1 hour at room temperature in PBS to form immune complexes. Immature moDC were then treated with the immune complexes in RPMI 1640 supplemented with 10% FBS at an MOI of 200 and incubated at 37°C with 5% CO₂ for 4 h. Cells were lysed on ice with RIPA buffer containing 1% Nonidet P-40 substitute (Sigma-Aldrich, USA), 50 mM Tris-Cl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich, USA), and 10% glycerol, supplemented with Protease Inhibitor Mini Tablets (Thermo Fisher Scientific, USA), 1 mM phenylmethylsulfonyl fluoride (PMSF; Roche, Switzerland), 10 mM MG132 (Sigma-Aldrich, USA), and 20 mM N-ethylmaleimide (NEM; Sigma-Aldrich, USA). Goat Fab anti-human Fab (Jackson ImmunoResearch, USA) was coupled onto M-270 Epoxy Dynabeads (Thermo Fisher Scientific, USA), following the manufacturer's instructions in the Dynabeads Antibody Coupling Kit. Immunoprecipitations (IP) were performed by incubating cell lysates from 1.5 million cells with 1 mg of Dynabeads (prebound with anti-human Fab) overnight at 4°C. Beads were then washed with RIPA buffer, and immunoprecipitates were eluted with 0.1 M Glycine buffer (pH 2.7).

2.4.9. Quantitative mass spectrometry and gene ontology analysis

Quantitative mass spectrometry was performed on the eluates from the co-immunoprecipitation as described [286]. Quantitative tandem mass tag 10-plex (TMT10plex) labeling and tandem mass spectrometry (LC-MS/MS) was done using a nanoAcquity UPLC System (Waters, USA) coupled to an Orbitrap Elite MS (Thermo Fisher Scientific, USA). MS spectra were searched

against the UniProt human proteome database using Proteome Discoverer 2.2 (Thermo Fisher Scientific, USA) with Sequest HT. Proteins were identified at a cutoff of false discovery rate < 1% with a minimum of two unique peptides. Identified proteins showing at least two times abundance difference between ‘Mod+Virus’ and ‘WT+Virus’ were selected for gene ontology analysis using GeneGo MetaCore (Clarivate Analytics, USA)

2.4.10. Illumina RNA sequencing and calculation of Molecular Distance to Medium (MDTM)

Immature moDCs from 5 donors were resuspended in fresh medium with ‘PBS’, ‘adenovirus only’ (Virus only), ‘WT antibody + adenovirus’ (Virus+WT) and ‘modified antibody + adenovirus’ (Virus+Mod) and incubated for 4 different time points – 1 hour, 4 hours, 12 hours and 24 hours. Total RNA was extracted following the double extraction protocol: RNA isolation by TRIzol followed by a Qiagen RNeasy Micro clean-up procedure (Qiagen, Germany). All RNAs were analyzed on Agilent Bioanalyzer (Agilent, USA) for quality assessment with RNA Integrity Number (RIN) range from 7.8-9.5 and median RIN 8.7. cDNA libraries were prepared using 2ng of total RNA and 1ul of a 1:50,000 dilution of ERCC RNA Spike in Controls (Ambion® Thermo Fisher Scientific, USA) using SMARTSeq v2 protocol [287] except for the following modifications: 1. Use of 20µM TSO, 2. Use of 250pg of cDNA with 1/5 reaction of Illumina Nextera XT kit (Illumina, USA). The length distribution of the cDNA libraries was monitored using DNA High Sensitivity Reagent Kit on the Perkin Elmer Labchip GX system (Perkin Elmer, USA). All samples were subjected to an indexed PE sequencing run of 2x51 cycles on an Illumina HiSeq 2000 (17 samples/lane).

Molecular Distance to Medium (MDTM) refers to the numerical score of a sample’s transcriptional changes relative to a control, as formulated by Banchereau et al. [288]. The

score of a sample for each time point is calculated by adding the absolute fold-changes of all differentially expressed genes (DEGs), which are genes with fold change $>+/-2$ compared to the control samples for the respective time points.

2.4.11. moDC: autologous CD14- PBMC co-culture assay (Enterovirus 71)

For EV71 model, to first expand EV71-specific lymphocyte population as a reporter cell pool, immature moDCs from healthy donors were incubated with EV71-B5 at MOI 3.6 in serum-free RPMI 1640 medium for 4 hours. The moDCs were then washed and co-incubated with autologous CD14-negative cells at 1:10 ratio for 6 days in RPMI 1640 medium supplemented with 10% FBS. After 6 days, EV71-B5 was pre-incubated with WT/Mod 5H7/1C6 antibody for 1 hour at room temperature in PBS to form immune complexes. A new round of autologous immature moDCs were treated with the immune complexes in serum-free RPMI 1640 at MOI of 10 for 4 hours. The moDCs were then washed and co-incubated with the pre-expanded lymphocytes at 37°C with 5% CO₂. Supernatant from the co-culture was collected on day 4, day 7 and day 11 to measure IFN- γ release, using Biolegend IFN- γ ELISA kit as per the manufacturer's instructions (Biolegend, USA). To measure T cell expansion, the co-cultured cells were harvested on day 11 and stained for the T cell markers CD3, CD4 and CD8. CD56 marker was included in the staining as well to exclude NKT cells.

2.5. In vivo mouse experiments

2.5.1. Ovalbumin antigen model

OT-I CD8 T cells were purified from single cell suspensions of inguinal lymph nodes and spleen cells by negative selection using CD8⁺ T Cell Isolation Kit (Miltenyi, Germany) following the manufacturer's protocol. The isolated OT-I cells were labeled with 10 μ M CFSE using the VybrantTM CFDA SE Cell Tracer kit (Life technologies, USA): briefly, 1×10^7 cells were labeled in 1 ml of 10 μ M CFSE in FBS-supplemented medium for 5 mins at room temperature. Excess dye was then removed by centrifugation at 10,000 $\times$ g for 1 min and the cells were washed three times with fresh medium. 2×10^6 CFSE-labeled OT-I CD8 T cells were injected intravenously into the tail veins of 8 to 10 weeks-old TRIM21-KI mice. 24 hours later, mice were injected subcutaneously into the loose skin over the neck with OVA-conjugated WT anti-mDEC205, OVA-conjugated modified anti-mDEC205 or a mixture of OVA + modified anti-mDEC205. 14 days later, the mice were sacrificed and the spleen, inguinal lymph nodes and blood were harvested and analyzed by flow cytometry.

2.5.2. GPC3 antigen model

Figure 2 shows the schematic layout of the protocol for GPC3 antigen model. Briefly, 6 weeks-old female TRIM21-KI mice were immunized subcutaneously by injecting into loose skin over the neck with 2 μ g of mGPC3₇₃₋₁₉₅-conjugated WT anti-mDEC205 (WT vaccine), mGPC3₇₃₋₁₉₅-conjugated modified anti-mDEC205 (modified vaccine) or saline. After 28 days, each mouse was inoculated subcutaneously with 5×10^5 MC38-null on the right flank and 5×10^5 MC38-GPC3 on the left flank. Tumor size was monitored 7 days after tumor injection and recorded twice a week. On day 30 post tumor challenge, the mice were sacrificed and the spleen, inguinal lymph nodes and blood were harvested and analyzed by flow cytometry.

Test GPC3 vaccine on TRIM21-KI mice

Figure 2.2. Timeline for anti-mDEC205 vaccine conjugated to mGPC3 antigen. Three vaccines/treatments were tested in the experiment: mGPC3₇₃₋₁₉₅-conjugated WT anti-mDEC205 (Wild-type vaccine), mGPC3₇₃₋₁₉₅-conjugated modified anti-mDEC205 (Fc-mod vaccine) or Saline. On Day -28, 6 weeks old TRIM21-KI mice were injected subcutaneously with 2 μg vaccine in 100 μl saline. On Day 0, each mouse was inoculated subcutaneously 5×10^5 MC38-GPC3. Tumor size was monitored 7 days after tumor injection and recorded twice a week. On day 30 post tumor challenge, the mice were sacrificed and the spleen, inguinal lymph nodes and blood were harvested and analyzed by flow cytometry.

2.5.3. Isolation of tumor cells from mouse

Both MC38-null (right flank) and MC38-GPC3 (left flank) tumors were surgically resected from mice. Fat, fibrous and necrotic areas were removed from the tumor sample and cut into small pieces of $\sim 2\text{mm}^3$. Single cell suspensions were prepared using mouse tumor dissociation kit (Miltenyi, Germany) on gentleMACS dissociator (Miltenyi, Germany) in C tubes following the manufacturer's protocol. The dissociated tumor samples were filtered through the 70μm

MACS SmartStrainer and erythrocytes were lysed with ACK buffer (Gibco, USA). Then, cells were analyzed by flow cytometry.

3. RESULTS

3.1. Biophysical properties of antibodies used in experiment

3.1.1. Modified anti-adenovirus antibodies possess improved affinity for human TRIM21, but abolished binding for mouse Trim21

Our published data showed that the affinity of chimeric IgG1 for human TRIM21 PRYSPRY domain was improved 300-fold from 125 nM to 0.425 nM when the Fc region was modified [210]. However, its affinity for mouse Trim21 PRYSPRY has not been measured. Since mouse embryonic fibroblasts (MEF) cells were previously used to demonstrate the function of Trim21 [289], we were also interested in verifying the affinity of our modified antibodies to mouse Trim21. Having an improved affinity for mouse Trim21 will allow studies to be conducted in mouse models. We therefore repeated the affinity measurements but for both human TRIM21 PRYSPRY and mouse Trim21 PRYSPRY. In this experiment, we immobilized the antibodies as ligands, flowed human or mouse recombinant TRIM21 PRYSPRY as analyte at multiple concentrations and measured the dissociation constant (K_D) using the ProteOn™ XPR36 Surface Plasmon Resonance (SPR) System. When the Fc region is modified, the affinity of chimeric IgG1 antibody for human TRIM21 PRYSPRY domain improved more than 200-fold from 98.3 nM to 0.4 nM (Figure 3.1a, 3.1c). Conversely, SPR analysis showed the same Fc modification has completely abolished the affinity of the antibody to mouse Trim21 PRYSPRY domain (Figure 3.1b). Taken together, these results suggest that the effect of the modified antibody can be studied only on human cells but not on mouse cells.

Figure 3.1. Surface Plasmon Resonance measurements of the binding between recombinant PRYSPRY and anti-adenovirus WT/Modified antibodies. **a–b**, The sensorgrams of recombinant human or mouse PRYSPRY binding to the indicated chip-immobilized WT or Modified antibodies are expressed in RU (response unit) versus time after subtracting the control signal. The PRYSPRY concentrations were 6.25, 12.5, 25, 50 and 100 nM (from bottom to top). Colored lines, SPR data from different concentrations of the analytes; bold red lines, model fits. **c**, The measured binding kinetics of the antibodies by SPR: k_a , k_d and dissociation constant (K_D).

3.1.2. Affinity of modified antibodies on Human and Mouse Fc receptors (FcRs)

As the modified antibodies were mutated in the Fc region, it could have introduced unintended perturbations on its affinity to classical FcRs like FcγR and FcRn. The capacity to engage FcRs plays a crucial role in antibody internalization into immune cells [215] and their subsequent FcR-dependent downstream effector functions. To clarify if any differences in immune functions triggered by WT and modified antibodies were attributed to FcR binding, we examined the binding between WT/modified antibodies and recombinant FcRs.

We first compared binding between WT or modified antibody to the five classical human FcγRs: FcγRI/CD64, FcγRIIa/CD32A, FcγRIIb/CD32b, FcγRIIIa/CD16A and FcγRIIIb/CD16b using ELISA (Figure 3.2a). Other than FcγRI, the assay was not sensitive enough to capture the binding to any other FcγRs. For FcγRI binding, we were able to observe a slight attenuation to its binding upon Fc modification. However, it was difficult to pinpoint the EC50 values due to the gentle inflection point for the binding curves. We proceeded to test the affinity of WT and modified antibody to the FcγRs using SPR. Our results showed a slight but definite drop in affinity when the Fc region was modified (Figure 3.2b, 3.2c), which was measured to be approximately two times lower than WT antibodies (Mod $K_D=0.69\text{nM}$ vs WT $K_D=0.34\text{nM}$) (Figure 3.2f). It was also interesting to note that the decline in affinity was due to a higher k_d (Mod $k_d= 6.75 \times 10^{-4} \text{ s}^{-1}$ vs WT $k_d= 3.06 \times 10^{-4} \text{ s}^{-1}$). Similarly, we inspected affinity of the antibodies to recombinant human FcRn by SPR assay. Although our results showed that the overall K_D was comparable (Figure 3.2d, 3.2e), we noted that Fc modification brought about a weaker k_a (Mod $k_a= 8.32 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ vs WT $k_a= 3.55 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$) but a stronger k_d (Mod $k_d = 8.75 \times 10^{-4} \text{ s}^{-1}$ vs WT $k_d = 4.28 \times 10^{-3} \text{ s}^{-1}$). All in all, we managed to show that the Fc modification do not significantly disrupt antibodies binding to human FcγRI and FcRn; giving a less than two-fold weaker binding to FcγRI and a similar binding to FcRn.

f

Analyte	hFcγRI			hFcRn		
	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	K_D (nM)	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	K_D (nM)
WT Anti-Ad5	8.91×10^5	3.06×10^{-4}	0.34	3.55×10^5	4.28×10^{-3}	12.1
Modified Anti-Ad5	9.79×10^5	6.75×10^{-4}	0.69	8.32×10^4	8.75×10^{-4}	10.5

Figure 3.2. Fc modification does not disrupt antibodies binding to human Fc receptors.

Difference in binding to **a**, human Fc gamma receptors between WT and Modified antibodies were measured by ELISA. Recombinant Fc gamma receptors were coated and 3-fold serial dilutions of WT or Modified antibodies were added into the wells. Data are expressed as the mean + SD of duplicates. **b**, Single-cycle kinetics sensorgram for recombinant human Fc gamma receptor I (FcγRI) binding to the indicated chip-immobilized WT anti-Ad5 (blue line) or Modified anti-Ad5 (Red line) are expressed in RU (response unit) versus time after subtracting the control signal. The PRYSPRY concentrations were serially injected in the order of: 1.25, 2.5, 5, 10 to 20 nM. Data are expressed as the mean of duplicate cycles. **c**, box plot shows the inverse log K_D value of FcγRI with WT anti-Ad5 (black box) or Modified anti-Ad5 (red box) as ligand. Data are expressed as the mean of two technical replicates (n=2). Single-cycle kinetics sensorgram for recombinant **d**, human neonatal Fc receptor (FcRn) binding to the indicated chip-immobilized WT anti-Ad5 (blue line) or Modified anti-Ad5 (Red line) are expressed in RU (response unit) versus time after subtracting the control signal. The PRYSPRY concentrations were serially injected in the order of: 2.2, 6.6, 20, 60 to 180 nM. Data are expressed as the mean of duplicate cycles. Data are expressed as the mean of five technical replicates (n=5). Box plots show the inverse log K_D value of **e**, human FcRn with WT anti-Ad5 (black box) or Modified anti-Ad5 (red box) as ligand. The measured binding kinetics of the antibodies to **f**, human FcRs by SPR: association rate (k_a), dissociation rate (k_d) and dissociation constant (K_D).

3.2. Immunological effect of modified antibodies in an adenovirus model

Many publications have demonstrated WT antibodies can induce TRIM21-dependent ADIN and immune signaling pathways [165, 187, 195, 197, 247]. We hypothesized that improving antibody affinity to TRIM21 will stabilize and encourage antibody-TRIM21 complex formation, which as a result promotes a higher extent of ADIN and immune signaling. As a proof-of-concept model, we used the established antibody (9c12)-bound adenovirus model and studied the effect on ADIN and immune signaling when Fc is modified (Figure 3.1) for enhanced TRIM21-binding, [187, 251].

3.2.1. WT and Modified antibodies demonstrated similar adenovirus neutralization efficacy in HeLa cells and moDCs

TRIM21-mediated ADIN has the potential to reinforce proteasomal degradation of antigens vital in DC cross presentation by supplementing the required MHC-I-restricted peptides [290, 291]. By improving antibody binding to TRIM21, we expect TRIM21-mediated ADIN to be boosted, which could further increase the efficacy of cross presentation in antigen-presenting cells such as DCs.

To assess if improving affinity of antibodies to TRIM21 enhanced its ability in ADIN induction, we conducted the viral neutralization assay in both non-immune (HeLa cells) and immune cells (moDCs) by infecting cells with adenovirus carrying eGFP transgene (Ad5-eGFP). The percentage of resulting eGFP-expressing cells was used as a readout of viral infection by Ad5-eGFP. Intracellular viral neutralization by TRIM21 should prevent viral infection and decrease the number of eGFP-positive cells. We found that in moDCs (Figure 3.3b), modified antibody gave very similar viral neutralization efficacy as that of WT antibody, analogous to the case of HeLa cells (Figure 3.3a). The reason for using a high MOI is because of the inefficient

adenoviral infection rate on moDC, for which only 45% at 100 MOI and 82% at 400 MOI were infected without the presence of antibodies (Figure 3.3c). Overall, this suggests that improved antibody affinity to TRIM21 did not lead to enhancement in ADIN for both Hela cells and moDCs.

Figure 3.3. Improving Fc affinity to TRIM21 did not affect adenovirus neutralization in HeLa cells and moDCs. Adenovirus which expresses enhanced green fluorescent protein (Ad5-eGFP) upon infection was used to infect **a**, HeLa cells at Multiplicity of Infection (MOI) of 1.2, or **b**, moDCs at MOI of 400 with either wild type or modified anti-hexon antibodies (WT and Mod respectively) at seven different antibody concentrations. The cells were then incubated for 48 hours for the eGFP to be expressed, before enumerating the percentage of eGFP-positive cells by Flow Cytometry. Relative percentage of eGFP-positive cells in the two treatments to control with 400 MOI virus alone were then calculated and plotted as a graph. **c**, Difference in adenovirus infection rate on moDCs between 100 MOI and 400 MOI without antibodies. Data are expressed as the mean $\pm$ SD of three biological replicates (n=3).

3.2.2. Modified immune complex (IC) significantly induced moDC maturation

To understand the immunostimulatory property of modified antibody and assess its direct impact on moDC maturation, we treated moDCs with WT or modified IC, which refers to adenovirus bound with either WT or modified anti-adenovirus antibodies. We also included negative control treatments with antibodies only, adenovirus only and positive controls with 1 μ g/ml LPS. We used a comprehensive panel of maturation markers (CD80, CD86, CD206, HLA-DR and CD40) and incorporated results from 20 healthy human donors. Unlike CD206 (mannose receptor) which is downregulated upon dendritic cell maturation [292, 293], human MHC II molecule HLA-DR and the three costimulatory molecules CD80, CD86 and CD40 necessary for T cell activation are upregulated upon DC maturation [294].

Results from 20 healthy donors demonstrated significantly higher expression of CD80 ($p < 0.05$), CD86 ($p < 0.05$), HLA-DR ($p < 0.01$), and lower expression of CD206 ($p < 0.01$) in moDCs treated with modified IC ('Virus+Mod') as compared to those treated with WT IC ('Virus+WT') (Figure 3.4b). We also observed an increase in CD40 expression in modified IC compared to WT IC although this was statistically not significant when multiple comparison-corrected statistical analysis was performed. The increase in CD40 expression in modified IC is still statistically significant relative to other control treatments. Of note, CD86 expression demonstrated up to 3-fold increase in MFI when treated with modified IC relative to PBS control and a 2.5-fold increase relative to WT IC. CD86 is a critical costimulatory molecule for priming naïve T cells, which drives their activation and differentiation into IL-2- and IFN- γ -secreting T cells [295, 296]. This indicates that modified IC can possibly lead to greater T cell activation.

To understand if the moDC maturation we observed depends on Fc receptor-mediated cell entry of immune complexes, we introduced L234A, L235A (LALA mutations) onto both the WT and modified antibody Fc regions, to eliminate binding to Fc γ R [226]. IC with LALA-mutated modified antibody ('Mod LALA+Virus') displayed similar levels of upregulation for CD80, CD86, HLA-DR and CD40 and downregulation for CD206 as compared to modified IC without LALA mutation ($p>0.05$; Figure 3.4c). This suggested that Fc receptor binding may not be required for the modified IC to trigger moDC maturation. Further investigation on LALA-mutants' affinity for TRIM21 and Fc receptors can help confirm this hypothesis.

a

b

Statistical analysis was done using repeated measure of Friedman with Dunnett multiple comparison non-parametric testing

Figure 3.4. Improving Fc affinity to TRIM21 stimulated the expression of moDC maturation markers. Immature monocyte-derived DCs (moDC) from healthy human donors were incubated for 24 hours with seven different groups of treatment: ‘PBS’, ‘WT antibody only’, ‘modified antibody only’, ‘adenovirus only’, ‘WT antibody + adenovirus’, ‘modified antibody + adenovirus’ and ‘LPS’. The cells were then stained with fluorescent dye-conjugated antibodies to measure the surface expression of five dendritic cell maturation markers CD80, CD86, HLA-DR, CD206 and CD40 by flow cytometry. **a**, the panels show the histogram charts: From bottom to top - ‘PBS’, ‘WT antibody only’, ‘modified antibody only’, ‘adenovirus only’, ‘WT antibody + adenovirus’, ‘modified antibody + adenovirus’ and ‘LPS’. **b**, the box charts show the median fluorescence intensity (MFI) fold change of all treatments relative to ‘PBS’ treatment values. Dotted line across the charts represents the baseline of 1. Data are expressed as the mean $\pm$ SD of 20 biological replicates (n=20). **c**, the box charts show the MFI fold change of ‘WT antibody + adenovirus’, ‘modified antibody + adenovirus’, ‘LALA-mutated WT

antibody + adenovirus', 'LALA-mutated modified antibody + adenovirus' treatments relative to 'Virus alone' treatment values. Dotted line across the charts represents the baseline of 1. Data are expressed as the mean $\pm$ SD of 4 biological replicates (n=4). Statistical analyses were performed using Dunnett multiple comparison non-parametric testing, and the adjusted p values are represented above the lines. N.S., $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

3.2.3. Modified IC stimulates moDC to secrete Th1-associated cytokines and chemokines

Knowing that modified IC induce moDC maturation, we were interested in finding out if these mature moDCs in question can secrete Th1-polarizing cytokines and chemokines. As we have previously mentioned in chapter 1.1.6., for vaccines against cancer or viral diseases to be effective, they need to foster the development of cellular immune response in the form of CTLs.

Using multiplexing technology, we identified cytokines and chemokines which were released by moDC after 24 hours of treatment with WT or modified IC. Of which, 10 proteins: CCL-3, CCL-4, CCL-5, CCL-7, CCL22, CXCL12, TRAIL, IL-6, TNF- α and IFN- γ , fall within the cut-off fold change-to-dose correlation of Spearman $\rho^2 > 0.6$ with $p < 0.05$ (yellow box, Figure 3.5a). Although DCs generally are not known to produce IFN- γ , it has been shown that cord blood moDCs have the capacity to secrete IFN- γ , which serves to further enhance its maturation in an autocrine manner [297]. We performed Dunnett multiple comparison parametric testing and revealed that CCL3 (MIP-1a), CCL4 (MIP-1b), CCL5 (RANTES), CCL7 (MCP-3), TNF- α , IL-6 and IFN- γ production was significantly higher following moDC incubation with modified IC in comparison to other treatments (Figure 3.5b). TNF- α and IL-6 are both components of classical cocktail for DC maturation [298], which serve to further propagate maturation signal to surrounding DCs [299]. *In vivo*, early maturing DC produce a large amount of CCL3, CCL4, and CCL5, which attract leukocytes that promote a Th1 response. Notably, adenovirus itself ('Virus alone') did not induce the upregulation of the cytokines and chemokines, affirming that cytokine release was not driven by the virus.

Figure 3.5. Improving Fc affinity for TRIM21 stimulates moDC to secrete Th1-associated cytokines and chemokines. Supernatant was collected and analyzed with Luminex multiplex assay using Human Milliplex MAP Kit immunology panel 1 and 2. **a**, Heatmap shows the fold change of cytokine and chemokine concentration in modified IC treatment compared to WT IC. relative to treatment with unmodified immune complex. Of the 64 targets analyzed, 35 are shown in the figure as cytokines with significant difference or moDC-related cytokines without significant difference. The color scale is log2 and ranges from red to green color, with red

representing 1×2^5 -fold upregulated and green representing 1×2^5 -fold downregulated expression. The cytokines and chemokines are ranked by the Spearman rho correlation (for fold change versus dose). The top 10 cytokines and chemokines (yellow box) are within the cut-off of Spearman $\rho^2 > 0.6$ at $p < 0.05$. **b**, Concentration of the seven chemokines and cytokines that are significantly upregulated by modified IC: CCL3, CCL4, CCL5, CCL7, IL-6, TNF- α and IFN- γ . Each donor is represented by a circle. Statistical analyses were performed between treatments with lines using Dunnett multiple comparison parametric testing, and the adjusted p values are represented above the lines. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2.4. Modified IC-treated moDCs promoted autologous T cell activation and expansion

One of our objectives was to find out if moDCs treated with modified IC can activate T cells. Antigen-specific CD4 T cells support and enhance immune response by cytokine production while antigen-specific cytotoxic CD8 T cells eradicate infected cells [300]. Of the cytokines secreted by activated T cells, IFN- γ is one of the more important immunomodulatory factors. It has pleiotropic effects on both immune and non-immune cells and is vital in the control of many infectious diseases, including mycobacteria tuberculosis, respiratory syncytial virus (RSV) [301] and human papillomavirus (HPV) [302].

To gauge the level of cellular immunity stimulated by our WT and modified antibodies, we incubated moDCs treated with WT or modified IC and co-cultured them with CFSE-labeled autologous CD14⁺ PBMCs for up to 12 days. On day 4, 8 and 12, supernatant was collected to measure IFN- γ release by the co-culture lymphocytes containing T cells. We found that modified IC treatment ('Virus+Mod') stimulated significantly higher IFN- γ secretion in the co-culture, reaching its peak concentration on day 8 with ~2-fold higher IFN- γ levels as compared to WT IC treatment (Figure 3.6a, $p < 0.0001$). After 12 days, we harvested the cells and gated for proliferated T cells (singlets/live/CD3⁺CD56⁻/CD4⁻CD8⁺/CFSE⁻). Flow cytometry results showed that modified IC treatment significantly increased the absolute count of proliferated CD8 T cell by ~3.5 times higher as compared to the negative controls (Figure 3.6b, c). Although the absolute count of proliferated CD4 T cell (singlets/live/CD3⁺CD56⁻/CD4⁺CD8⁻/CFSE⁻) was also ~2 times higher in modified IC treatment as compared to the negative controls, statistical analysis showed that it was not a significant increase relative to WT IC treatment. Altogether, experimental data from 13 donors demonstrated significant CD8 T cell expansion by modified immune complex treatment relative to WT immune complex treatment, but not for the case of CD4 T cell expansion (Figure 3.6d).

From this experiment, we managed to show that modified antibody which has higher affinity to TRIM21, generated mature moDCs potent enough to stimulate IFN- γ release from autologous lymphocytes and indirectly promoted CD8 T cell expansion.

a

IFN-g release

b

c

d

Figure 3.6. Immune complex with modified antibody promoted CD8 T cell expansion. **a**, IFN- γ concentration of supernatant collected on day 4, 8 and 12 from moDC:CD14- PBMC co-culture cells were measured by ELISA. Data are expressed as the mean + SD of three replicates. **b**, Histogram showing CFSE staining of CD4 and CD8 T cells from the co-culture on Day 12: From bottom to top - 'PBS', 'WT antibody only', 'modified antibody only', 'adenovirus only', 'WT antibody + adenovirus', 'modified antibody + adenovirus', 'LPS' and CD3/CD28 agonist 'TransACT'. **c**, Absolute number of CD4 T cells and CD8 T cells in the co-culture after 12 days. Each dot is a replicate of the experiment. Error bar shows the SEM of the replicates. Statistical analyses were done using one-way ANOVA multiple comparison parametric testing. **d**, Fold change in CD4 T cell count and CD8 T cell count of 'Virus only', 'WT antibody + adenovirus' and 'modified antibody + adenovirus' treatments, relative to 'PBS' treatment for 13 donors (n=13). Each dot represents one donor. Statistical analyses were performed using Dunnett multiple comparison non-parametric testing. The adjusted p values are represented above the lines. N.S., $p > 0.05$, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

3.2.5. Antigen-specific CD8 T cells were promoted by modified antibody

Our third objective is to find out if moDCs treated with modified IC can activate antigen-specific T cells. To verify if the CD8 T cell response was antigen-specific, we re-stimulated day 12 moDC:CD14-PBMC co-culture, in which the moDCs were treated with modified IC on day 0, with another batch of autologous moDC. This time round, the moDCs were treated with TNF- α and pulsed with Ad5 hexon protein 15-mer peptide library. The negative controls were pulsed with no peptides or an irrelevant protein NY-ESO-1 peptide library, whereas the positive controls were PMA/Ionomycin and CD3/CD28 agonist (TransACT) which indiscriminately activates all T cells. The cells were incubated for 16 hours with brefeldin A to block the release of IFN- γ as the activation goes on.

Upon re-stimulation with moDCs pulsed with Ad5 Hexon peptide Library, there was a significantly larger CFSE-IFN- γ ⁺ CD8 T cell population relative to the negative controls (1.84% vs 0.46%; ~3.5-fold, $p < 0.0001$), revealing an increase in adenovirus-specific CD8 T cell population (Figure 3.7). Unlike CD8 T cells, the IFN- γ ⁺ population in CD4 T cells are not adenovirus-specific (Figure 3.8). The positive control PMA/Ionomycin attest to the fact that CD4 T cells are capable of responding to stimulation and are not stupefied by overnight treatment with brefeldin A. Taken together, we have demonstrated *in vitro* the ability of modified antibodies in indirectly activating cellular immunity.

Figure 3.7. Immune complex with modified antibody promoted adenovirus-specific CD8 T cell expansion. **a**, Co-culture cells harvested from wells treated with Virus+Mod antibody was re-stimulated for 16 hours with autologous moDC pulsed with Ad5 Hexon or NY-ESO-1 peptide Library, which are a collection of 15-mer peptides of the respective proteins, in the presence of Brefeldin A. Cells were then stained for flow cytometry analysis. The cells in the gate are CD8 T cells (singlets/live/CD3+CD56-/CD4-CD8+). Figure shows the results of all triplicate wells. **b**, Frequency of CFSE-diluted IFN- γ + CD8 T cells after re-stimulation. **c**,

Absolute cell count of CFSE-diluted IFN- γ ⁺ CD8 T cells after re-stimulation. Error bar shows the SEM of the replicates. Statistical analyses were done using one-way ANOVA multiple comparison parametric testing, and the adjusted p values are represented above the lines. N.S., $p>0.05$, **** $p<0.0001$.

Figure 3.8. Immune complex with modified antibody did not promote adenovirus-specific CD4 T cell expansion. **a**, Co-culture cells harvested from wells treated with Virus+Mod antibody was re-stimulated for 16 hours with autologous moDC pulsed with Ad5 Hexon or NY-ESO-1 peptide Library, which are a collection of 15-mer peptides of the respective proteins, in the presence of Brefeldin A. Cells were then stained for flow cytometry analysis. The cells in the gate are CD4 T cells (singlets/live/CD3+CD56-/CD4+CD8-). Figure shows the results of all triplicate wells. **b**, Frequency of CFSE-diluted IFN- γ + CD4 T cells after re-stimulation.

c, Absolute cell count of CFSE-diluted IFN- γ ⁺ CD4 T cells after re-stimulation. Error bar shows the SEM of the replicates. Statistical analyses were done using one-way ANOVA multiple comparison parametric testing, and the adjusted p values are represented above the lines. N.S., $p > 0.05$.

3.3. Mechanistic study of the activity of modified Abs on DCs

We designed a series of experiments to help us understand the inner workings of how modified antibody mediates its immunostimulatory action on DCs. To do that, we first addressed the importance of TRIM21 as a mediator in augmenting moDC maturation. Although the modified antibody was engineered with the intention to improve its affinity to TRIM21, the mutation might have also introduced unexpected perturbations in its interaction with other intracellular factors.

3.3.1. Co-immunoprecipitation of antibodies confirmed intracellular interaction between TRIM21 and antibodies

To validate the presumption that antibody enters moDC and interacts with cytoplasmic TRIM21, we lysed the moDCs after 4 hours of IC treatment and performed co-immunoprecipitation (co-IP) using magnetic beads conjugated with anti-human Fab. We made sure to include extra washes (5 times PBS wash) before cell lysis to thoroughly remove antibodies which were not internalized. Western blot analysis of eluate fractions from lysates of IC-treated moDCs showed that both WT and modified antibodies were indeed binding to TRIM21 protein in the cell (Figure 3.9). We also noticed an apparently higher amount of modified antibody as compared to WT antibody, in the lysates and eluates of the treated samples. However, western blot does not give an accurate quantification of the antibodies and only provides limited information on modified antibody's interactome within the cell. To delve deeper on the amount of antibody within the cells and what modified antibody binds to, we proceed to analyze the eluate samples with quantitative mass spectrometry.

Figure 3.9. Co-immunoprecipitation of antibodies in moDCs 4 hours after immune complex treatment (Figure 2D from [210]). Western blot detection for human Fab (hFab) and TRIM21 in the lysate (L), unbound (U) or eluate (E) fractions of untreated or immune-complex treated moDC following co-immunoprecipitation with beads conjugated with anti-human Fab. GAPDH was used as housekeeping control. Results shown is from Donor C103, which is representative of 5 independent experiments with separate donors.

3.3.2. Internalized modified antibody interacted with ribosomal proteins and ISG15 ligase TRIM25

Since the modified antibody exhibits stronger binding to TRIM21 (Figure 3.1), we expected a quantitative difference in TRIM21 protein bound to modified antibody, relative to WT antibody (i.e. modified antibody binds to more TRIM21 protein compared to WT antibody). However, the Western Blot assay was not sensitive enough to accurately compare the protein levels in the samples. In addition, we were unsure if the Fc modification have led to any qualitative difference in protein interactome which could have contributed to the eventual moDC maturation. To answer both questions, we performed quantitative proteomic analysis on the protein eluates which were pulled down from immune complex-treated moDCs. The protein abundances were normalized by iRT (indexed retention time) peptide standards, which was added to each sample according to the number of cells lysed. First, we found that TRIM21 protein level was 2.6-fold higher in the co-immunoprecipitates with the 'Mod+V' treatment as compared to that in the 'WT+V' treatment (Figure 3.10c). Next, we identified a total of 228 proteins with more than two-fold difference in abundance between 'WT+Virus' and 'Mod+Virus' treatment. The proteins regulate cell metabolism (31.14%), translation (26.75%), transport (15.79%), immune defense (6.14%) and other biological functions (20.18%) (Figure 3.10a). Gene ontology analysis revealed that the list was enriched with RNA-binding proteins and ribosomal proteins (Figure 3.10b), suggesting that modified antibody might have participated in the regulation of protein translation. What is of particular interest to us are the proteins involved in immune defense (13 upregulated and 1 downregulated; Figure 3.10a). We found that both TRIM21 and its fellow TRIM family member, TRIM25, were pulled down in higher abundance with modified antibody (Figure 3.10a). In fact, TRIM25, which is known to activate the anti-viral RIG-I/MAVS pathway and immune activation [303], showed a much higher relative abundance to 'PBS' treatment in 'Mod+Virus' treatment than

TRIM21 (TRIM25 – 32x vs TRIM21 – 2.6x when normalized by iRT peptide standard; Figure 3.10c). This implies that modified antibody might also have improved binding for TRIM25, which promoted immune activation and moDC maturation. As this result was found towards the end of the project, future investigation on TRIM25's involvement will be proposed and described in the discussion section. Interestingly, we observed that the antibody protein signal was stronger for modified antibodies than WT antibodies in both western blot and mass spectrometry (albeit only to a small extent of 1.5-fold for mass spectrometry; Figure 3.10c), indicating a greater amount of modified antibodies than WT antibodies in the cells. This suggested that the modification on Fc might have introduced an auxiliary advantage in retainment within cells.

a **Proteins with 2x abundance difference between Mod+V and WT+V treatments**

b **Molecular Functions**

Figure 3.10. Proteomics and gene ontology analysis of co-immunoprecipitates with internalized antibodies. **a**, pie chart shows the 228 proteins exhibiting at least 2-fold difference in abundance between ‘Mod+Virus’ and ‘WT+Virus’ treatments ($\uparrow$: Mod+Virus > WT+Virus; $\downarrow$: Mod+Virus < WT+Virus). The proteins were categorized based on their biological functions and the 14 proteins involved in ‘Immune Defense’ were listed. The percentage of proteins under each biological function category is shown. **b**, bar chart shows the top 10 molecular functions with the lowest p-value, enriched in the 228 proteins. **c**, Normalized abundance of TRIM21 and TRIM25 relative to ‘PBS’ treatment in ‘WT+Virus’ and ‘Mod+Virus’ treatments. For antibody, the bar graph shows the normalized abundance of ‘Mod+Virus’ treatment relative to ‘WT+Virus’ treatment.

3.3.3. Modified antibody was strongly retained in moDCs after IC treatment

Since co-IP results showed higher intensity of modified antibodies in moDCs as compared to WT antibodies following IC treatment, we went on to gauge antibody internalization using flow cytometry and immunofluorescence. After 24 hours of incubation with WT or modified IC or other control treatments, moDCs were fixed and permeabilized before staining for intracellular antibodies. Flow cytometry result revealed that modified antibodies were significantly retained upon modified IC treatment relative to other controls (Figure 3.11a, b). This is supported by the immunofluorescence data (Figure 3.11c) which showed strong intracellular staining for antibodies in modified IC ('Virus+Mod') treatment. It is particularly interesting to see a dispersed and speckled distribution of antibodies in moDC treated with WT IC ('Virus+WT'), whereas for the case with modified IC, antibodies come across as being clumped and accumulated evenly throughout the cell. However, we do note that the degree of difference in antibody amount measured in the mass spectrometry results (1.5-fold; Figure 3.10c) was incongruous to that displayed in the immunofluorescence images (Figure 3.11). This will be inspected with repeat experiments in the future. Nonetheless, all results pointed to the fact that more modified antibody than WT antibody was retained within the moDCs.

In view of this new data, it appears that modified antibody might have initiated moDC maturation not only by stronger interaction with TRIM21, but also by stronger influx of antibodies which further amplified TRIM21-antibody interaction. This raises the question of whether the modified antibody retainment was the result of faster rate of internalization, or slower rate of degradation. To measure internalization rate, we pre-incubated immune complexes with single-labeled anti-IgG Fab-AF647 and track the amount of internalized antibody by enumerating with flow cytometry. For measuring degradation rate, we followed the protocol by Li et al. [285] by pre-incubating the immune complexes with a dual-labeled

anti-IgG Fab-AF647 conjugated with the quencher dye QC-1. QC-1 is a non-fluorescent, dark quencher dye that quenches fluorescence at approximately the wavelength of 550–950 nm by Förster resonance energy transfer (FRET). The dual-labeled antibody does not emit fluorescence outside the cells. Signal can only be detected following internalization and subsequent degradation to separate the AF647 dye and the quencher. We went on to incubate the moDCs with the indirectly-labeled immune complexes for 6 different time points from 15 mins to 24 hours. Our result shows that modified antibody promoted more rapid internalization compared to WT antibody throughout the entire course of 24 hours (red line with shaded circle vs blue line with shaded circle; Figure 3.11d). In fact, immune complex with modified antibody also displayed a slightly faster rate of degradation than immune complex with WT antibody (red line with hollow circle vs blue line with hollow circle; Figure 3.11d), but given the much lower degree of increment compared to internalization, it is most likely brought about by the presence of more internalized antibodies to be degraded. This allows us to safely conclude that rapid internalization was the reason for the antibody retainment.

Altogether, our data revealed that modified antibody might have initiated moDC maturation not only through stronger interaction with TRIM21, but also by increased entry of antibodies which further amplified TRIM21-antibody interaction.

Figure 3.11. Modified antibodies improved immune complex internalization rate into moDCs. **a, b, c,** Immature monocyte-derived DCs (moDC) from healthy human donors were incubated for 24 hours with seven different groups of treatment: ‘PBS’, ‘WT antibody only’, ‘modified antibody only’, ‘adenovirus only’, ‘WT antibody + adenovirus’ and ‘modified antibody + adenovirus’. Following that, the cells were fixed and permeabilized with methanol,

then stained with fluorescent dye-conjugated anti-human Fab to measure the amount of antibody internalized into the moDCs. **a**, the histogram shows from bottom to top - 'PBS', 'WT antibody only', 'modified antibody only', 'adenovirus only', 'WT antibody + adenovirus' and 'modified antibody + adenovirus', **b**, the bar graph illustrates the distribution of antibody Fab median fluorescence intensity (MFI) for all treatments at 4-hour timepoint. Data were derived from 3 technical replicates. Statistical analyses were performed using one-way ANOVA multiple comparison parametric testing, and the adjusted p values are represented above the lines. $p > 0.05$, * $p < 0.05$, *** $p < 0.001$. **c**, Immunofluorescence staining of DAPI, TRIM21 and antibody in moDCs for different treatment. Cells were directly labeled using AF488-conjugated anti-TRIM21 (green) and AF647-conjugated anti-human Fab (red) antibodies; nuclei were stained blue with DAPI. Images taken using confocal microscopy images were analyzed and processed on the Imaris software. For each channel and each field of view, laser intensity, photomultiplier gain and offset were individually modified to optimize the signal/noise ratio. **d**, moDCs were incubated for 6 timepoints: 15 mins, 30 mins, 1h, 2h, 4h and 24h with 'WT antibody + adenovirus' or 'modified antibody + adenovirus' which were pre-labeled with anti-IgG Fab-AF647 (measure internalization) or anti-IgG Fab-AF647-QC1 (measure degradation). MFI values of duplicate wells are plotted, and error bars represent mean $\pm$ SD.

3.3.4. TRIM21 is imperative in NF- κ B signaling by internalized antibodies and modified antibodies induced higher levels of NF- κ B signaling than WT antibodies

Since NF- κ B signaling is mediated by IgG-TRIM21 interaction [195-197] and plays a crucial role in DC maturation [198, 231-233], we deduced that modified antibody with increased affinity to TRIM21 could have influenced moDC maturation through escalated NF- κ B signaling. To make sure that any changes in NF- κ B signaling levels is solely the result from IgG-TRIM21 interaction, we knocked down TRIM21 in HEK293T cells and tested the capacity of WT and modified IC to induce NF- κ B activation. Ad5 alone, or in complex with WT or modified antibody, was added to HEK293T cells pre-transfected with NF- κ B luciferase reporter vector and TRIM21 siRNA or mock siRNA. Western blot results show that TRIM21 was unreservedly knocked down when transfected with TRIM21 siRNA (Figure 3.12a). Ad5 alone did not stimulate signaling, while both WT and modified IC induced an increase in NF- κ B activity, with modified IC promoting significantly higher signaling levels than the other two treatments (Figure 3.12b). When TRIM21 expression was knocked down, NF- κ B upregulation by IC treatment was completely abolished, hinting at the indispensability of TRIM21 for antibody-triggered NF- κ B signaling (Figure 3.12b). As a side note, WT or modified antibodies alone did not stimulate any increase in NF- κ B activity (data not shown). In part of our preliminary data, we also studied the levels of in NF- κ B downstream gene transcripts: BCL2A1, BIRC3, CCL5, CXCL10 and IFNB [304-308] and found that they are upregulated in modified IC-treated moDCs (supplementary figure 4). To sum it up, TRIM21 is required for NF- κ B signaling by both WT and modified antibodies, and increasing Fc affinity to TRIM21 further boost the signaling levels. Taking a step further to give a more detailed picture of the mechanism, we went on to assess the overall gene expression profile of moDCs stimulated by modified IC. The data can guide us to screen for implicated genes and pathways that we might not have expected to influence its phenotypic difference.

Figure 3.12. TRIM21 is essential for NF-κB upregulation by modified antibody in HEK293T cells. **a**, Western blot detection for TRIM21 in the lysate of HEK293T cells following treatment with no transfection (UT), lipofectamine transfection without siRNA (No siRNA), lipofectamine transfection with negative control siRNA (Mock siRNA) and TRIM21 siRNA (T21 siRNA). **b**, Fold change in NF-κB induction relative to untreated in response to Ad5 infection (Virus Alone), immune complex with WT antibody (Virus+WT) or immune complex with modified antibody (Virus+Mod) in HEK293T NF-κB reporter cells pre-transfected with Mock or TRIM21 siRNA. Error bars represent mean $\pm$ SD of seven experiments. Statistical analyses were performed using Wilcoxon non-parametric T test, and the adjusted p values are represented above the lines. * $p < 0.05$.

3.3.5. Modified immune complex induced drastic transcriptional changes in human moDCs

Having established that modified IC stimulates TRIM21-mediated NF- κ B signaling (Figure 3.12), we further characterized the function of our modified antibodies by defining the transcriptional changes elicited in moDC over time. To this end, immature moDCs from five healthy donors (Donor 118, 119, 120, 123 and 124) were treated *in vitro* for 1, 4, 12 and 24 hours to PBS, adenovirus only (Virus), WT IC (WT+V) or modified IC (Mod+V). All selected donors displayed an increase in surface CD86 marker upon modified IC treatment (Supplementary Figure 5). One-way ANOVA multiple comparison analyses between all four conditions (6 comparisons of the form B|A where A and B can be one of PBS, Virus, WT+V, Mod+V) yielded a total of 7495 differentially expressed genes (DEGs) across all four time points. Using these genes, a heatmap was generated using log₂ transcripts per million (log₂TPM) values, by grouping the data according to time point, followed by treatment and lastly by donor (Figure 3.13a). For each time point, the data was normalized by deducting log₂(Treatment) with log₂(PBS control). Thus, the data shows log₂ fold change relative to PBS. Figure 3.13a shows the overview of gene expression profiles arranged by hierarchical clustering.

From 4 h time point onwards, modified IC treatment began to stimulate a more dramatic transcriptional difference relative to PBS control in comparison to Virus and WT IC. This variance among the treatment groups is nicely represented by principal component analysis (PCA; Figure 3.13b), which we observed that at 1 h time point there is little effect of treatment on gene expression. Yet, as time progresses, the treatment effect becomes stronger, with samples in the same treatment group positioned at close proximity from each other at 4, 12 and 24 h, indicating that gene expression is increasingly dependent on the treatment. To quantitate

the global transcriptional difference between each stimulus at various timepoint, we calculated the molecular distance to medium (MDTM) formulated by Banchereau et al [288]. MDTM is the sum of absolute transcriptional fold changes compared to PBS control for all differentially expressed genes. MDTM can be interpreted as the numerical representation of a sample's overall difference in transcriptional kinetics relative to the PBS-treated control for each time point. In simpler terms, the higher the MDTM, the more the transcriptional difference is between the treated sample and the PBS control. From the calculated MDTM, moDCs demonstrated the highest magnitude of transcriptional response to modified IC relative to the controls for all the time points (Figure 3.13c). From the result, we deduced that the combination of better quality and quantity in TRIM21 binding, fueled by higher TRIM21 affinity and stronger retainment of modified antibodies respectively, led to the huge transcriptional disparity between WT IC and modified IC.

Figure 3.13. Modified immune complex induced drastic transcriptional changes in human moDCs. **a**, Heatmap of the 7495 genes differentially expressed in moDCs stimulated *in vitro* with PBS, adenovirus alone (Virus), WT immune complex (WT+V) or modified immune complex (Mod+V) for 1, 4, 12 or 24 hours. Log2 Fold change of transcript per million (TPM) relative to PBS control was represented as red (positive) or blue (negative) color as shown by the color scale. **b**, Principal component analysis of the 7495 genes differentially expressed in this data set, colored by moDCs with different treatment for 1, 4, 12 and 24 hours. Samples of the same treatment were marked out in dotted circles if they were at close proximity to each other. **c**, Bar graph representing the mean molecular distance to medium (MDTM) value induced by the three treatments at all time points.

3.3.6. Modified immune complex induced upregulation of proinflammatory transcriptional response while promoting asynchronous metabolic pathway relative to PBS control

Using the RNA-seq data, we zoomed in to examine the critical pathway/mechanism which delineated modified IC and WT IC in the stimulation of moDC maturation. We identified a total of 634 DEGs between the two treatments across all time points and plotted them as a heatmap (Figure 3.14a). We grouped the genes into two clusters and inspected their biological processes by gene ontology (GO) enrichment analysis. Figure 3.14b shows five gene function of interest and their p-value associated to each cluster. Unsurprisingly, cluster 1, which was upregulated for modified IC treatment, was enriched with genes pertaining to inflammation and immune-related responses. This includes NF- κ B, TNF and IFN signaling responses (Figure 3.14b). The upregulation of these pathways was barely noticeable at 1h and 4h timepoint, but exponentially surged at 12h and 24h time point. This implied that the supplementary signaling activity by modified antibody occurred only after the 4h time point. I suspect that the lapse of time required for the change could very well be the duration needed for overwhelming quantity of modified antibodies to enter the cell, which in this case was limited by the rate of adenovirus infection.

Interestingly, for cluster 2, relative gene expression of treatments to PBS control were pendulating between positive and negative values as time progressed. The oscillation seemed to be systematic, with modified IC treatment consistently exhibiting a more dramatic swing in relative gene expression. The issue was further complicated by fluctuating transcript levels in PBS control, meaning that transcript levels were also undulating in untreated cells. Gene cluster 2 is generally involved in non-immune functions, such as regulation of metabolic processes, transcription initiation, signaling, ion transport and localization (Figure 3.14b). To give a more

in-depth view of the gene expression profile, the transcript per million (tpm) values of three representative DEGs from each cluster are shown in Figure 3.14c. All three genes stated for cluster 1 likely contributed to maturation in modified IC-treated moDCs: C-X-C Motif Chemokine Ligand 10 (CXCL10) is an inflammatory chemoattractant regulated by both IFN- γ and NF- κ B pathways, which recruits T cells, eosinophils, monocytes and NK cells [309]. Interferon-Induced Protein with Tetratricopeptide Repeats 1 (IFIT1) is an antiviral protein regulated by IFN pathway, which sequesters viral nucleic acids to defend the cell from infection, and also reciprocally modulates additional inflammatory pathways [310, 311]. Antigen Peptide Transporter 1 (TAP1) is the transporter protein heavily involved in the delivery of cytosolic peptides across the endoplasmic reticulum to MHC-I molecules [312]. The participation of the three genes in spurring moDC maturation is more convoluted due to the incoherent progression of gene expression levels: Ornithine Decarboxylase 1 (ODC1) is an enzyme that catalyzes ornithine to putrescine in the polyamine biosynthesis pathway, which has been implicated in tumorigenesis and attenuation of inflammatory responses in macrophages [313, 314]. Dual Specificity Phosphatase 1 (DUSP1) expression can be induced by proinflammatory stimuli like LPS, which restrains further inflammation by downregulating the mitogen-activated protein kinase (MAPK) pathway [315]. Alpha-L-Iduronidase (IDUA) regulates lysosomal degradation of two glycosaminoglycans, dermatan sulfate and heparan sulfate, by hydrolyzing the terminal alpha-L-iduronic acid residue [316]. We observed that the oscillating gene expression for cluster 2 is reminiscent of the circadian rhythm and thus, speculated that IC treatment could have threw the cadence of the biological clock off, resulting in misaligned circadian rhythm relative to PBS control. However, the expression of several genes regulating the circadian rhythm did not show significant difference between treatments at 1h and 4h timepoint time point (Supplementary Figure 6). This implied that the differential expression of these oscillating genes was not caused by the circadian genes

Figure 3.14. Modified immune complex induced an inflammatory transcriptional response but staggered the oscillation of metabolism-related gene expression relative to control. a, Heatmap of the 634 genes differentially expressed in moDCs stimulated *in vitro* with WT immune complex (WT+V) or modified immune complex (Mod+V) for 1, 4, 12 or 24 hours. The genes were arranged by hierarchical clustering and two clusters of genes were identified. Cluster 1 contains 262 genes which were collectively upregulated by Mod+V treatment. Cluster 2 contains 372 genes which their expression oscillated over time. Log₂ Fold change of transcript per million (TPM) relative to PBS control was represented as red (positive) or blue (negative) color as shown by the color scale. **b,** Gene ontology (GO) enrichment analysis of the two gene clusters. Five biological functions which were significantly enriched were shown for each cluster and ranked according to their -log(p-value). **c,** Expression of three representative genes over time is shown for each cluster. Cluster 1 – CXCL10, IFIT1 and TAP1. Cluster 2 – ODC1, DUSP1 and IDUA.

3.3.7. Transcriptional profile of modified IC-treated moDCs is analogous to PAMP-stimulated moDCs

One notable aspect of DC maturation is the distinctive functional properties it endows depending on the stimulus they experience. Steady-state dendritic cells exhibit a low surface expression of MHC-II and co-stimulatory molecules. In response to PAMPs such as LPS, they undergo immunogenic maturation which renders them capable of secreting inflammatory cytokines and subsequently induce the antigen-specific effector T cell activation. On the other end of the spectrum, constitutive maturation [317, 318] can increase MHC-II expression as well, but grants tolerogenic properties to DCs instead. Tolerogenic DCs stimulated in this case preferentially drive the generation of Tregs instead of effector T cells, which results in tolerance against the primed antigen [35, 36]. Dalod and colleagues observed that PAMP-stimulated moDCs which have underwent immunogenic DC maturation, express a list of genes which were typically upregulated or downregulated [319]. They were termed ‘Core Up’ and ‘Core Down’ genes respectively (Supplementary Table 1). To distinguish whether moDC maturation promoted by modified IC belonged to immunogenic or tolerogenic maturation, heatmap showing the relative expression of ‘Core Up’ and ‘Core Down’ genes to PBS control at 24 h time point was generated (Figure 3.15). Overall, the course of gene expression by modified IC-treated moDCs followed that of the immunogenic DC maturation, as most ‘Core Up’ genes were upregulated. Although not as apparent for ‘Core Down’ genes, but most of them were also downregulated for modified IC treatment, especially when the result was juxtaposed with that of the controls.

Figure 3.15. Modified IC stimulated moDCs to adopt a transcriptional profile resembling that of immunogenic maturation. Heatmap of the 'Core Up' and 'Core Down' genes expressed in moDCs stimulated *in vitro* with WT immune complex (WT+V) or modified immune complex (Mod+V) for 24 hours. Log2 Fold change of transcript per million (TPM) relative to PBS control was represented as red (positive) or blue (negative) color as shown by the color scale. List of 'Core Up' and 'Core Down' genes were derived from Dalod and colleagues' paper and can be found in supplementary table 1 [319]

3.3.8. CD8 T cell proliferation stimulated by modified IC-treated moDCs is dependent on proteasomal activity

Proteasome was speculated to be involved in the degradation of intracellular immune complexes and liberating unanchored polyubiquitin chains for inducing TRIM21-mediated immune signaling pathways [187, 195, 197, 247]. In view of this, we tested if the CD8 T cell proliferation observed in the 12-days modified IC-treated moDC:CD14- PBMC co-culture (Figure 3.7) was dependent on proteasomal activity. We made use of MG132, a synthetic peptide aldehyde which is well-documented as a potent and cell-permeable proteasome inhibitor [320, 321], to stop proteasomes from activating. MG132 does not only block the proteasomal degradation of substrate proteins, but also prohibits the disassembly of polyubiquitin chains by the proteasome-associated deubiquitylating enzymes. Therefore, polyubiquitin chains cannot be released by substrate proteins under the influence of proteasomes [322]. moDCs were pretreated with MG132 for 1 hour, washed, treated with modified IC or other controls for 4 hours, and then co-cultured with CFSE-labeled autologous CD14- PBMC for 11 days. Flow cytometry analysis revealed that prior treatment of moDCs with MG132 prevented the expansion of CD8 T cells, as shown by the absence of CFSE-diluted population (Figure 3.16a; population in red box). To make certain that the loss of proliferation is not because of MG132 toxicity to moDC, we analyzed moDC viability after MG132 treatment. At 22 hours post-treatment, the viability of moDC with MG132 treatment was 61% of that without MG132 treatment (Figure 3.16b). Next, we analysed the frequency of CFSE-low (proliferated) T cells with or without MG132 pre-treatment. If the remaining ~61% live moDCs were able to mediate T cell proliferation, we should observe only a congruous drop in T cell proliferation. Upon addition of MG132, we can see that for the 'Fc-mod + virus' treatment, the proliferated CD4 T cells decreased from 77% to 20%, demonstrating a 74% drop in CD4 proliferation, whereas the proliferated CD8 T cells decreased from 37% to 6%,

demonstrating 83% drop in CD8 proliferation (Figure 3.16c). The drastic drop in T cell proliferation (74% for CD4 and 83% for CD8) in contrast to the drop in viable moDCs (~39%), suggested that the CD8 T cell-priming ability of the surviving moDCs was handicapped by proteasome inhibition. This implied that proteasomal activity is vital to the enhancement of antigen cross-presentation by modified IC.

Figure 3.16. Proteasome inhibitor MG132 suppress CD8 T cell expansion by immune complex with modified antibody (Adapted from Figure 6E and Supplementary figure 3D from [210]). **a**, Histograms show flow cytometry analysis of CFSE-dilution in CD8 T cells following 11 days of co-culture with moDC. The moDCs were either pre-incubated with or without MG132 for 1 hour and then subjected to a 4-hour treatment with ‘PBS’, ‘unmodified antibody only’, ‘Fc-modified antibody only’, ‘adenovirus only’, ‘unmodified antibody + adenovirus’, ‘Fc-modified antibody + adenovirus’ or CD3/CD28 agonist ‘TransACT’. The

cells in the gate are CD8 T cells (singlets/live/CD3+CD56-/CD4-CD8+). **b**, Viability of moDCs were measured 22 hours after MG132 treatment. The cells were incubated with or without MG132 for 1 hour. After incubation, the cells were then washed and resuspended in fresh medium without MG132 and cultured for 22 hours before analysis using Live/Dead stain on flow cytometry. The graph shows the mean number of live cells from triplicate wells. **c**, Frequency of proliferated CD4 and CD8 T cells after 11 days of co-culture with treated moDCs, with or without MG132 pre-treatment. Error bars show the SEM. The number of live cells in MG132-treatment is also expressed as a percentage of the no-MG132 treatment.

3.3.9. Modified anti-human DEC205 (hDEC205) antibodies do not induce moDC maturation

So far, we have provided evidence that modified antibody trafficked into moDC by adenovirus can stimulate DC maturation and subsequently antigen-specific CD8 T cell activation. This is likely the result of upregulated TRIM21-mediated immune signaling pathways like NF- κ B, due to improved affinity for TRIM21 and extensive retainment of antibodies. Our next goal is to examine if the same effect can be observed for modified antibodies internalized by DC receptors such as DEC205. First, we probed for cell surface expression of hDEC205 on moDCs by flow cytometry to ensure the presence of receptor for the anti-hDEC205 to bind to. Unexpectedly, only ~66% of the moDCs generated in our experiment were stained positive for hDEC205 (Figure 3.17a), which was inconsistent with previous report that showed more than 90% of moDCs were hDEC205+ [323]. Despite the low hDEC205 expression, we found that both WT and modified antibodies still managed to interact with intracellular TRIM21 through co-IP experiment using magnetic beads conjugated with anti-human Fab (Figure 3.17b). We went on to incubate moDCs with titrated concentrations of WT or modified anti-hDEC205 for 24 hours, and included WT and modified antibody-adenovirus IC as positive controls. To our disappointment, anti-hDEC205 antibodies, be it WT or modified, did not demonstrate any indication of moDC maturation even at 32 ug/ml concentration, which was four times the antibody concentration used in adenovirus model (Figure 3.17c). On the contrary, the positive control treated with modified IC exhibited strong moDC maturation. To confirm that the failure to promote moDC maturation was not because of slower antibody internalization resulting in incomplete activity at 24 h timepoint, we allowed the moDCs to be incubated with the modified anti-hDEC205 treatment for a longer period of time. However, moDCs treated with modified anti-hDEC205 remained inert even after the 72 hours of incubation (Data not shown). This insinuated that modified anti-hDEC205 antibody do not promote moDC maturation. We

suspected that the lack of response could have stemmed from the difference in antibody internalization route between the adenovirus model hDEC205 model. Unlike adenovirus which has been reported to transport antibodies into the cytosol, there were no reported evidence that supports antibody entry into cytosol via the DEC205 receptor. By immunofluorescence staining, we confirmed that internalization of both WT and modified anti-DEC205 antibodies were comparatively paltry in relation to that shown in modified IC treatment (Figure 3.17d). Based on our current findings, we postulated that modified anti-hDEC205 was unable to promote moDC maturation due largely to the inefficacy of hDEC205 receptor to mediate antibody entry into cytosol. This could have stymied the retainment of antibodies within the moDC, which unexpectedly played an indispensable role in the induction of moDC maturation.

Figure 3.17. Anti-human DEC205 antibodies did not stimulate moDC maturation. a, Immature moDCs from healthy human donor were stained for cell surface DEC205 expression on ice. Representative data of 3 independent experiments is shown. Singlets/Live cell/CD11c+ cells were gated and shown in the graph. Grey histogram represents isotype control and red histogram represent moDCs stained with anti-DEC205 antibody. **b,** Western blot detection for human Fab (hFab) and TRIM21 in the lysate (L), unbound (U) or eluate (E) fractions of moDCs incubated with WT or modified anti-DEC205 antibodies for 4 hours, then co-immunoprecipitated with beads conjugated with anti-human Fab. GAPDH was used as

housekeeping control. **c**, immature moDCs were incubated for 24 hours with six different groups of treatment: 'PBS', WT adenovirus immune complex ('Virus+WT'), modified adenovirus immune complex ('Virus+Mod'), 'LPS', 'WT anti-DEC205' and 'Modified anti-DEC205'. Two-fold serial dilutions starting from 32 µg/ml concentration was performed to titrate the anti-DEC205 antibodies. The cells were then stained with fluorescent dye-conjugated antibodies to measure the surface expression of five dendritic cell maturation markers CD80, CD86, HLA-DR, CD206 and CD40 by flow cytometry. **d**, Immunofluorescence staining of DAPI, TRIM21 and antibody in moDCs for different treatments. Cells were directly labeled using AF488-conjugated anti-TRIM21 (green) and AF647-conjugated anti-human Fab (red) antibodies; nuclei were stained blue with DAPI. Images taken using confocal microscopy images were analyzed and processed on the Imaris software. For each channel and each field of view, laser intensity, photomultiplier gain and offset were individually modified to optimize the signal/noise ratio.

3.3.10. Modified anti-mouse DEC205 antibodies do not induce TRIM21-KI BMDC maturation

Since modified anti-human DEC205 do not promote moDC maturation, we went on to test if the same can be said for modified anti-mouse DEC205 on mouse DCs. To do so, we acquired the TRIM21-KI C56BL/6 mouse that has its mouse TRIM21 PRYSPRY domain sequence replaced with the human homologue. This was to ensure modified Fc which do not bind mouse TRIM21 (Figure 3.1) could seamlessly work on mouse DCs. Then, we confirmed that ~48% of CD11c⁺ BMDCs from transgenic mice expressed mDEC205 in our experiment (Figure 3.18a), which should be sufficient for us to test the immunogenicity of WT/modified anti-mDEC205. On account of the uncertainty if modified Fc can in fact induce mouse DC maturation, we applied antibody-adenovirus IC as a positive control. Hirschowitz et al., has reported that Ad5 can infect mouse BMDCs [324], which we authenticated by infecting BMDCs with Ad5 with GFP transgene (Figure 3.18b). At 400 MOI, up to half the BMDCs were successfully infected and expressed GFP. We affirmed that modified IC stimulated BMDC maturation after 24 hours of incubation, which boosted the surface expression of CD80, CD40 and MHCII (Figure 3.18c). However, both WT and modified anti-mDEC205 antibodies were ineffective in spurring any sign of BMDC maturation, similar to the case in human moDC. In addition, intracellular staining of antibodies revealed that modified IC treatment led to massive retainment of modified antibodies, achieving ~8-fold and ~10-fold antibody MFI fold relative to WT IC and WT/modified anti-mDEC205 treatments respectively (Figure 3.18c). Percentage of hFab-positive BMDCs was a staggering 87.7% after treatment with modified IC, while WT IC (30.7%), WT anti-mDEC205 (22.1%) and modified anti-mDEC205 (26.2%) were considerably lower (Figure 3.18d). The difference in antibody retainment is corroborated with immunofluorescence imaging by confocal microscopy. Detailed inspection of the images disclosed that antibodies were internalized into the BMDCs for IC treatments, whereas

mDEC205-targeting antibodies were not found within the cells but instead remained bound on the cell surface (Figure 3.19).

To summarize, modification of Fc on anti-mouse DEC205 antibodies was not able to instill the ability to stimulate mouse DC maturation, very likely due to its inability to result in antibody retainment within DCs as observed in adenovirus infection model.

Figure 3.18. Anti-mouse DEC205 antibodies did not stimulate mouse BMDC maturation.

a, Immature BMDCs from TRIM21-KI C57BL/6 mouse were stained for cell surface DEC205 expression on ice. Singlets/Live cell/CD11c⁺ cells were gated and shown in the graph. Grey

histogram represents isotype control and red histogram represent BMDCs stained with anti-DEC205 antibody. **b**, BMDCs were infected with 50, 100, 200 and 400 MOI of Ad5-GFP for 48 hours and analyzed for expression of GFP by flow cytometry. Grey filled histogram represents uninfected BMDC (0 MOI), unfilled histograms represent infected BMDCs, with blue – 50 MOI, green – 100 MOI, orange – 200 MOI and red – 400 MOI. Legend on the right shows the frequency/percentage of GFP+ cells. **c**, immature BMDCs were incubated for 24 hours with six different groups of treatment: ‘PBS’, WT adenovirus immune complex (‘Virus+WT’), modified adenovirus immune complex (‘Virus+Mod’), ‘LPS’, ‘WT anti-DEC205’ and ‘Modified anti-DEC205’. The cells were then stained with fluorescent dye-conjugated antibodies to measure the surface expression of three dendritic cell maturation markers CD80, MHCII and CD40 by flow cytometry. For intracellular staining of hFab, the BMDCs were fixed and permeabilized with methanol at -20°C before staining with fluorescent dye-conjugated anti-hFab. **d**, Histogram shows intracellular antibody staining of BMDCs after different treatments. Grey filled histogram represents PBS control. For unfilled histograms: black – Virus+WT, red – Virus+Mod, green – WT anti-mDEC205, blue – Modified anti-mDEC205. Legend on the right shows the frequency/percentage of hIgG+ cells.

Figure 3.19. Intracellular staining of antibodies in BMDCs. BMDCs were treated for 24 hours with PBS (A, B, C, D), WT anti-Ad5 + Ad5 (E, F, G, H), Mod anti-Ad5 + Ad5 (I, J, K, L), WT anti-mDEC205 (M, N, O, P) and Mod anti-Ad5 + Ad5 (Q, R, S, T). Immunofluorescence staining of BMDCs were conducted with DAPI (Blue; A, E, I, M, Q), AF488-conjugated anti-CD11c (Green; B, F, J, N, R) and AF647-conjugated anti-human Fab (Red; C, G, K, O, S). The last panel of each row shows the merged images (D, H, L, P, T). White arrows (O, P, S, T) show hFab staining on cell surface and not within the cells. Images were taken using 100x/1.40 Oil objective on Olympus FV1000 Confocal microscope. The images were analyzed and processed on the Imaris software. For each channel and each field of view, laser intensity, photomultiplier gain and offset were individually modified to optimize the signal/noise ratio.

3.4. Vaccine test of antigen-conjugated modified antibody in *in vivo* mouse model

Although modified anti-DEC205 antibodies did not stimulate DC maturation in our *in vitro* experiments, we were still interested to understand its immune effects in an *in vivo* setting. It was reported that anti-DEC205 antibodies carrying an antigen were found to be ~100-fold more efficient in activating antigen-specific T cells than isotype antibodies in mice [263]. We aimed to make use of this exceptional ability in stimulating cellular immunity *in vivo* [97, 262-264] to test if modified anti-mDEC205 antibody with improved affinity for TRIM21 can further promote antigen-specific T cell activation and tumor cytotoxicity. To do that, we immunized TRIM21-KI mice (Mouse TRIM21 PRYSPRY domain substituted with the human homologue) with anti-mDEC205 conjugated with ovalbumin or mGPC3 in the absence of adjuvants.

3.4.1. Ovalbumin-targeting to DCs by modified anti-mDEC205 induces OT-I CD8 T cell expansion in the lymph nodes

Using ovalbumin as an antigen, we have the advantage of using ovalbumin-specific CD8 T cells from the commercially available OT-I mice as a tool to measure antigen-specific CD8 T cell activation. In this experiment, we isolated CD8 T cells from OT-I mice and stained them with CFSE to track their proliferation. These CFSE-labeled CD8 T cells were then injected into TRIM21-KI mice to act as *in vivo* reporter cells. Following that, the mice were immunized with ovalbumin-conjugated anti-mDEC205 (WT/Mod anti-mDEC205-Ova) or a mixture of ovalbumin and modified anti-DEC205 (Mod Ab + Ova Mix). 14 days after administering the antibodies – blood, lymph nodes and spleen were harvested from the mice for flow cytometry analysis. Without the administration of adjuvants, we expected WT anti-mDEC205-Ova to instigate tolerogenic immune response. According to the study by Laura Bonifaz et al., the immune tolerance would wipe out the ovalbumin-specific CD8 T cells via clonal deletion by the end of two weeks post-immunization [325]. Conversely, by using modified antibody which

doubles up as an ‘adjuvant’ that encourages CD8 T cell activation, we predict it will instead provoke immunostimulatory response and preserve the survival of antigen-specific T cells.

We focused on quantifying the expansion of ovalbumin-specific OT-I CD8 T cells by gating for CFSE^{dim} CD8 T cells stained positive by H2Kb Pentamers with ovalbumin₂₅₇₋₂₆₄(SIINFEKL) peptide (singlets/live/CD3+CD8+/Pentamer+/CFSE^{dim}; Supplementary Figure 7). Our results showed that modified anti-mDEC205-Ova promoted significant increase in absolute cell count of proliferated CD8 T cells in the lymph nodes, but not in blood and spleen (Figure 3.20b). Contrary to our expectation, CD8 T cells of mice treated with WT anti-mDEC205-Ova were not entirely deleted and remained detectable. This result deviated from the previous report by Laura Bonifaz et al., but we realize the incongruity might have happened as we did not re-challenge the mice like they did, on day 12 after immunization, with ovalbumin antigen + complete Freund’s adjuvant (CFA). This additional step would have accelerated the induction of peripheral tolerance, eliminating residual OT-I T cells in WT anti-mDEC205-Ova treatment. At the same time, it might also help expand the OT-I cells and increase the number of proliferated cells in modified anti-mDEC205-Ova treatment.

Because of the small sample size in this preliminary experiment (n=6; 2 mice for each treatment) and the miniscule cell count detected, we were not fully confident of the results. It is also important to take note that the evidence for anti-DEC205 internalization remains inconclusive, which warrants further investigation to verify the case for modified anti-DEC205 antibody promoting antigen-specific CD8 T cells. However, due to COVID-19 circuit breaker, we were not able to probe further and repeat the experiment. In the future, we would conduct an in vitro mouse dendritic cell treated with anti-mDEC205-Ova : CFSE-labeled OT-I CD8 T cell experiment to measure cross presentation by the modified anti-mDEC205-Ova antibody. In addition, we would also include the ovalbumin re-challenge step to not only delete the antigen-

specific T cells for WT vaccine group, but also possibly boost the antigen-specific CD8 T cells in the modified vaccine group.

Figure 3.20. Modified anti-mDEC205-Ova expanded OT-I CD8 T cells in lymph nodes, but not in blood and spleen. CFSE-labeled OT-I CD8 T cells were injected intravenously into TRIM21-KI mice, before the mice were immunized with ovalbumin-conjugated WT anti-mDEC205, ovalbumin-conjugated modified anti-mDEC205 or a mixture of ovalbumin + modified anti-mDEC205. **a**, Histograms show the CFSE expression of singlets/live/CD3+CD8+/Pentamer+ cell population in blood, lymph nodes and spleen after 14 days: From bottom to top – Mouse 5 and 6 belong to modified anti-mDEC205 + ovalbumin mix treatment (Shaded grey - ‘Mod Ab + Ova Mix’), Mouse 1 and 2 belong to WT anti-

mDEC205-Ova (Shaded blue - 'WT anti-mDEC205-Ova'), Mouse 3 and 4 belong to modified anti-mDEC205-Ova (Shaded red - 'Mod anti-mDEC205-Ova'). **b**, Bar graphs show the absolute cell count (Top panel) and percentage (bottom panel) of proliferated CD8 T cells (singlets/live/CD3+CD8+/Pentamer+/CFSE^{dim}). Data from two mice for each treatment group are expressed in mean + SD. Statistical analyses were done using one-way ANOVA multiple comparison parametric testing, and the adjusted p values are represented above the lines. * p<0.05.

3.4.2. Modified anti-mDEC205-GPC3 immunization before tumor challenge delayed the onset of tumor growth

We decided to first test the function of modified antibody as a prophylactic/preventive cancer vaccine. We immunized TRIM21-KI mice with 2 μ g mGPC3 fragment-conjugated modified anti-mDEC205 (modified vaccine) 4 weeks before tumor inoculation to prime the immunity against mGPC3 antigen. The negative controls were WT anti-mDEC205 (WT vaccine) and saline. After 4 weeks, we challenged the mice with mGPC3-expressing mouse colon carcinoma cell line MC38 and monitored tumor growth for 28 days.

We observed that vaccination with WT vaccine accelerated tumor growth as compared to the saline control (Figure 3.21a). We suspect this is due to the stimulation of tolerogenic response DEC205-targeting vaccines in the absence of adjuvants [326]. On the other hand, modified vaccine conferred a minor protection by controlling tumor growth initially, but relented after Day 17 and allowed the tumor to match the growth in saline controls (Figure 3.21a). This result suggests that modified vaccine prompted some form of immune defense against MC38-GPC3, but was not potent enough to completely eradicate the tumor.

As we were interested to know if GPC3-expressing tumors were selectively eradicated, we sacrificed the mice on Day 30 post-tumor injection and extracted the tumors for flow cytometry to inspect GPC3 expression. Flow cytometry results showed that there was no significant difference in GPC3 and PDL1 expression among the different treatments. This implied that there was no selective killing of GPC3+ tumor cells by the immune response raised by modified vaccine. However, we noted that the tumors extracted from the mice uniformly plummeted in GPC3 expression as compared to the tumor cells in culture (~6-fold decrease in MFI; Figure 3.21c). We surmise that tumor cells with high GPC3 expression could have been spontaneously rejected by our TRIM21 C57BL/6 mouse model, or somehow down-regulated its GPC3

expression *in vivo*. Thus, the lowered GPC3 expression likely brought down the tumor-killing efficiency of any GPC3-specific CTLs promoted by modified vaccine.

We also attempted to measure the expansion of GPC3-specific T cells by MHC-peptide multimer staining. Since there were no commercial tetramers available for GPC3 peptides, we tetramerized recombinant biotinylated H2-Db monomers made by our collaborator, then exchanged their UV-cleavable peptides with the previously identified most-inducible epitope peptide in mGPC3 - mGPC3₁₂₇₋₁₃₆ AMFKNNYPSL peptide [274]. Using the tetramer, we stained for GPC3-specific CTLs in the inguinal lymph nodes, spleens and tumors extracted from mice 36 days post tumor inoculation. The results showed that the absolute cell count of tetramer+ CD8 T cells was found to be higher in lymph nodes from mice vaccinated with modified vaccine (Figure 3.22a). Despite this encouraging result, we were disappointed to find that the tetramer staining was non-specific, as it also binds to CD8-negative T cells (highlighted in red box; Figure 3.22b; Gating strategy shown in supplementary figure 8). This severely discounted the reliability of the tetramer staining results and made us decided not to use its data to evaluate the efficacy of the vaccines in expanding GPC3-specific CTLs.

Figure 3.21. Prophylactic treatment of modified vaccine moderately delayed the onset of tumor growth. TRIM21-KI mice were vaccinated with mGPC3₇₃₋₁₉₅-conjugated WT anti-mDEC205 (WT vaccine), mGPC3₇₃₋₁₉₅-conjugated modified anti-mDEC205 (modified vaccine) or saline. After 28 days, all mice were injected with MC38-GPC3 (GPC3-positive

tumor). **a**, Size of tumor up to 28 days after tumor inoculation. **b**, individual plots of mouse tumor growth for each treatment. **c**, Median fluorescence intensity (MFI) of GPC3 and PDL1 on cell-cultured MC38-GPC3 and tumors extracted from mice on Day 36. Data from all mice for each treatment group are expressed in mean + SD.

Figure 3.22. Tetramer for GPC3-specific CTLs indiscriminately stains both CD8 and non-CD8 T cells. a, absolute cell count and frequency of tetramer+ CD8 T cells in lymph nodes, spleens and tumors 36 days post tumor inoculation. The mice were vaccinated with saline (blue box) or mGPC3₇₃₋₁₉₅-conjugated modified anti-mDEC205 (modified vaccine) (red box). The staining control with no tetramers was also shown (black box). **b,** dot plot of tetramer staining for CD3+ CD8+/- T cells. Tetramer staining for non-CD8 T cells is highlighted in red box.

4. DISCUSSION

Since the initial characterization of TRIM21 function in intracellular immunity [187, 247, 327], numerous reports have emerged to support its essential role in neutralizing antigens within the cell [190-192, 289, 328]. These approaches centered on verifying the neutralization in non-immune cells by either knocking down TRIM21 itself, or eliminating antibody's affinity to TRIM21. To our knowledge, there were none that attempted to inspect the effects on immune cells and enhance TRIM21-mediated immunity by improving antibody affinity to TRIM21. We hypothesized that doing so may tap on the potential of TRIM21-mediated immunity to help improve existing immuno-therapeutics.

4.1. **Biophysical effects of Fc engineering on modified antibody**

4.1.1. **Modified antibody displayed a strong affinity for human TRIM21 but not mouse TRIM21**

In this study, we engineered the Fc sequence of human IgG1 by introducing 5 amino acid mutations in the CH2 and CH3 domain. The resulting modified antibody showed a 200-fold improvement in affinity to human TRIM21. Since we were interested in progressing to mouse model studies, we also examined its binding to mouse TRIM21. However, our SPR analysis displayed an abolished binding of modified antibodies to mouse PRYSPRY, the binding domain of TRIM21 to antibody Fc. Alignment of human and mouse TRIM21 PRYSPRY domain sequences (UniProtKB Accession Number P19474 and Q62191) revealed 84.1% sequence homology, which might have led to intricate differences in the binding site. As a result, modified antibody was able to bind human TRIM21, but not mouse TRIM21. To further scrutinize the reason, we predicted the structure of modified human and mouse antibody Fc using Robetta protein structure prediction server [329]. We then aligned the predicted models with existing protein database files uploaded for both the human (2IWG) and mouse (3zo0) Fc-

TRIM21 crystal structure (Supplementary Figure 9). Despite the mutations introduced in modified Fc, we discovered that WT and modified Fc superposed remarkably closely when interacting with human TRIM21 PRYSPRY. When interacting with mouse TRIM21 PRYSPRY, WT and modified Fc were slightly misaligned, showing structural rearrangements. More importantly, the mutated residues H433T and N434R conformed exceptionally well within the binding pocket with human TRIM21. However, when interacting with mouse TRIM21, the side chain of mutated residue N434R jut out towards the TRIM21 hotspot residue M331, infringing on the space occupied by the TRIM21 protein side chains. Non-bonded atom pairs exhibit strong repulsion at short distances. We measured the distance between Fc R434-C γ and TRIM21 M331-N ϵ to be 2.5Å, which is much lower than the general cut-off distance of 4Å [330]. This possibly led to major steric hindrances at the binding site and abolished the binding affinity of modified human Fc to mouse TRIM21.

Nevertheless, to overcome the abolished affinity to mouse trim21, we mutated the Trim21 gene sequence in the C57BL/6 mouse strain. The TRIM21 knock-in (TRIM21-KI) mice used in our mouse model experiments were generated by replacing the mouse Trim21 PRYSPRY sequence with the human homologue. This would preserve the binding between modified antibody and human PRYSPRY portion. Concurrently, it allowed the unchanged mouse RING domain, which is responsible for the ubiquitin ligase activity of TRIM21, to interact with its downstream partners of mouse origins and mediate the subsequent immune signaling responses.

4.1.2. Fc modification did not drastically affect the binding to classical human Fc receptors

As the Fc domain was mutated in modified antibody, we investigated if the binding to various Fc receptors were altered. We found that binding to Fc γ RI was slightly affected by the Fc

mutation. Since the affinity for Fc γ RI is attenuated by two-fold upon Fc mutation, any enhancement in biological/immunological response initiated by modified antibody on moDCs is not brought about by FcRn- and Fc γ RI-mediated internalization. However, we failed to measure the binding of other Fc γ Rs due to the lack of ELISA binding signal from monomeric antibodies to low-affinity Fc γ Rs. From the advice of the examiner, we learned that complexing antibodies with antigens to increase the binding signal is a pre-requisite for detectable binding to low-affinity Fc γ Rs [331]. To troubleshoot the Fc γ R-binding ELISA assay, WT or modified anti-Ad5-adenovirus immune complex will be used in the future.

4.2. Immunological effects of modified antibody in adenovirus infection model

Having confirmed modified antibody's improved binding to human TRIM21 PRYSPRY domain, we proceeded to investigate its effects on TRIM21-associated immunological functions, which are antibody-dependent intracellular neutralization (ADIN) of adenovirus particles and the induction of immune signaling pathways.

4.2.1. Increase in Fc affinity to TRIM21 improved immune signaling but not ADIN

TRIM21-mediated ADIN has been consistently demonstrated to protect the host from infection [191, 192, 289]. One of our initial rationale of generating a modified antibody was to augment the effect of ADIN. To clarify if ADIN was boosted, we compared the levels of viral neutralization evoked by WT or modified IC. Virus neutralization entails the reduction of viral infectivity and there are many ways that antibody can mediate this effect. This includes preventing attachment to receptor, prohibiting penetration through host cell membrane, interfering virus uncoating and inhibiting the process of viral replication [332]. To exclude the element of inhibition by entry-blocking, we chose to use the non-entry-blocking anti-adenovirus antibody clone 9C12 for our experiment. Confocal studies reported by Varghese and colleagues [251] has confirmed that adenoviral attachment and subsequent virus entry were not impeded by 9C12. Our results revealed that ADIN efficacy by antibodies in both non-immune and immune cells remained unperturbed with enhanced binding to TRIM21. In corroboration to our finding, Foss and colleagues reported ADIN can be mediated with suboptimal binding and was insensitive to a 100-fold drop in Fc affinity to TRIM21. To remove ADIN activity, Fc binding to TRIM21 had to be entirely abolished by introducing H433A mutation [197]. In addition, ADIN activity is also tightly regulated by the intracellular amount of TRIM21 and functionality of proteasomes [187]. In stark contrast to the unchanged rate of ADIN, it was noted that tuning down the Fc affinity to TRIM21 significantly diminished

immune signaling [197]. Likewise, we have provided multiple evidence that strengthening Fc binding to TRIM21 led to the augmentation of immune signaling pathways. For instance, TRIM21-dependent NF- κ B signaling response level was bolstered in modified IC-treated HEK293T cells (Figure 3.12) and direct downstream transcripts of NF- κ B and AP-1 pathways were upregulated in moDCs (Supplementary Figure 4). Moreover, our RNA sequencing data showed that genes associated with NF- κ B, TNF and IFN signaling pathways were upregulated by modified IC treatment (Figure 3.14). All of these pathways have been previously described by others induce DC maturation [233, 333, 334], which corroborates with the maturation of moDC seen in our result.

4.2.2. Activation of moDCs by modified IC enabled efficient cross presentation to CD8

T cells

Activation of moDCs by modified IC was verified by increased expression of cell surface CD80/CD86/CD40 costimulatory molecules and the secretion of Th1-associated cytokines and chemokines. These two criteria functioned as signal 2 and signal 3 respectively and were paramount to effective cross presentation and priming of antigen-specific T cells [335, 336]. In addition, gene expression of specific genes elucidated by RNA sequencing also closely matched the transcriptomic fingerprint of DC which had undergone immunogenic maturation. The similarity in gene expression outlook indicated an analogous functional behavior, of it being a potent stimulator of T cell response.

True enough, moDCs treated with modified IC remarkably stimulated antigen-specific CD8 T cells (Figure 3.6 and 3.7), but failed to expand CD4 T cells despite the hike in surface HLA-DR (MHC II molecule) expression (Figure 3.4). This substantiated the fact that adenovirus was not degraded by endosomal pathway, but by proteasomal-dependent ADIN which degrades

peptides for loading onto MHC I. On top of that, this process was mediated by enhanced moDC cross-presentation and not classical direct MHC-I presentation, since the adenovirus was exogenous in origin and not intracellularly expressed. Given the reaction's inadequacy in promoting antigen-specific CD4 T cell activation (Figure 3.8) and its reliance on proteasome (Figure 3.16), we construed that increasing Fc affinity for TRIM21 endowed DCs with added propensity for cross-presentation.

Having demonstrated the potential of modified immune complex to induce and enhance adenoviral-specific CD8 T cell responses, we tried to extend the work to another infectious virus model – EV71. We tested the affinity of anti-EV71 antibodies, kindly given to us from our collaborator as gifts (Supplementary Figure 1), and expressed the Fc-modified variants (Supplementary Figure 2). In the EV71 infection model, T cell expansion was similarly induced by modified IC ('EV71+Mod')-treated moDC. However, this result was muddled by the observation of T cell expansion by 'EV71 only'-treated moDC (Supplementary Figure 3b, 3c). The reason for the discrepancy in result was because, unlike adenovirus infection which suppresses the activation of DCs to induce T cell proliferation [337], EV71 infection is immunostimulatory – capable of stimulating moDC to promote T cells expansion [338]. Therefore, to investigate and show the edge of modified antibody in activating cellular immunity, we deemed that EV71 infection model was not the best alternative model to proceed with.

4.3. Mechanism of modified antibody in spurring DC cross presentation

Having confirmed that modified antibody can lead to improved DC cross presentation, mainly led by the stimulation of DC maturation, we proceeded to investigate its mechanism of action.

4.3.1. Modified antibody requires adenovirus to increase its rate of internalization, which is critical for efficient DC maturation

In this study, we used both adenovirus and anti-DEC205 Fab to initiate antibody internalization. Adenovirus-Antibody IC enters the cytosol to interact with TRIM21 because adenovirus releases protein VI to lyse the endosomal membrane [250], whereas DEC205-targeted antigen were associated with entry into late endosomal compartments, colocalizing with LAMP1 marker in immunofluorescence images [237]. Co-IP experiment authenticated the direct interaction of TRIM21 and antibody in both models (Figure 3.9 and 3.17b), but the result hinted to the fact that more modified antibody than WT antibody was present in equal volume of cell lysate only for adenovirus model. Intracellular staining of internalized antibodies verified that Fc modification bestowed the additional advantage of antibody retainment within cells treated with adenovirus-antibody IC. We also confirmed that this was the result from a boost in internalization rate, which can be observed very early at 15 mins after addition of immune complexes to the moDCs (Figure 3.11d). On the other hand, no apparent increase in antibody retainment was observed for cells treated with anti-DEC205 antibodies in both human and mouse model (Figure 3.17c and Figure 3.18c). By juxtaposing both results, we can see that the presence of adenovirus dictated the occurrence of stronger internalization. On that basis, we have several likely scenarios that could have led to this happening.

Firstly, we surmised that adenovirus-mediated entry into the cytosolic compartment [339] helped facilitate the improvement in internalization, by sequestering internalized modified

antibody to cytosolic TRIM21 through its enhanced binding. This will be investigated in the future. Secondly is the ability of adenovirus to carry multiple antibodies. McEwan and colleagues have shown that each unit of immune complex can comprise of one virion binding up to 2.05×10^{12} antibody molecules [340]. This implies that internalization of a single immune complex unit can bring in 205 times more antibody than the internalization of a single unit of anti-DEC205. The exponential difference in the theoretical amount of internalized antibody might justify the huge discrepancy of antibody retained in the cells between the adenovirus and anti-DEC205 models. Thirdly, for DEC205-mediated endocytosis, proteins that were taken in were known to be subjected to greater degradative forces due to endosomal-lysosomal proteolysis, which led to its dissipation over time. This is supported by experiments showing that inhibitors against lysosomal protease and acidification were able to rescue the internalized anti-DEC205 antibody and improve DC cross presentation [237].

Correspondingly, the incidence of DC maturation by modified antibody in our experiments also correlated with the amount of retained antibodies (Figure 3.4b, 3.17c and 3.18c). This suggested that quantity of intact/detectable antibody retained within the cell, regardless of TRIM21 affinity, is already a major determinant in the induction of DC maturation by modified immune complexes. We hypothesize that the internalization of more antibodies could have led to more antibodies interacting with intracellular TRIM21, thus resulting in a stronger immune signaling response which propelled DC maturation. This will require further investigation in the future. Nevertheless, this casted a sense of doubt on our original hypothesis that DC maturation was solely the result of enhanced antibody binding to TRIM21, and alluded to the fact that sheer quantity of retained antibodies also contributed significantly to DC activation. In view of this new discovery, we can deduce that moDC maturation arose from elevated immune signaling levels and it was dependent on the interaction between TRIM21 and

internalized modified antibody (Figure 3.12). One additional experiment we can consider in the future is to deliver anti-DEC205 or anti-adenovirus antibodies (without virus) directly into the moDC cytosol by electroporation or microinjection [341], then inspect the state of DC maturation. This will clarify the dispensability of an infectious agent (i.e., adenovirus) in the DC maturation stimulated by modified antibody.

4.3.2. Possible involvement of TRIM25 in immune response by modified antibody

By analyzing the protein interactome of modified antibody, we identified TRIM25 protein as one of the most enriched proteins that was involved in immune response (Figure 3.10). Along with TRIM25, we also found a strong presence of RNA-binding and ribosomal proteins pulled down from the eluate with modified antibody. This observation can be explained by the ancillary RNA-binding activity of TRIM25 PRYSPRY domain, which has been documented to co-immunoprecipitate other RNA-binding proteins due to their association with RNA strands [342, 343]. TRIM25 regulates RIG-I signaling by conjugating K63-polyubiquitin chains to RIG-I, which facilitates the interaction to mitochondrial antiviral signaling protein (MAVS) and subsequent downstream antiviral signaling like NF- κ B and IRF3/7 pathways [344-346]. Activation of RIG-I is essential for inflammatory cytokine production by moDCs and supports its maturation [347]. We propose a possible mechanistic route which involves the engagement of both TRIM21 and TRIM25 by the modified antibody in cytosol (Figure 4.1). This hypothesis is supported by our RNA-seq data, which show upregulation of several NF- κ B and IRF3/7 downstream genes such as TNF, CCL5, CXCL10, IFNB, IFIT1/2/3 and ISG15 in the 'Mod+Virus'-treated samples. The aforementioned genes encode for proteins involved in immune response, such as encouraging inflammatory cytokine secretion, recruiting other leukocytes and promoting further immune signaling pathways, which serve to induce DC

maturation. The involvement of TRIM25 in the modified antibody-mediated moDC maturation will be explored in the future.

Figure 4.1. Modified antibody mediates immune response through both TRIM21 and TRIM25. Adenovirus transports modified antibody into the cell cytosol, which engages TRIM21 to initiate viral neutralization and immune signaling pathways. Likewise, modified antibody may also engage TRIM25 protein to polyubiquitinate and switch on the RIG-I pathway, which leads to the activation of NF-κB and IRF3/7 pathways.

4.3.3. Signaling pathways stimulated by modified antibody

Previous study has reported that TRIM21 activated by antibody undergo polyubiquitination and proteasomal degradation, which releases unanchored polyubiquitin chains that mediate NF-κB signaling [197]. The difference in NF-κB signaling between WT and modified IC can be very well regarded as the result of improved binding between modified antibody and

TRIM21 (Figure 3.12b). However, less was described for TRIM21's impact on other inflammatory pathways such as IFN. NF- κ B plays a key role in the early stages of virus infection by rapidly binding to IFNB enhancer and recruiting the co-activator CBP/p300 to initiate IFN β expression [348]. Furthermore, RelA knockout cells were demonstrated to be impaired in early type I IFN induction after virus infection, illustrating the importance of NF- κ B in driving type I IFN response [349]. Indeed, our RNA-seq data showed that IFNB1 gene was significantly upregulated by modified IC at 24 h timepoint (22-fold upregulation relative to Virus treatment and 45-fold upregulation relative to WT IC treatment; Data not shown). Later in the infection, feed-forward activation of IRF-7 by the released IFN β will then accelerate and further amplify the IFN response [349, 350]. In essence, upregulation of NF- κ B pathway made possible by stronger TRIM21-antibody binding had escalated IFN signaling response (Figure 3.10). According to several other studies, NF- κ B-dependent transcriptome was also demonstrated to be enriched for genes encoding chemokine signaling and proteins involved in controlling antigen processing and presentation [351, 352], again emphasizing its importance for the rise of DC maturation.

In response to different activation state in immune cells, distinct cellular metabolism patterns have to be tailored to satisfy their bioenergetic and biosynthetic requirements [353-355] (Figure 4.2). For instance, moDC differentiation in response to IL-4 and GM-CSF depended on fatty acid synthesis [356], which is regulated by mitochondrial biogenesis through peroxisome proliferator-activated receptor- γ (PPAR γ) and PPAR γ co-activator 1 α (PGC1 α) [357, 358]. Resting and quiescent DCs face low anabolic demands as they are less secretory and do not have to perform T cell activation duties. Instead, they have higher tendency for catabolic events and were shown to perform fatty acid oxidation to fuel oxidative phosphorylation [359]. Upon TLR-driven activation, DCs display two phases of glucose metabolism – an early phase of

increased flux in aerobic glycolysis pathway, followed by the engagement of anaerobic glycolysis pathway, also known as Warburg metabolism [360, 361]. Fatty acid synthesis is also necessary during DC activation for the formation of ER and Golgi to facilitate protein translation [362]. Indeed, our mass spectrometry data showed that almost a third of the moDC proteins that co-enriched with internalized modified and not WT complexes, had a metabolic function, suggesting a perturbation in metabolism. In terms of transcripts, our RNA-seq data also revealed that a large group of metabolic genes were oscillating with a great amplitude in both directions when moDCs are treated with modified IC compared to WT IC, which further supports the case that metabolism was intricately attuned by the modified IC. However, this set of oscillating genes (Gene Cluster 2 in Figure 3.14a) was a puzzle, given the fact that unstimulated samples also exhibited an undulating gene expression (Cluster 2 genes in Figure 3.14c). We reasoned that this perplexing result was not due to technical error, since the undulation of gene expression in unstimulated samples was observed only for specific gene clusters and not globally (Figure 3.13a). In addition, the phenomenon was not caused by a disturbance in circadian rhythm, as the expression of canonical circadian genes were very similar across treatments (Supplementary Figure 6). After browsing through publications, the closest documented evidence to the oscillating genes observed in our case was the microarray data by Olex et al., [363]. The author identified 32 genes which exhibit the expression pattern of early upregulation, followed by significant and abrupt downregulation during the span of 24 hours after poly(I:C) stimulation. Biological functions associated with this gene group included immune response, signal transduction and membrane transport. The author noted that the unique expression profile shows that these genes have potent effects, are tightly regulated, and may have deleterious effects if expressed in large quantities or over long periods of time. However, the authors did not mention observing the control group undulating in a smaller amplitude, presumably because it did not. Hence, on top of the sizable difference in number of

oscillating genes (32 DEGs in case study vs 372 DEGs in Gene cluster 2; Figure 3.42c), this case study did not exactly provide a resolution to our issue. More research must be conducted to resolve the conundrum.

Figure 4.2. Changes in dendritic cell metabolism through development, quiescence and activation [364]. The development of dendritic cells (DCs) from progenitor cells is associated with mitochondrial biogenesis, which is driven by peroxisome proliferator-activated receptor- γ (PPAR γ) co-activator 1 α (PGC1 α) and promoted by PPAR γ , mammalian target of rapamycin (mTOR) and MYC. Differentiated DCs populate their niches as immature DCs. Immature DCs use fatty acid oxidation as a core metabolic process. Activation of DCs by Toll-like receptor (TLR) agonists leads to a rapid increase in flux through glycolysis and the associated pentose phosphate pathway, with an accompanying increase in spare respiratory capacity and fatty acid synthesis. These metabolic changes are initiated by a pathway downstream of TLRs that involves AKT, TANK-binding kinase 1 (TBK1), inhibitor of nuclear factor- κ B kinase subunit- ϵ (IKK ϵ) and hexokinase 2 (HK2), and they are crucial for DC activation. After being activated, DCs remain glycolytic. This process is essential for continued DC survival and is controlled by mTOR and hypoxia-inducible factor 1 α (HIF1 α). CDP, committed DC progenitor; OXPHOS, oxidative phosphorylation.

4.4. The application of modified DEC205-targeting antibody as cancer vaccine

4.4.1. Inability of modified anti-DEC205 to stimulate DC maturation *in vitro*

In our experiments, we found that modified anti-DEC205 was not able to stimulate maturation for both human and mouse DCs *in vitro* (Figure 3.17b and 3.18b) likely because anti-DEC205 antibodies (both wildtype and modified) were hardly internalized into the cells, based on both the immunofluorescence imaging (Figure 3.17d and Figure 3.19) and Flow cytometry (Figure 3.18d) results. However, this starkly juxtaposed with the Western Blot result (Figure 3.17b), which showed high levels of antibodies in the lysate and eluates. This can be attributed to an inherently slow internalization rate of the DEC205 receptor [237], or rapid degradation rate as DEC205-mediated internalization has been demonstrated to direct antigens into the lysosomal pathway [365], which deterred detection by fluorescently-labeled secondary antibodies used in our flow cytometry and immunofluorescence experiments.

Chatterjee and colleagues successfully showed internalization of anti-DEC205 (clone 3G9 - the same antibody clone as what we used) into moDCs, after incubation with fluorescently pre-labeled anti-DEC205 [237]. In comparison, in our experiment, unlabeled anti-DEC205 was first incubated with the DCs, before staining them with a fluorescently-labeled anti-Fab antibody. To replicate their experiment, we adopted their method of directly labelling the anti-DEC205 to avoid the issue of failed detection by secondary antibodies. We managed to show that PE-conjugated anti-DEC205 were indeed internalized into the moDCs (Supplementary Figure 10). However, the antibody internalization was not detected by fluorescently labeled secondary anti-human IgG antibodies, as we have previously shown.

To ensure that the signal detected was not entirely due to staining of cell surface DEC205, we incubated the moDCs at 4°C or 37°C. At low temperature (4°C), rate of antibody internalization

is severely hampered, thus any difference in signal between 4°C and 37°C conditions will be due to antibody internalization. Our results showed internalization of both WT and modified anti-DEC205 were strong and unexpectedly comparable (Supplementary Figure 10b), which is contrary to what was shown in adenovirus model, where modified antibody displayed significantly higher internalization rate. The lack of a more rapid internalization rate for modified anti-DEC205 probably led to its inability to stimulate DC maturation *in vitro*.

4.4.2. Prophylactic use of antigen-conjugated modified anti-DEC205 in mouse model

Although modified anti-DEC205 was not shown to stimulate DC maturation *in vitro*, we demonstrated that antigen-conjugated modified anti-DEC205 (modified vaccine) was able to promote a certain degree of antigen-specific CD8 T cell proliferation (Figure 3.20b) and early tumor regression when applied as a prophylactic cancer vaccine (Figure 3.21a). However, we could not observe any antigen-specific tumor killing, partly due to constitutive downregulation of antigen by MC38-GPC3 tumor within the mice (Figure 3.21d). We also noted that there was no drastic drop in weight of mice after vaccination (data not shown), illustrating the safety of the modified vaccines. Regrettably, due to the lack of a functional tetramer to measure GPC3-specific T cells, we were unable to gauge the true worth of the vaccines in stimulating cellular immunity. One possibility is that the tetramer binding is inhibited by the anti-CD8 antibodies used during staining [366]. However, the anti-mouse CD8 clone 53-6.7 used for the experiment was documented to enhance background tetramer staining on CD8 T cells, not inhibiting it [367]. In addition, the non-specific tetramer staining occurred on non-CD8 T cells (Figure 3.22b), which anti-CD8 antibodies should not have bound to. As a result, we concluded that the use of anti-CD8 clone 53-6.7 was not the reason for the failed tetramer staining, and other methods of troubleshooting will have to be explored to solve the issue. As a side note, Holman et al has discovered that anti-CD8 clone CT serves as a better antibody choice for tetramer

staining, as it inhibits non-specific tetramer staining on CD8 T cells [367]. In the future, clone CT will be used for CD8 staining in conjunction with tetramer staining.

The purpose of a cancer vaccine is to augment T cell responses to improve anti-tumor immunity. In the future, we would like to re-test the ability of modified vaccine in stimulating antigen-specific T cells in the ovalbumin model. Although it is an artificial model, all the tools needed to appraise the CTL-induction capability of a vaccine are well-established and commercially available (e.g., tetramers, B16/OVA melanoma cell line, OT I mouse). In addition, immunization in combination with immune checkpoint blockade (anti-PD-1/PD-L1/CTLA-4) can also be considered to boost T cell response.

5. CONCLUSION

In conclusion, we have presented *in vitro* evidence that modified antibody with enhanced affinity to TRIM21 is able to transform the humoral activity of internalized antibodies into an adaptive cellular immunity, i.e., the proliferation of antigen-specific CD8 T cells. This was made possible by improving cross presentation through stimulating DC maturation, which eventually leads to the activation of antigen-specific CD8 T cells. The mechanism is likely mediated through the escalation of TRIM21-dependent immune signaling, which was reinforced by massive antibody internalization. Preliminary data from *in vivo* mouse model have indicated that administration of antigen-conjugated modified DEC205-targeting antibodies was able to activate a certain degree of antigen-specific T cell expansion and tumor killing. These findings form the basis for further investigations into the mechanism and therapeutic function of Fc-modified antibodies in both cancer and infectious diseases.

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Supplementary Figures

Supplementary Figure 1. Enterovirus 71/ Coxsackie Virus A16 Binding ELISA of chimeric 5H7 and 1C6 antibodies. Sandwich binding ELISA of chimeric anti-EV71 antibodies 5H7 and 1C6, and anti-adenovirus antibody 9C12. **a**, ELISA binding absorbance of chimeric antibodies to EV71 or CVA16. Data are expressed as the mean $\pm$ SD and results shown are representative of 3 independent experiments. **b**, Half maximal effective concentration (EC₅₀) of the chimeric antibodies to EV71 viral particles.

Supplementary Figure 2. Surface Plasmon Resonance measurements of the binding between recombinant PRYSPRY and anti-EV71 WT/Modified antibodies. a–b, The sensorgrams of recombinant human or mouse PRYSPRY binding to the indicated chip-immobilized WT or Modified antibodies are expressed in RU (response unit) versus time after subtracting the control signal. The PRYSPRY concentrations were 6.25, 12.5, 25, 50 and 100 nM (from bottom to top). Colored lines are SPR data from different concentrations of the analytes; bold red lines are the curve fitting using Langmuir 1:1 model. **c,** The calculated dissociation constants (K_D).

Supplementary Figure 3. Effect of modified antibodies + virus in Enterovirus 71 model.

a, b, c, Lymphocyte activation assay in Enterovirus 71 model. **a,** Schematic workflow of the experiment: moDC was first exposed to the virus, then washed and added to autologous CD14-negative cells to pre-expand the EV71-specific lymphocytes for 6 days. Next, another batch of moDC was treated with seven different groups of treatment: ‘PBS’, ‘WT antibody only’ (WT only), ‘modified antibody only’ (Mod only), ‘EV71 only’, ‘EV71 + WT antibody’ (EV71+WT), ‘EV71 + modified antibody’ (EV71+Mod) and ‘LPS’ and incubated for 4 hours. The cells were then washed and added to the pre-expanded EV71-specific lymphocytes. Supernatant was collected on Day 4 and 7, and concentration of interferon gamma (IFN- γ) released was

measured by ELISA. The absolute counts of CD4 and CD8 T cells was measured by flow cytometry by normalization with counting beads. The anti-EV71 antibodies used were either **b**, neutralizing (5H7) or **c**, non-neutralizing (1C6). Separate moDCs treated with six groups of treatments: 'PBS', 'Virus only', '5H7 WT + Virus', '5H7 Mod + Virus', '1C6 WT + Virus' and '1C6 Mod + Virus', were washed after 4 hours and incubated for another 20 hours, before collecting the supernatant and isolating the RNA using TRIzol. **d**, mRNA levels of EV71 VP1 (Viral protein 1) gene transcript were measured by qPCR and normalized internally to GAPDH expression. **e**, Amount of EV71 viral particles in the supernatant was measured by EV71 sandwich ELISA. Data are expressed as the mean + SD of three technical replicates.

Supplementary Figure 4. Treatment with modified IC stimulates moDC to upregulate NF- κ B and AP-1 signaling downstream transcripts (Adenovirus model). moDCs from healthy human donors were treated with ‘adenovirus only’ (Virus only), ‘WT antibody + adenovirus’ (Virus+WT) and ‘modified antibody + adenovirus’ (Virus+Mod) and incubated for 6 different time points: 1h, 2h, 4h, 6h, 8h and 24h. The cells were then lysed with TRIzol and the RNA was isolated for quantitative PCR (qPCR). mRNA levels of six direct gene transcripts of NF- κ B and AP-1: BCL2A1, BIRC3, CCL5, CXCL8, CXCL10 and IFN γ were measured and normalized internally to GAPDH expression. The relative fold change of ‘Virus+WT’ and ‘Virus+Mod’ groups to ‘Virus only’ group was then calculated. Data are expressed as the mean $\pm$ SD of three biological replicates (n=3).

CD86 Expression

Supplementary Figure 5. moDCs from Donor 118, 119, 120, 123 and 124 undergo maturation upon modified IC treatment (Samples sent for RNA-sequencing). Immature monocyte-derived DCs (moDC) from donor 118, 119, 120, 123 and 124 were incubated for 24 hours with seven different groups of treatment: ‘PBS’, WT antibody only (‘WT only’), modified antibody only (‘Mod only’), adenovirus only (‘Virus only’), WT antibody + adenovirus (‘WT + Virus’), modified antibody + adenovirus (‘Mod + Virus’) and ‘LPS’. The cells were then stained with fluorescent dye-conjugated antibodies to measure the surface expression of dendritic cell maturation marker CD86, HLA-DR by flow cytometry. The bar graphs show the median fluorescence intensity (MFI) values. Data are expressed as the mean + SD of three replicates.

Supplementary Figure 6. Expression levels of circadian genes in RNA-seq data. moDCs were stimulated in vitro with PBS, adenovirus alone (Virus only), WT immune complex (Virus+WT) or modified immune complex (Virus+Mod) for 1, 4, 12 or 24 hours. Expression of seven genes associated in the regulation of circadian rhythm over time is shown.

Supplementary Figure 7. Gating strategy for proliferated OT-I CD8 T cells. The proliferated OT-I CD8 T cells were gated by Singlets-FSC/Singlets-SSC/Live cells/CD8 T cells/OT-I cells/CFSE- cells. Absolute number of OT-I CD8 T cell was calculated by dividing its cell count to the number of counting beads acquired, then multiplying by 10000, which is the number of counting beads added to each well.

Supplementary Figure 8. Gating strategy for tetramer+ CD8 T cells. The tetramer+ CD8 T cells were gated by Singlets-FSC/Singlets-SSC/Lymphocytes/Live cells/CD3 T cells/CD8/Tetramer.

HUMAN TRIM21

MOUSE TRIM21

Supplementary Figure 9. Comparison of human WT and modified Fc interaction with Human/Mouse TRIM21. Top figure shows human IgG1 Fc-hTRIM21 PRYSPRY complex, bottom figure shows human IgG1 Fc-mTRIM21 PRYSPRY complex. Superposition of WT Fc (blue) and modified Fc (cyan) in complex with TRIM21 PRYSPRY domain (orange). Structure of modified Fc was predicted using Robetta protein structure prediction server[329], which was then aligned with human (2IWG) or mouse (3zo0) Fc-TRIM21 complex using PyMOL. WT residues (green) and mutated residues (red) making important contributions are annotated. TRIM21 hotspot residues (magenta) previously identified are also labelled.

Supplementary Figure 10. Internalization of pre-labeled anti-human DEC205 antibody into human moDCs. a, moDCs were incubated with unlabeled or PE-conjugated WT/modified anti-human DEC205 antibodies for 24 hours. For moDCs incubated with unlabeled antibodies, the cells were fixed and permeabilised, before staining with a secondary PE-conjugated anti-human IgG antibody. **b,** moDCs were incubated with PE-conjugated WT/modified anti-human DEC205 at 4°C or 37°C for 3 hours. The bar graphs show the median fluorescence intensity (MFI) values. Data are expressed as the mean + SD of two replicates.

‘Core Up’ genes	‘Core Down’ genes
Cxcl9	Ramp1
Jdp2	IL16
CD86	Mxd4
CD80	Cryl1
Gch1	Exosc5
Ccl4	Pdlim2
Tnfsf9	Rgl2
Ccl2	Rfng
Sdc4	Fuca1
Ptgs2	Apex1
Rtp4	Crtap
Tank	Cerk
Irf1	Mast3
Gadd45b	Mfng
Cflar	Trit1
Tmcc3	St6gal1
Marcksl1	Mycl1
Traf1	Cyb5r1
Rel	Impa2
Tnfaip3	Ppm1m
Gpd2	Mical1
Rbbp8	Rcbtb2
Ripk2	Sfxn3
Tlk2	Glud1
Dnaja2	Gltf
Nfkbia	Hexa
Dusp2	Fes
Peli1	Hagh
Parp9	Smpd2
Tdrd7	Ncor2
Clic4	Bnip3l
Xrn1	Hmha1
Nmi	Tk2
Pi4k2b	Xpc
Rgs1	Hacl1
Zfp281	Mphosp9
Tap1	Zmat3
Azi2	Prkdc
Sos1	Xpot
B3gnt2	Vps26b
Ccdc50	Grk6
Stat2	Wdr81
Pml	Pnpo
Eif2ak2	Dhrs1
Tnfsf4	Top2b

Nck2	Lyl1
Cd38	Add3
Stat1	Hexb
Trim21	Ifngr1
Ifih1	Inpp5d
Nt5c3	Mapk14
Isg20	Scarb1
Oas2	Arhgap18
Ifi44	Lat2
Pla1a	Rgs18
Lta	Tm6sf1
Tnfsf10	Idh1
Mx1	Cat
Il15	
Gbp2	
Il15ra	
Serpib9	
Ube2l6	
Cd200	
Il2ra	
Cfb	
Cxcl10	
Il6	
Oasl1	
Rsad2	
Ifit2	
Ifit3	

Supplementary Table 1. List of ‘Core Up’ and ‘Core Down’ genes during dendritic cell maturation according to Dalod et al. [319, 368]. Table shows two sets of genes previously reported to be decreased (“CORE DOWN”) or increased (“CORE UP”) upon TLR-induced ‘immunogenic’ maturation.

Author's publications

1. Jia, Q., Q. Ng, **W. Chin**, T. Meng, V. T. K. Chow, C.-I. Wang, J. Kwang and F. He (2017). "Effective in vivo therapeutic IgG antibody against VP3 of enterovirus 71 with receptor-competing activity." 7: 46402.
2. Ng, P.M.L., Kaliaperumal, N., Lee, C.Y., **Chin, W.J.**, Tan, H.C., Au, V.B., Goh, A.X.-H., Tan, Q.W., Yeo, D.S.G., Connolly, J.E., Wang, C.-I., 2019. Enhancing Antigen Cross-Presentation in Human Monocyte-Derived Dendritic Cells by Recruiting the Intracellular Fc Receptor TRIM21. The Journal of Immunology, ji1800462.

Posters and Conference talks

<u>Conference name</u>	<u>Date</u>	<u>Achievements</u>
Antibodies as Drugs: New Horizons in the Therapeutic Use of Engineered Antibodies (Keystone Symposia)	7 April 2019 - 11 April 2019	Oral Presentation (10 mins short talk) and Poster Presentation