Lung-resident, M2e-specific CD4 T cells critically protect during influenza infection

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Cover illustration:
$CD4^+$ T cells (blue) residing in the lung of a mouse immunised intranasally with CTA1-3M2e-DD and subsequently infected with influenza X47 (H3N2) virus.

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ABSTRACT

Memory $CD4^+$ T cells are a critical component of the adaptive immune response to fight infection. However, current vaccines against influenza only focus on the other arm of adaptive immunity—triggering B cells to produce antibodies. These antibodies are directed against viral surface proteins that are extremely heterogeneous and rapidly mutate, meaning that current vaccines offer neither broad protection against different strains, nor long-term protection as a result of mutation. In this thesis we aim to develop a mechanistic understanding of how memory $CD4^+$ T cells directed against the conserved influenza protein, M2e, mediate protection in a mouse model of immunisation and infection.

We administer M2e intranasally within an adjuvant vector, CTA1-DD, which enables us to generate large populations of M2e-specific $CD4^+$ T cells in lung. Having identified the critical recognition sequence for the M2e peptide, we established an MHC class II tetramer assay that enables detection, extraction and characterisation of M2e-specific $CD4^+$ T cells from the lungs and lymphoid tissues of immunised mice. In paper 1, we utilise in-vitro antigen restimulation and flow cytometry to broadly characterise these lung-resident $CD4^+$ T cells as having a predominantly Th17 phenotype. We identify that their capacity to protect mice from different strains of influenza virus is independent of antibody, by using knock-out and congenic mouse models. In paper 2, we identify that intranasal delivery is critical for generating lung-resident $CD4^+$ T cells, and confirm that tissue-resident memory cells do not require support from circulating cells during infection. We also characterise the M2e-specific $CD4^+$ T cell population further by profiling their transcriptome at the single-cell level during the time-course of infection. We identify a previously unreported cytotoxic population of M2e-specific $CD4^+$ T cells that rapidly expand to control virus propagation during the early phase of infection. Characterisation of this subset is of significance to the influenza vaccine field, where the search for correlates of $CD4^+$ T cell mediated protection have thus far yielded no robust biomarkers. In paper 3, we show how M2e-specific $CD4^+$ T cells can work synergistically with $CD8^+$ T cells targetting another conserved influenza protein to confer improved protection from influenza infection. Together, these findings highlight the critical role played by M2e-specific $CD4^+$ T cells in protection from influenza, underlining its usefulness as a component of future universal vaccine candidates.

Keywords: influenza, vaccine, universal, heterosubtypic, M2e, CD4, Th17, ThCTL, tissue-resident, cytotoxic, single-cell RNA-seq.

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SAMMANFATTNING PÅ SVENSKA

Minnes CD4 T celler är centrala i skyddet mot många typer av infektionssjukdomar. Trots detta är de flesta vacciner framtagna för att stimulera specifik antikroppsproduktion även om också T celler aktiveras vid vaccinationen. Detta är även fallet för vacciner mot influensa, då skyddet mot influensainfektion framförallt riktar sig mot ytproteiner på viruset och syftet är att genom antikroppar neutralisera och eliminera virus. Det har dock visat sig att för ett bredare och bättre skydd mot viruset, som kan genomgå snabba mutationer och byta skepnad, behövs T celler som reagerar på konserverade interna strukturella proteiner. I denna avhandling har jag studerat specifika CD4 T celler som reagerat på ett prototypvaccin, CTA1-3M2e-DD, som bär på en peptid från virusets M2 jonkanals-protein, och som givits intranasalt till möss. M2e-peptiden är en konserverad epitop i väldigt många influensa A virusstammar. Genom olika experimentella metoder har jag sedan försökt få en uppfattning om på vilket sätt dessa CD4 T celler ger ett brett skydd mot influensa virus. Särskilt har jag fokuserat på deras placering i lungorna och hur aktiverade CD4 T celler vid en infektion griper in och försvarar mot infektionen. Deras egenskaper och arsenal av gener som utnyttjas för att försvara mot infektion har kartlagts.

Efter vaccination återfinns ett stort antal minnes CD4 T celler specifika för M2e i lungorna och i de dränerande lymfknutorna. Genom en tetramer, som utgörs av M2e-peptid och delar av MHC klass II molekylen, har vi kunnat hitta dessa celler och därmed kunnat isolera cellerna ur vävnaden. Tetrameren, som är märkt med en fluorochrom, binder till receptorn på M2e-specific $CD4^+$ T celler och kan därmed identifieras i en flödescytometer. I artikel 1 beskriver jag hur vi tagfit fram tetrameren och isolerat CD4 T cellerna ur lungorna. Jag beskriver möjliga skyddande egenskaper som kan förklara varför dessa celler är centrala i skyddet mot influensa och beskriver dessa framförallt som Th17 celler. Vidare visar jag på att dessa celler även utan närvaro av antikroppar kan skydda mot infektion genom att använda mig av gen-defekta möss. I den andra artikeln går jag ett steg längre och tydliggör att endast genom intranasal vaccination kan dessa celler stimuleras till att bli residenta CD4 T celler i lungorna och att vid en infektion räcker dessa celler som skydd genom att dom snabbt svarar med expansion av populationen i lungorna. Isolerade minnes-M2e-specifika T celler karakteriserades därefter på enskild cell nivå (single cell RNAseq) och deras gen-använding monitorerades över tid vid en infektion. På detta sätt identifierade jag en tidigare inte beskriven cytotoxisk egenskap hos dessa celler vilken jag kunde sätta i samband med ett tidigt brott i virusexpansionen i lungorna. I artikel 3 försöker utvidga komponenterna som används i ett brett skyddande influensavaccin genom att också kunna stimulera cytotoxiska CD8 T celler med ett annat strukturellt protein, nukleoproteinet (NP). Två fusionsproteiner, ett med M2e och det andra med NP, ger förstärkt skydd mot influensa. Detaljerade studier av dessa CD4 T cellers funktion under en infektion är mycket betydelsefulla, dels för vår förståelse av mekanismerna bakom ett brett infektionskydd mot influensa, och som vägledning av hur framtida brett skyddande universal-vacciner mot influensa skall utformas för att vara effektiva.

PAPERS

This thesis is based on three studies, referred to in the text as Papers I, II and III:

I. Eliasson DG, Omokanye A, Schön K, Wenzel UA, Bernasconi V, Bemark M, Kolpe A, El Bakkouri K, Ysenbaert T, Deng L, Fiers W, Saelens X, Lycke N. **M2e-tetramer-specific memory CD4 T cells are broadly protective against influenza infection.**

Mucosal Immunology. 2018; 11(1):273-289.

II. Omokanye A, Lebrero C, Ågren R, Proux-Wéra E, Ong LC, Kolpe A, Bernasconi V, Wenzel UA, Schön K, Bemark M, Saelens X, Lycke N. **Single-cell transcriptome analysis of lung-resident, M2e-specific T cells identifies cytotoxic sub-populations that rapidly expand to control influenza virus replication.**

Manuscript.

III. Omokanye A, Ong LC, Bernasconi V, Strömberg A, Schön K, Saelens X, Lycke N.
Lung-resident, M2e-specific CD4 T cells act synergistically with NP-specific CD8 T cells to confer improved protection from influenza infection.

Manuscript.

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ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cell
BCR	B cell receptor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
GISRS	Global Influenza Surveillance and Response System
HA	Haemagglutinin
IIV	Inactivated influenza vaccine
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
LAIV	Live attenuated influenza vaccine
M	Matrix protein
M2e	Matrix protein 2 ectodomain
MHC	Major histocompatibility complex
NA	Neuraminidase
NF-κB	Nuclear factor kappa-B
NK	Naturall killer
NLRP3	NOD-like receptor pyrin domain-containing-3
NP	Nucleoprotein
NS	Non structural
PA	Polymerase acidic
PAMP	Pathogen associated molecular patterns
PB	Polymerase basic
PBMC	Peripheral blood mononuclear cell
PRR	Pattern recognition receptor
RIG-I	Retinoic acid inducible gene I
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
SAA	Serum amyloid A
TCR	T cell receptor
Tfh	T follicular helper
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Treg	T regulatory
WHO	World Health Organization

1 INTRODUCTION

This thesis centres on findings presented in 3 papers, which explore the potential value of a vaccine targeting the evolutionarily conserved M2 protein—using the M2e-peptide—on the surface of influenza viruses. This introduction offers a context for this work by detailing the unique challenges posed to the vaccine research community by influenza viruses. It outlines the rationale for an approach targetting conserved influenza proteins, and evaluates the key approaches taken across the 3 papers, before summarising critical findings in the context of existing research.

1.1 Influenza: a perpetual public health concern

1.1.1 Virological classification, life cycle and ecological reservoirs

Influenza viruses are enveloped, negative-sense, RNA viruses belonging to the family *Orthomyxoviridae*. Four genera of influenza virus have been reported to date, each giving rise to a single eponymous species, or type: A, B, C and D (Table 1). These obligate intracellular parasites primarily attach to—and replicate within—epithelial cells of the respiratory tract in a wide variety of vertebrate organisms [1]. This typically results in passive carriage and shedding of virus within airway secretions, but can lead to severe respiratory disease and sudden death depending on multiple virus- and host-specific factors [2, 3].

Type	Reported	Identified hosts	Human disease	Pandemics
A	1931 [4]	Multiple	Mild - severe	Yes
B	1940 [5]	Primarily human	Mild - severe	No
C	1949 [6]	Primarily human	Mild	No
D	2013 [7]	Primarily cattle	None	No

Table 1. Influenza virus types.

Whilst all 4 influenza types infect humans, influenza A and B are of particular concern as they cause the majority of influenza disease. Both circulate persistently, resulting in seasonal, localised epidemics that are unpredictable in terms of duration, severity and at risk populations [8]. Approximately 25% of seasonal infections can be attributed to influenza B viruses, which circulate as two distinct lineages (Victoria and Yamagata) [9]. Influenza A viruses cause 75% of seasonal epidemic cases, but that considerable threat pales in comparison to their exclusive ability to cause global, pandemic outbreaks of severe disease. 2018 marks the centenary of the "Spanish" influenza A pandemic, a devastating outbreak that killed up to 100 million people worldwide between 1918 and 1920 [10]. As a result of their epidemic and pandemic threat, effective vaccines against influenza A viruses are vital. However, they remain elusive—in part—because of rapid viral adaptation [11].

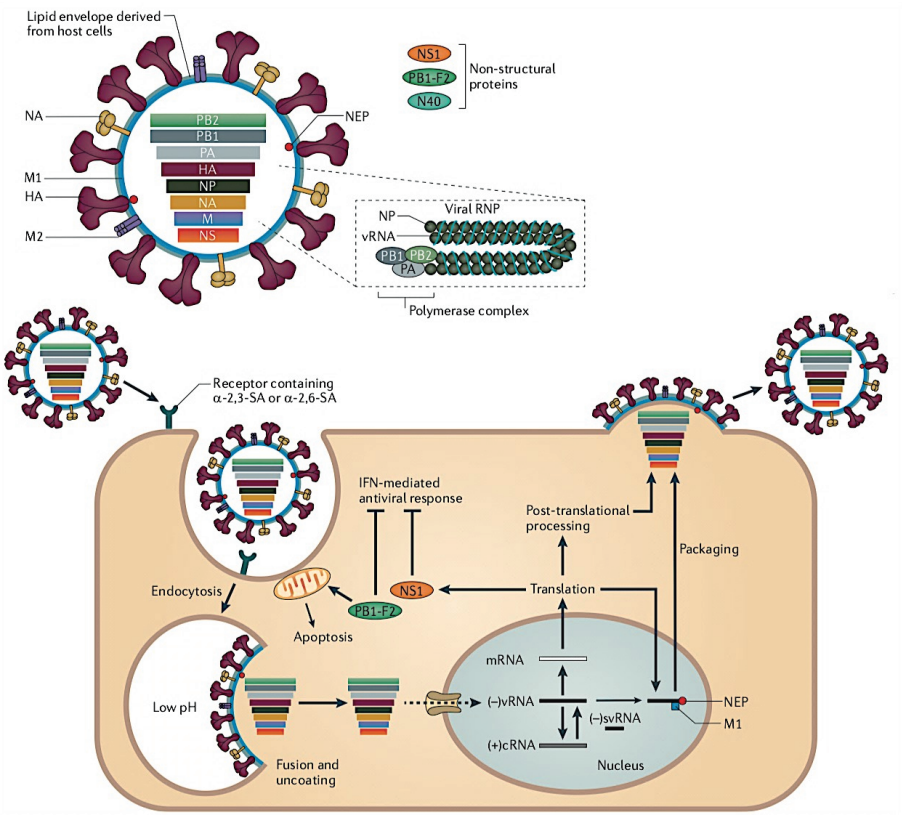


Figure 1. Influenza A virus architecture and replication cycle. Medina *et al.* [2]

To appreciate the challenge posed by this adaptability, an understanding of influenza virus architecture and replication is required. Both are detailed in Figure 1 [2]. Influenza A viruses are typically spherical and 100 nm in diameter, roughly 100 times smaller than the respiratory tract epithelial cells they infect [12, 13]. Their genome comprises 8 segments, encoding 11 proteins: haemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix proteins M1 and M2, non-structural proteins NS1 and NS2 (NEP), RNA polymerase subunits PA, PB1 and PB2, and the pro-apoptotic accessory protein PB1-F2. The viral envelope is made up of a lipid bilayer containing transmembrane glycoproteins HA, NA, and M2. HA is the most abundant envelope protein with approximately 500 copies per virion, followed by NA with 100 copies and M2 with around 20 copies (~3%) [14]. Beneath the lipid bilayer, M1 forms a matrix that encases the viral ribonucleoproteins (vRNPs), anchoring to NEP. These vRNPs form the virus core and are made up of negative-sense viral RNA (vRNA) wrapped around NP. The polymerase complex proteins PB1, PB2 and PA sit at one end of the vRNPs. Influenza viruses initiate entry to epithelial cells via attachment of the head domain of HA (HA1) to membrane glycoprotein-bound sialic acid residues, bearing either α(2,3) or α(2,6) carbohydrate linkages. This triggers endocytosis and translocation to an endosome in the epithelial cell. Despite its low abundance, M2 plays a critical role in viral replication. It functions as a pH-sensitive ion channel that selectively permits entry of protons from the endosome into the virus core, resulting in disassembly of the vRNPs. Meanwhile, the acidic environment also triggers conformational changes that enable the stalk domain of HA (HA2) to fuse with the endosome membrane, releasing vRNPs into the cytoplasm before their translocation to the nucleus. Once there, vRNA is replicated via a positive-sense intermediate (cRNA) and transcribed into viral mRNAs that are exported and translated into viral proteins in the cytoplasm. Some viral proteins re-enter the nucleus to assemble new vRNPs, whilst others are directed to the cell membrane, where they form lipid rafts. Replicated vRNPs are directed to these rafts, where budding of new viral particles occurs. Budding is facilitated by NA, which cleaves sialic acid residues, freeing HA from attachment to the cell membrane [12, 15]. Influenza polymerase lacks proofreading activity, resulting in a mutation rate of approximately one error per replicated genome [16]. Consequently, each infected host cell is capable of producing thousands of viral mutants, some of which may confer a survival advantage within the host [17, 18]. In addition, different influenza viruses co-infecting the same cell are able to readily exchange whole RNA segments. These random reassortment events confer additional adaptability to the

virus, allowing it to thrive even when faced with demanding selection pressures, such as vaccination. Influenza A viruses mutate much more readily than influenza B viruses, a factor that may explain their adaptation to a wider host range [19]. Whilst influenza B and C co-circulate almost exclusively in humans, the natural reservoir for influenza A viruses are wild waterfowl—in whom viral replication and shedding readily occur in both the respiratory and gastrointestinal tract. Influenza A viruses also infect domestic poultry, pigs, dogs, cats, horses, and a wide range of other terrestrial and aquatic mammals (Figure 2) [1, 20, 21]. This extraordinary range of highly mobile hosts leads to perpetual reassortment of animal- and human-adapted influenza A viruses. In particular, surface glycoproteins HA and NA are prone to mutation. As a result, multiple subtypes exist, classified by their different HA and NA structures—presently 18 HA and 11 NA subtypes have been documented. The most common influenza A subtypes that circulate seasonally in humans are H1N1 and H3N2.

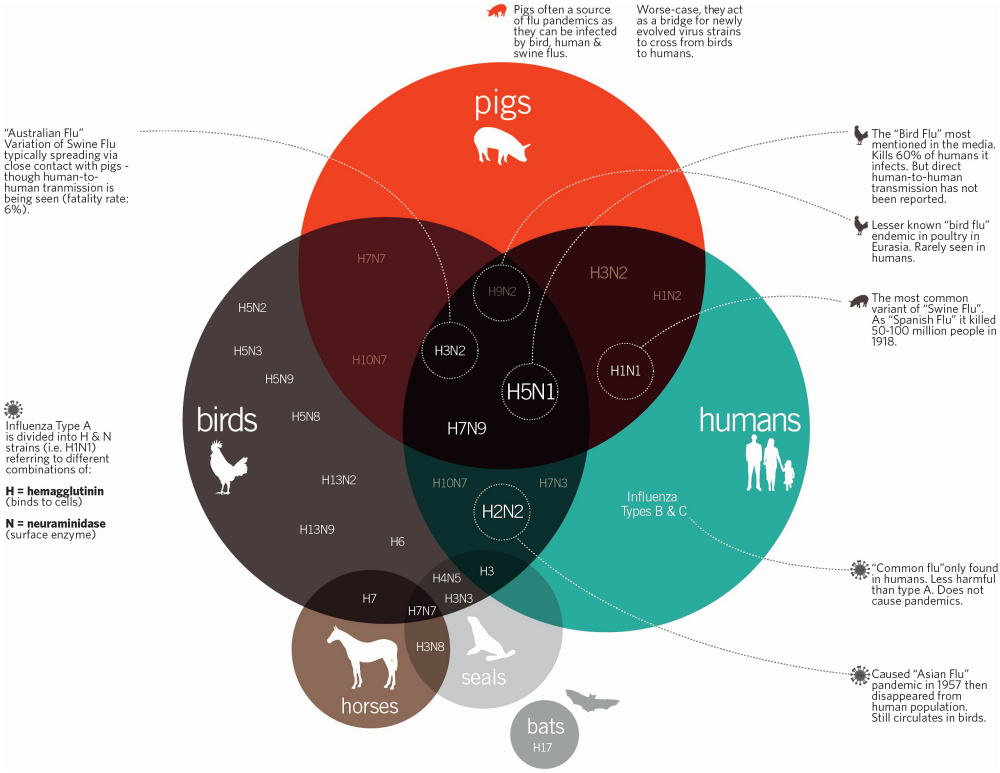


Figure 2. Influenza A host range and subtype pathogenicity.
text size = human fatality rate;greyed text = rarely infects humans
McCandless *et al*. [20]

Attachment of HA to sialic acid residues on epithelial cell membranes is a critical step for initiating influenza virus entry to host cells. Two major linkages are found between sialic acids and the carbohydrates they are bound to, described as α(2,3) or α(2,6) sialosides. Viruses from humans primarily recognise the α(2,6) linkage, whereas avian-adapted viruses typically recognise α(2,3) linkages. Those from swine recognise both, and a population of epithelial cells bearing α(2,3)-linked sialic acids also exist in humans, but primarily in the lower respiratory tract. This explains the importance of both humans and pigs as possible mixing vessels for reassortment between avian and human-adapted influenza viruses (Figure 3) [22]. As we will go on to explore, current vaccines against influenza primarily target HA and NA. However, influenza virus composition is subject to continuous mutation across multiple host organisms via polymerase-mediated errors (antigenic drift) or reassortment of whole gene segments (antigenic shift), which ultimately enable viruses to evade immune protection generated via natural infection or vaccination [23].

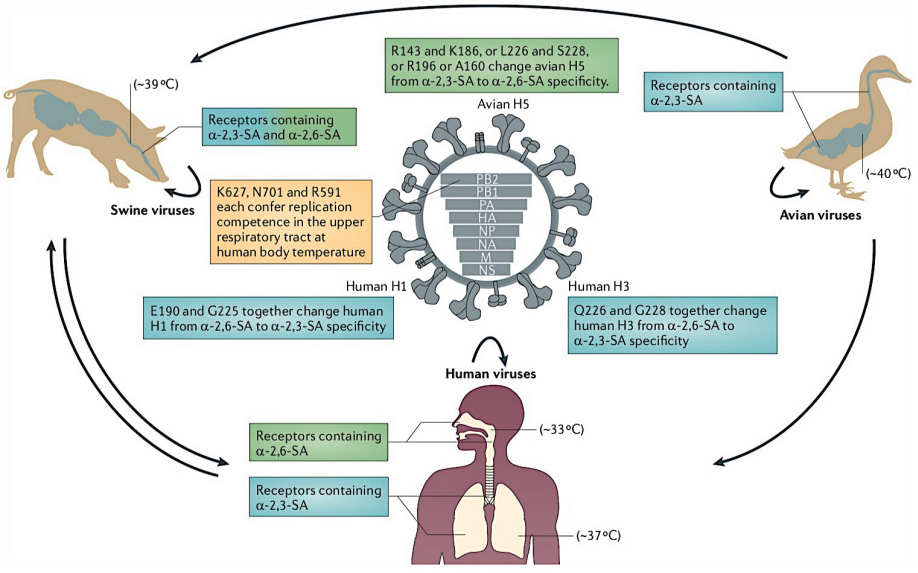


Figure 3. Influenza sialoside binding and mutations conferring host adaptation.
Medina *et al*. [2]

1.1.2 Societal and economic burden

Approximately 40% of individuals infected with influenza are asymptomatic or experience subclinical disease, although they can still transmit the virus [24]. Transmission occurs via direct conact with an infected person, exposure to infectious droplets (coughing and sneezing) or fomites (materials and surfaces exposed to droplets) [25]. Of those that do experience symtpoms, they are typically mild, self-limiting, and restricted to the upper respiratory tract. Infection is hallmarked by a dry and unproductive cough, fever, sore throat, coryza, generalised muscle ache and lethargy [26]. However, infection can lead to considerably more severe symptoms in young children, the elderly, those with concomitant cardiovascular, metabolic or respiratory disease and pregnant women [8, 27-30]. In these at risk groups, primary influenza infection may additionally predispose individuals to secondary bacterial infections of the lower respiratory tract. Both primary influenza infections and secondary bacterial infections can lead to acute respiratory distress, hypoxia and death [31].

Seasonal outbreaks of influenza occur worldwide [32]. Estimating the burden of influenza across different geographical regions can be difficult and depends on adequate healthcare infrastructure that can reliably record all cases of respiratory illness, whilst documenting the presence or absence of laboratory-confirmed influenza infection via patient throat swabs [33, 34]. The most comprehensive study of global mortality associated with seasonal influenza was published this year, and identified that previously reported calculations heavily underestimated influenza burden. Iuliano *et al.* modelled country-specific, influenza-associated excess respiratory mortality data from 1999-2015 and estimate that up to 650,000 deaths per year are caused by influenza-associated respiratory illness worldwide [35]. What is striking about their data, is that it highlights the greater burden of disease experienced by young children (<5 years) and the elderly (>75 years), but also low-income countries in sub-Saharan Africa and southeast Asia. This staggering mortality figure does not include deaths attributable to non-respiratory disease, which would undoubtably increase the estimate [36]. Examining the wider impact of influenza-related illness; including direct health costs related to hospital attendance or admission, loss of workforce productivity, and increased care needs in the community, Molinari *et al*. estimate that the annual cost of seasonal influenza to the US economy alone is $81 billion [37].

The health and economic burden of influenza increases dramatically in a pandemic outbreak [38]. When a virus that typically infects birds acquires the ability to co-infect a mammalian species harbouring mammal-adapted viruses, recombination events can yield novel strains to which the global human population has no immunity. These recombination events lead to devastating pandemics if the resulting viruses cause severe disease in those infected, and transmit effectively from person to person. Over the past 100 years, four major influenza A pandemics have occured (Table 2) [10, 39, 40]. These pandemics have lead to significant social and political disruption, again disproportionaltely affecting low- and middle-income countries [41]. Critically, they have killed millions of seemingly fit and healthy young adults, as opposed to the typical at risk populations for seasonal epidemics (Figure 4) [42]. A number of theories have been mooted to explain this phenomenon. They include heightened immune responses in the young ("cytokine storms"), cross-protective immunity from multiple previous infections in the elderly ("antigenic history"), and lifetime immune responses being shaped by an individual's first influenza exposure ("antigenic imprinting").

Year	Name	Deaths	Subtype (reassortments)
1918-1920	"Spansih" flu	100 million	H1N1 (direct avian to human transmission)
1957-1958	"Asian" flu	2 million	H2N2 (avian H2N2 and human H1N1)
1968-1969	"Hong Kong" flu	1 million	H3N2 (avian H3 and human H2N2)
2009-2010	"Swine" flu	500,000	H1N1 (multiple; swine, avian and human)

Table 2. Pandemic influenza outbreaks 1918-present. [10, 39, 40]

Over the past decade, outbreaks of avian H5 and H7 influenza viruses amongst wild and domestic birds have lead to castrophic losses in the farming industry, resulting in the destruction of hundreds of millions of domestic poultry across Asia, Africa, Europe and North America to halt virus spread [43]. Alarmingly, these viruses have successfully transmitted to humans—typically following attendance at live poultry markets or farms—causing several hundred human infections in recent years, with consistently high mortality rates around 40% [21, 44]. Although these viruses have not spread widely beyond infected individuals, should they evolve the ability to transmit effectively from person to person, specifically via the airborne route, they

will initiate a pandemic [45, 46]. As a society, the question repeatedly being asked is, are we prepared? A recently published simulation by the Institute for Disease Modeling predicts that if a highly contagious and virulent, avian-origin pathogen—like the 1918 influenza—were to appear today, it could lead to the death of 30 million people worldwide within just 6 months [47]. That is a staggering figure, but reflects current limitations in public heatlh response mechanisms and mounting resistance to front line antiviral therapies, to which many influenza viruses have evolved resistance [48, 49]. Given the escalating industrialisation of animal farming—which requires that animals be intensively housed, increasing transmision risk—and reduced separation between the habitats of highly mobile human and wild animal populations, opportunities for both zoonotic and reverse-zoonotic transmissions are bound to increase [25, 50]. The now widely adopted "one health" approach advocates for interdisciplinary collaboration between all stakeholders working across the human-animal interface, with efforts to intensify surveillance of influenza virus evolution in the environment coupled with efforts to develop new therapies [1, 21, 51, 52]. Novel vaccine strategies capable of protecting humans and livestock from the wideranging threats posed by circulating influenza A viruses are chief amongst these priorities. What has emerged from the 4 major pandemics of the last 100 years, is that our immune system retains a memory for previous encounters with influenza viruses, and this shapes future responses and susceptibility [53, 54]. What follows is a breakdown of current understanding of the key actors in the development of immune memory to influenza virus infection, which we go on to leverage in a vaccine platform whose mechanism of action is explored in papers I-III.

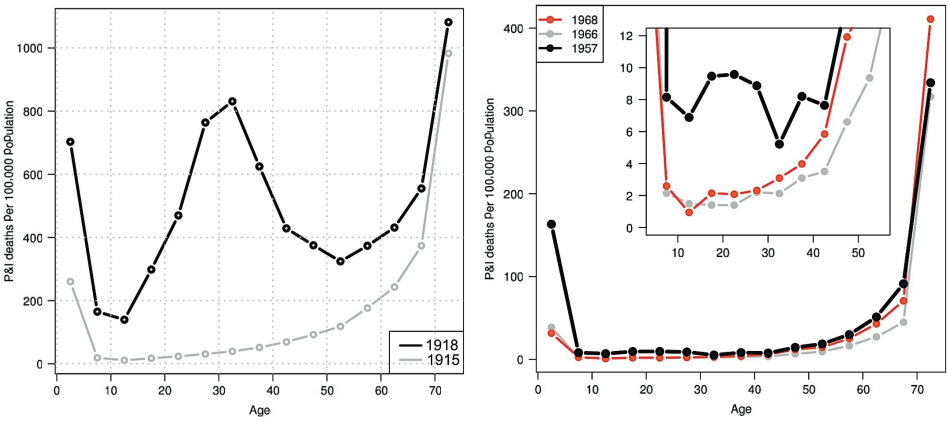


Figure 4. Age-specific mortality for pneumonia and influenza in the United States (L) and Canada (R), during influenza pandemics in the 20th Century. Ma *et al.* [42]

1.2 Influenza immunity

Our immune system is divided into innate and adaptive immune functions generated by a wide range of specialised cells that work synchronously to ensure not just our health, but also our survival [55]. These sentinal immune cells face the challenging task of balancing appropriately hostile responses to pathogens—invading organisms that cause harm—with tolerance to non-pathogens such as food, dust, or our own cells and tissues. Viral and bacterial pathogens bear a number of structural elements, named antigens, which trigger responses from infected cells or patrolling cells of the immune system. Typically these responses are categorised as innate or adaptive, although these distinctions are not mutually exclusive [56, 57]. Innate responses provide an immediate and non-specific barrier against pathogens. They include physical structures, such as mucus, and chemical sensors (pattern recognition receptors; PRRs) that identify conserved antigenic components of viruses and bacteria (pathogen associated molecular patterns; PAMPs). Cells of the innate immune system are critical for the initiation of an immune response and these are able to attract and modulate cells of the adaptive response, which are B and T cells. The latter carry receptors that are highly specific and unique for single epitopes of the pathogen and both B and T cells can retain a memory that ensures responses of greater immediacy and magnitude if the pathogen is re-encountered.

1.2.1 Innate response to influenza viruses

Influenza viruses initiate entry to cells via HA-mediated binding to sialic acid residues, only after successful NA-mediated penetration of constitutive mucus barriers [58-60]. Multiple PRRs activate the innate immune system at the onset of influenza virus infection. These include endosomal recognition in infected cells via Toll-like receptor 7 (TLR7), phagosomal recognition of dying cells via TLR3, extracellular recognition of damaged cells via TLR4 and cytosolic recognition through retinoic acid inducible gene I (RIG-I) [61, 62]. Downstream signaling triggered by these PRRs results in activation of transcription factors nuclear factor kappa-B (NF-κB) and interferon regulatory factors (IRFs) 3 and 7. This leads to the expression of type I interferons (IFNα and IFNβ), type III interferons (IFNλ) and pro-inflammatory cytokines such as IL6, IL8 and TNFα [61]. Type I and III IFNs

secreted by infected cells trigger cognate receptors in autocrine or paracrine loops, activating signalling pathways that govern the expression of multiple IFN-stimulated genes (ISGs) [63]. IFNs, as well as several of these ISGs, directly limit influenza virus replication [64-67]. In response to this intitial wave of distress signals, mucus production increases rapidly [68, 69]. Circulating cells of the innate system are also drawn to the lung, including natural killer (NK) cells, neutrophils, macrophages, and dendritic cells (DCs). NK cells identify and lyse infected host cells, macrophages and neutrophils phagocytose lysed cells and regulate adaptive immune responses, whilst DCs collect, process and present viral antigens loaded onto major histocompatibility complex (MHC) molecules to naive $CD4^+$ and $CD8^+$ T cells, initiating the adaptive immune response [61, 70]. Type I IFNs directly modulate innate NK cells, as well as adaptive $CD4^+$ and $CD8^+$ T cell responses by boosting production of IFNγ (a type II IFN) and proteasomes such as granzymes, which block viral replication and induce lysis of infected cells. Additionally, NOD-like receptor pyrin domain-containing-3 (NLRP3) activation is mediated by a range of stimuli dependent on the influenza virus M2 protein ion channel, and results in recruitment of ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), which then interacts with pro-caspase-1 to form the NLRP3 inflammasome. Subsequent activation of caspase-1 induces pyroptosis and the cleavage of pro-inflammatory cytokine precursors pro-IL1β and pro-IL18 to their mature forms ready for secretion. These cytokines promote the recriuitment, activation and proliferation of both innate and adaptive immune cells recruited to the site of infection [61, 62].

1.2.2 Adaptive response to influenza viruses

Antigen presentation

Adaptive responses are broadly categorised into two arms: humoral and cellular. The humoral response is mediated by B cells that—following engagement of their receptor and extensive cell division—mature to plasma cells that produce antibodies primarily directed at antigens on the surface of the virus. The cellular response is mediated by $CD4^+$ and $CD8^+$ T cells that bear receptors capable of recognising unique epitopes on external and internal influenza antigens. These cells are activated indirectly by specialised antigen presenting cells (APCs) or infected cells, in which antigenic peptides from the virus are processed and loaded onto MHC molecules. For

MHC class I presentation, influenza virus peptides are degraded by cytosolic proteasomes in infected cells, before being transported to the endoplasmic reticulum, where they associate with MHC class I molecules and are then transported to the cell membrane via the Golgi apparatus [71]. MHC class I molecules can be expressed by all nucleated cells following infection and typically present endogenous antigen to the $CD8^+$ T cell receptor. A limited number of specialised APCs—chiefly DCs—are capable of processing exogenous antigen via endosomes or lysosomes, loading them onto MHC class II molecules ready for engagement with the $CD4^+$ T cell receptor. Multiple distinct subsets of respiratory DCs exist. In particular, two myeloid or "classical" subsets exist, namely cDC1 and cDC2 cells, which are subdivided on the basis of CD103 and CD11b expression, such that cDC1 cells are $CD103^+CD11b^-$, while cDC2 cells are $CD103^+CD11b^+$ or $CD103^-CD11b^+$. These DC subsets play central roles in antigen presentation in the lung and respiratory tract [72]. In the context of influenza infection, DC activation and maturation is triggered by TLR7 or RIG-I signaling, under the influence of type I IFNs. Mature DCs capture antigens in the lung, before migrating to the draining lymph nodes (LNs)—a specialised organ housing naive $CD4^+$ T cells, $CD8^+$ T cells and B cells—where they either present directly to T cells via MHC class I/II, or transfer antigens to LN-resident $CD8\alpha^+$ cDCs. The transfer of antigen between migratory cDCs from lung and LN-resident $CD8\alpha^+$ cDCs has been mooted as a mechanism to successfully overcome infection-induced impairment of MHC class I presentation [73]. The cDC1 cells are also capable of acquiring exogenous antigen and presenting it on MHC class I molecules via a process called cross-presentation [74].

Macrophages and B cells play critical auxilliary roles as professional APCs, primarily expressing exogenous influenza antigens to $CD4^+$ T cells via MHC class II. Multiple studies confirm that respiratory epithelial cells also express antgens via MHC class II constitutively, with expression upregulated by IFNs [75-78]. Whilst these cells may not play a significant role in T cell priming, this work suggests that B cells as well as epithelial cells have the capacity to participate in the effector phase of adaptive $CD4^+$ T cell responses.

B cells

B cells recognise unprocessed surface antigens from the influenza virus via their B cell receptor (BCR); essentially a transmembrane immunoglubulin with an adjoining signal transduction moeity (CD79). In the bone marrow (BM) the antigen recognition domain of the BCR is acquired through extensive gene recombinations during progenitor cell development, resulting in a circulating naive B cell pool with innumerable BCR specificities. In secondary lymphoid organs, such as the draining lymph node and spleen, B cells expand following activation in clusters in the B cell follicle called germinal centres (GCs). These GCs comprise of intensely dividing B cells in close proximity to $CD4^+$ T follicular helper (Tfh) cells that exert an essential control over the GC reaction. In this way the dividing IgM B cells undergo somatic hypermutations (SHM) and class switch recombination (CSR) to down-stream isoypes in the GC. In addition, ectopic tertiary lymphoid structures known as induced bronchus-associated lymphoid tissue (iBALT) form in the lung [79]. Antigens binding to cross-linked BCRs are endocytosed and processed, before presentation on the surface of the B cell via MHC class II to CD4 T cells in a cognate restricted fashion. The activated Tfh cells express surface protein CD40L and engage in critical CD40L-CD40 interactions with the activated B cells to form and maintain the GC reaction [71]. The Tfh cells also produce cytokines such as IL4 and IL21, which are important for B cell expansion and differentiation leading to CSR and clonal selection guided by SHM. Whereas naive and newly activated B cells carry IgM BCRs the dividing B cells may undergo CSR to IgA, IgE or IgG subclasses in the GC, a process which is governed by cytokine signals delivered by the Tfh cells [80]. IgM antibodies are the hallmark of primary infection and initiate complement-mediated neutralisation of influenza viruses [81]. HA-specific IgA antibodies secreted into mucus in the respiratory lumen work alongside transduced IgG antibodies from serum to neutralise influenza viruses [82]. B cell responses mainly target viral HA and NA glycoproteins [83]. In particular, antigenic sites on the HA head domain (HA1) appear to be immunodominant as compared to other epitopes of the virus, however, the mechanisms that underpin immunodominance remain unresolved [53, 84]. Antibodies targetting HA1 are typically neutralising as they block binding to epithelial cell sialosides, preventing virus entry. High serum anti-HA1 antibody titres generally correlate with protection. Antibodies targeting NA and M2 are not neutralising, but do prevent release of replicated viruses from infected cells [85].

NK cells serve as an important bridge between innate and adaptive immune responses. Their cytotoxicity receptors (NCRs) NKp44 and NKp46 directly bind to HA expressed on the surface of infected host cells, triggering lysis [86, 87]. Antibodies generated by vaccination or previous virus exposure bind to viral proteins—particularly NA and M2—expressed on the surface membranes of infected host cells. NK cells as well as alveolar macrophages express surface Fc receptors (FcRs) that bind to the Fc region of these antibodies, again inducing lysis in a process called antibody-dependent cell-mediated cytotoxicity (ADCC). This is the mechanism by which anti-M2e antibodies mediate protection [88].

T cells

The T cell receptors (TCRs) of most naive $CD4^+$ and $CD8^+$ T cells comprise of glycoprotein TCRα and TCRβ chains. During early development of progenitor T cells in the BM, the TCRβ chain is formed after gene recombinations and a circulating naive T cell pool featuring innumerable TCR specificities is achieved. Within the lymph node, mature DCs engage their MHCs with $CD4^+$ or $CD8^+$ T cells expressing cognate TCRs, and via expression of co-stimulatory molecules CD80 and CD86, engage CD28 on the T cells. The combination of TCR/CD3 signalling and CD28 co-stimulation—in the context of specific cytokines—provide potent activation, proliferation and differentiation signals following antigen recognition. The resulting activated $CD4^+$ and $CD8^+$ T cells migrate from LNs back to the lung and respiratory tract, where they can remain as resident memory T cells or re-engage as effector T cells with antigen-bearing innate cells and secrete effector molecules that help to control virus spread [89, 90].

Naive $CD8^+$ T cells respond to signalling from type I or III IFNs, IFNγ, IL2, and IL12 to differentiate into cytotoxic T lymphocytes (CTLs) [91]. Their transcriptional programs are goverened by T-box transcription factor 21 (Tbx21/Tbet), eomesodermin (Eomes), and PR domain zinc finger protein 1 (Prdm1/Blimp1) [92-94]. Activated $CD8^+$ T cells express XCL1, a chemokine that attracts XCR1-expressing, cross-presenting, cDC1 cells and is required for maximal priming and expansion of CTLs [95, 96]. CTLs lyse influenza-infected cells via secretion of perforin and granzymes. Perforin permeabilises the membrane of infected cells,

facilitating passive entry of granzymes—of which A and B are best described—to induce apoptosis [97]. Uptake of granzymes into cells can occur in the absence of perforin, or when perforin levels are sub-lytic, via receptor-mediated endocytosis [98]. Surprisingly, in the absence of granzymes A and B, influenza virus-specific CTLs can still lyse target cells *in vivo*, with granzymes K and M amongst suggested accessory pathways [97, 99]. In addition to cell lysis, granzymes directly cleave viral or host cell proteins to arrest virus replication, and induce production of pro-inflammatory cytokines IL1β, TNFα, IL6 and IL8 by APCs [100]. Independent of perforin and granzyme activity, CTLs can also induce apoptosis of infected cells via Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL) interactions [101-103]. In addition, FoxP3-positive, IL17-secreting $CD8^+$ T cells generated *in vitro* have been shown to mediate protection against lethal influenza via an IFNγ-dependent mechanism [104].

Naive $CD4^+$ T cells differentiate into a wide range of subtypes—Th1, Th2, Th17, Treg and Tfh—depending on a combination of cytokine signalling and transcription factor regulation during DC activation and upon arrival at terminal effector sites. Th1 cells develop in the context of IFNγ and IL12 signalling. They secrete IFNγ and IL2 under the regulation of transcription factor T-bet (Tbx21). Th2 cells develop in the context of IL2 and IL4 signalling. They secrete IL4, IL5 and IL13 under the regulation of transcription factor GATA3. Th17 cells develop in the context of TGF-β, IL6, IL-21 and IL23 signalling. These T cells secrete IL17A, IL17F, IL21 and IL22 under the regulation of transcription factors RORα and RORγt. Treg cells develop in the context of IL-2, IL-15 and TGFβ signaling. They secrete IL10 and TGFβ under the regulation of transcription factor Foxp3. Tfh cells develop in the context of IL6 and IL21 signalling. They secrete IL4 and IL21 and express bcl-6 and the chemokine receptor CXCR5, which facilitates homing to the B cell follicle in the draining lymph node [105, 106].

Following infection with influenza, Th1 and Th2 effector subtype responses dominate. Th1 cytokines enhance pro-inflammatory cellular immunity, stimulating $CD8^+$ T cells to become cytotoxic. They also enhance the activities of innate NK cells and macrophages. Th1, Th2 and Tfh cells support the maturation of activated B cells into antibody-secreting plasma cells. Treg cells are responsible for maintaining homeostasis of the immune system and secure tolerance to self-antigens, but during the course of an immune response they suppress antigen-specific effector $CD4^+$ and

$CD8^+$ T cell activities to dampen tissue destruction. Although the specific role of Th17 cells is still being explored in the context of influenza, Th17 responses have been shown to play a key role in preventing opportunistic bacterial pneumonia, an important sequale of primary influenza infection [107]. Influenza viruses appear to have evolved mechanisms to inhibit Th17 responses, however, IL10 deficiency can rescue Th17 responses, improving survial after a high dose viral challenge [108-110]. In addition, acting through IL17 production, Th17 cells attract neutrophils to the site of infection, which among other things can draw influenza-specific CTLs to the site of infection [107, 111-113]. Th17 cells and IL-17-signalling is also critical for the development of iBALT structures, with their formation constraining inflammation in the lung during infection [114, 115]. IL-17 also plays an essential role in regulating transport of dimeric IgA across the mucosal membrane via up-regulation of the polyIg-receptors on epithelial cells.

The activity of terminally differentiated effector cells is negatively regulated by immune checkpoint signal transducers that downregulate effector cytokine production. These include cytotoxic T-lymphocyte–associated antigen 4 (CTLA4), programmed death 1 (PD1) and lymphocyte-activation gene 3 (LAG3), effectively preventing sustained activation, and possible pathology once virus-infected cells have been cleared [116-118].

Although Th subset designations were initially thought of as independent states representing terminally differentiated cells, a number of studies have demonstrated that expression of "master" transcription factor regulators and cytokines is surprisingly flexible. For example, the co-expression of Tbet or RORγt by Foxp3-regulated cells; IL10 production by Th1, Th2 and Th17 cells; IFNγ production by Th17 cells; and IL13 production by Th1 cells, are just some of the reported examples of this plasticity [119]. The mechanisms that drive this plasticity of $CD4^+$ T cell function are still largely unkown, although they appear to be heavily context dependent and may be ascribed to tissue-specific factors. An example of this CD4 T cell plasticity is the Th17-skewed memory CD4 T cell responses at peripheral sites, with cells exhibiting a mix of Th1, Th2, Th17, Treg and even cytotoxic features in response to viral infection and inflammation [120, 121].

1.3 The emerging importance of lung-resident memory

1.3.1 Seasonal vaccines: good, but not good enough

Vaccination is the most effective of available methods to reduce morbidity and mortality from seasonal outbreaks of influenza A and B virus infections [122, 123]. Licenced influenza vaccines can be broadly classified as either inactivated (IIV) or live-attenuated (LAIV). These vaccines are formulated to protect recipients from infection by the most prevalent influenza viruses in circulation, which must be predicted approximately 8 months prior to the onset of seasonal outbreaks. Selection of these reference strains is determined by consensus amongst multiple laboratories engaged in the World Health Organization Global Influenza Surveillance and Response System (WHO GISRS) [32]. IIVs are administered by intramuscular injection and typically contain 15μg each of the hemagglutinin glycoprotein from two influenza A subtypes (H1N1 and H3N2) and one or two influenza B lineages (Yamagata and Victoria). These antigenically representative strains are reassorted with an influenza A (H1N1) virus adapted for high yield growth in eggs. Reassorted viruses with HA and NA proteins from representative strains and internal components from the egg-adapted virus are then amplified. Following amplification, viruses are inactivated, split into constituent proteins and purified to enrich primarily for the HA component in the final injectable vaccine. IIVs are licenced for individuals over 6 months of age. LAIVs are developed by reassorting representative strains with cold-adapted viruses, which replicate at low temperature (25°C). Although this vaccine delivers replication-competent viruses to recipients, this temperature sensitivity restricts replication to the nasal cavity (~25°C) rather than higher temperatures elsewhere in the respiratory tract (~35°C). LAIVs are licenced for healthy individuals aged 2-49 [124, 125].

Currently available vaccines focus on inducing neutralising antibodies directed against surface HAs. The HA1 head domain that these antibodies primarily target is prone to antigenic drift, so annual adjustments to vaccine composition are required. If circulating viruses drift sufficiently from those selected for the vaccine, protective efficacy can drop dramatically. At least 5 such mismatch events have been documented over the past 15 years [126]. For example, during the 2003/2004 season

A/Fujian/411/2002 emerged as a new and unanticipated antigenic H3N2 variant, with only 25% of characterised isolates during the seasonal outbreak bearing antigenic similarity to the widely used vaccine strain A/Panama/2007/99 (H3N2) [126]. Mutations introduced during egg- or cold-adaptation can also lead to mismatches between strains selected by the WHO and those that appear in the vaccine, further impacting efficacy [126, 127]. Even in years when influenza vaccines are well matched to circulating viruses, estimates of vaccine effectiveness can be disappointingly low, ranging between 30 and 60% [127, 128]. In addition, vaccine effectiveness estimates vary significantly by age group and also by recent vaccination status, with the poorest responses observed in the elderly [129-131]. Current vaccines are further limited by their lengthy time frame for manufacture. In a pandemic setting, where the sudden emergence of a reassorted, antigenically shifted virus triggers an immediate need for a vaccine, egg-based production methods are simply too slow [126].

1.3.2 Memory T cells confer heterosubtypic protection

Analyses of morbidity and mortality data collected during pandemic outbreaks over the past century highlight important age-specific differences in susceptibility to influenza, with outbreaks disproportionally affecting younger individuals (Figure 4) [42]. Multiple factors likely contribute to these observations, but a substantial body of evidence indicates that the cumulative life-course increase in memory T cells that can respond to conserved influenza antigens plays a key role in limiting severity of disease in the absence of neutralising antibodies [132-135]. For example, in instances where seasonal vaccines are mismatched with circulating strains, LAIVs offer better protection when compared to IIVs [136]. Unlike IIVs, replication-competent LAIVs generate strong $CD4^+$ and $CD8^+$ T cell responses to conserved internal viral antigens [136, 137]. The importance of heterosubtypic immunity mediated by $CD4^+$ and $CD8^+$ T cells has been demonstrated in a small number of human prospective cohort studies (Table 3), however, these studies do not clarify whether one type of T cell response offers greater protection than the other, or if they confer protection independently of one another [138].

Reference	Study type	Influenza A strain(s)	NtAb-ve	T cell correlate	Protection against	
					Severe symptoms	Virus shedding
McMichael *et al*. [139]	EC	H1N1	Y	CD8+IFNγ+	N	Y
Wilkinson *et al*. [140]	EC	H1N1 H3N2	Y	CD4+IFNγ+	Y	Y
Sridhar *et al*. [141]	CAI	H1N1 pdm09	Y	CD8+IFNγ+	Y	Y
Hayward *et al*. [142]	CAI	H1N1 H3N2 H1N1 pdm09	N	CD3+IFNγ+	Y	Y

Table 3. Human prospective cohort studies demonstrating heterosubtypic protection. EC - experimental challenge; CAI - communty aacquired infection; NtAb-ve – confirmed absence of neutralising HA antibody.
Adapted from Sridhar *et al.* [137]

Whilsts $CD8^+$ T lymphocytes directly clear virus-infected cells through cytolysis or by inducing apoptosis, the contributions of $CD4^+$ T cells are more diverse. $CD4^+$ T cells have the capacity to regulate high affinity antibody production against conserved viral components via cognate interactions with antigen-specific memory B cells. They also have an indirect role in cytotoxicity, supporting the activation and maintenance of CTLs [143]. In recent years, the independent role that influenza-specific $CD4^+$ memory T cells play in mediating protection has gained more recognition [144]. McKinstry *et al.* demonstrated this excellently in a mouse adoptive transfer model of influenza-specific CD4 T cells. They found that transfer of *in vitro*-polarised, HA-specific Th1 or Th17 memory $CD4^+$ T cells into naive hosts conferred 100% protection from lethal virus challenge, whereas transfer of naive or Th2 $CD4^+$ T cells conferred no protection. Although transferred $CD4^+$ T cells did support B cells and $CD8^+$ T cell responses in recipient hosts, this study also highlighted a perforin-dependent mechanism for viral control mediated by $CD4^+$ T cells [145]. Initially thought to be an abberent *in vitro* finding, the capacity of $CD4^+$ T cells to become cytotoxic *in vivo* has now been demonstrated more widely in the context of viral infections, and it appears to be an important feature of tissue-resident memory (Trm) CD4 T cells close to epithelial surfaces [146-148].

1.3.3 Tissue-resident, memory $CD4^+$ T cells and cytotoxicity

Infection with influenza virus generates both circulating (Tcm) and lung-resident Trm cell populations, which can be discriminated by flow cytometric analysis of CD44, CD62L, CD103 and CD69 surface marker expression [144]. Using a highly informative parabiosis model, Teijero *et al*. underlined the importance of Trm for protection against influenza. They observed that unlike spleen-derived Tcm cells, lung-resident influenza-specific $CD4^+$ Trm cells preferentially homed to the lungs of conjoined naive mice, resulting in superior protection from infection [149]. Despite a growing appreciation of their importance for protection, the signalling and transcriptional pathways that regulate $CD4^+$ T cell residency in lung and cytotoxic differentiation during infection remain unclear [150]. Much of what we understand about cytotoxicity in T cells comes from studying $CD8^+$ T lymphocytes, and whilst there are seemingly clear overlaps between cytotoxic $CD4^+$ T cells (ThCTL) and $CD8^+$ CTLs, more CD4- and disease-specific studies are required [151, 152]. Figure 5 summarises some of the key transcriptional factors implicated in ThCTL effector programming [150]. $CD4^+$ and $CD8^+$ T cells share a common progenitor, both developing in the thymus initially as CD4/8 double positive precursors. The transcription factors T helper-inducing POZ/Kruppel-like factor (ThPOK/Zbtb7b) and Runt-related transcription factor 3 (Runx3) work antagonistically, controlling the lineage fates and stability of $CD4^+$ and $CD8^+$ T cells respectively after exiting the thymus. Runx3 co-operates with Eomes and T-bet, to induce transcription of cytolytic effectors, however, ectopic expression of ThPOK by $CD8^+$ T cells has been shown to reduce expression of transcription factor Eomes, resulting in a blunted CTL effector phenotype [153]. In a colitis model of infection—induced by the transfer of naive Thpok-GFP or Runx3-YFP $CD4^+$ T cells—Mucida *et al.* were able to demonstrate that a subset of expanded $CD4^+$ T cells downlregualted Thpok expression after migrating to the intestinal environment where IFNγ and IL17 were expressed in abundance. These cells increased expression of Runx3, resulting in the production of cytotoxic effector molecules such as granzyme B, IFNγ and TNF. The authors also showed that TGFβ—a critical regulator of Th17 differentiation—can suppresses ThPOK, resulting in increased Runx3 and T-bet expression, and reduced IL17 expression [154]. Their team expanded this model to assess the impact of T cell specificity, by analysing the effector differentiation of transferred ovalbumin p323 peptide-specific TCR Tg OT-II $CD4^+$ T cells. At steady state, $CD4^+$ T cells that downregulated ThPOK were quiescent even in the continuous presence of p323-

peptide feeding. However, in the context of IL15 administration p323-peptide feeding resulted in dramatically increased inflammatory and cytolytic functions in Runx3-expressing $CD4^+$ T cells [155]. Whilst no similar studies have been performed in lung, we hypothesise that a similar antigen-specific mechanism might also drive the differentiation of memory $CD4^+$ T cells towards cytotoxicity during influenza infection, whereby infected epithelial cells express cognate antigen via MHC class II in the context of type I/II IFN signalling and DC-derived IL15 [75, 77, 102, 121, 156, 157]. B lymphocyte-induced maturation protein 1 (Blimp1/Prdm1) has been shown to facilitate the binding of T-bet to the granzyme B and perforin promoters in CD4 T^+ cells [157]. Interestingly, T-bet, which drives the differentiation of $CD8^+$ T cells, also induces expression of Runx3 in $CD4^+$ T cells [158]. Blimp1 and T-bet are regulated by upstream signals that can be modulated by cytokines in the cell microenvironment (Figure 5) [157]. More recently, Kotov *et al*. demonstrated that multiple Th cell subsets may in fact be able to lyse target cells via a combination of cognate MHC class II and Fas-FasL interactions in the context of infection [159].

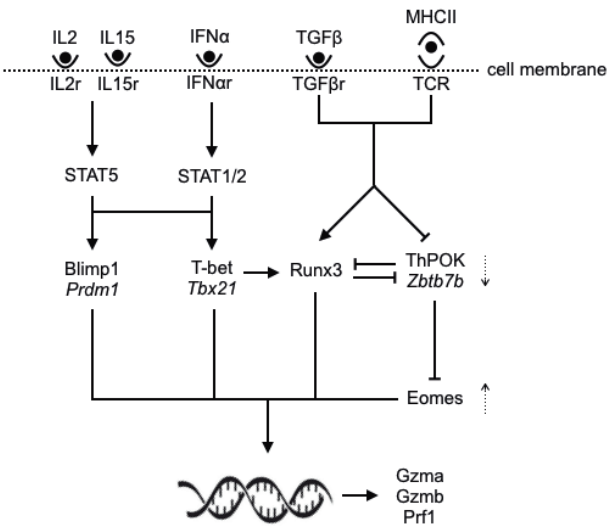


Figure 5. Regulation of cytotoxic $CD4^+$ T cell differentiation.
Adapted from Tian *et al.* [150]

1.4 Towards a universal, mucosal vaccine solution

Multiple research groups are now striving to develop vaccines that generate cross-protective $CD4^+$ and $CD8^+$ T cells against conserved peptides in the influenza virus [126, 160]. Whilst vaccines based on small peptides are advantageous as they have excellent safety profiles, and can be efficiently mass-produced using molecular techniques; peptides alone are not sufficiently immunogenic, and require effective adjuvants to mount immunising responses, particularly if delivered mucosally. Studies that have thus far reached clinical trials largely focus on expressing peptides from NP, M1, M2 and the haemagglutinin HA2 domain via different delivery platforms, most often with the goal of boosting an already existing memory response [161]. Highly promising results have been achieved with vaccines utilising self-adjuvanting modified vaccinia Ankara and simian adenovirus vectors, which induce strong cellular responses when administered parentally or via intradermal injection [162-165]. By contrast, little progress has been made with mucosal delivery systems for influenza vaccination with subcomponent vaccines. In our group we utilise a strong mucosal adjuvant CTA1-DD, which is a fusion protein consisting of the enzymatically active cholera toxin (CT) A1-subunit (CTA1), and a D-fragment from *Staphylococcus aureus* protein A [166, 167]. This adjuvant is non-toxic and safe when delivered intranasally (i.n.). Hence, CTA1-DD provides a platform for needle-free administration of a candidate influenza vaccine, with the added benefit of inducing mucosal IgA and lung-resident memory $CD4^+$ T cells [168]. The ectodomain of matrix protein 2 (M2e) is a 24 amino acid peptide sequence that is highly conserved across influenza strains [169]. Although a large number of animal studies demonstrate that M2e-based vaccines confer protection via induction of non-neutralising anti-M2e antibody, the independent protective contribution of M2e-specific $CD4^+$ T cells has only recently been identified but not yet evaluated [170, 171]. We sought to investigate this by merging M2e with the mucosal adjuvant CTA1-DD to generate in a single fusion protein, CTA1-3M2e-DD, for use in immunisation-challenge experiments.

2 AIMS

The overall aim of this thesis was to explore the mechanisms by which M2e-specific $CD4^+$ T cells mediate protection from influenza virus challenge following mucosal immunisation with CTA1-3M2e-DD.

Specific aims were as follows:

1. Determine the phenotype, localisation and function of M2e-specific $CD4^+$ memory T cells following i.n immunisation.

2. Assess the impact of a selective disruption of specific humoral or cellular immune responses on protection.

3. Evaluate the expansion of memory $CD4^+$ Trm cells upon challenge infection and their importance for protection.

4. Establish a temporal transcriptional profile at the single cell level for M2e-specific memory $CD4^+$ Trm during the timecourse of infection and correlate their activities to the recovery from infection.

5. Assess whether combining M2e with conserved epitopes from selected influenza antigens, i.e the haemagglutinin stalk domain (HA2) and nucleoprotein (NP) could improve immune protection against influenza virus infections.

3 METHODS

Here we summarise important considerations regarding the main tools and methods used to investigate M2e-specific $CD4^+$ T cells in papers 1-III. Specific protocol details can be found in the methods section of each paper.

Mouse models of influenza infection

The primary model organism used in our studies is the BALB/c mouse. Inbred mice are the most frequently used animal model for influenza virus research [172]. The model is practical in terms of animal size, cost, and breeding requirements. Mouse-specific reagents are widely available, and multiple genetic modifications have been characterised to facilitate functional exploration if immune responses *in vivo.* We utilised BALB.B, JHD, DBA/1 and IL17KO strains that introduced specific immune system modifications to our vaccination-challenge model, to help understand the unique contribution of M2e-specific $CD4^+$ T cells to protetion. A limitation of the mosue model is that, owing to a defective Mx1 gene, inbred strains are highly susceptible to infection with mouse-adapted viruses [173, 174]. As such, the primary readouts from challenge infection are survival or death. Influenza infection is much more nuanced in humans, with a wider spectrum of disease. Heterosubtypic protection mediated by T cell vaccines do not prevent infection, as they are not designed to generate neutralising anti-HA antibodies. Their primary aim is to limit morbidity or severity of disease, and this is poorly captured when simply monitoring weight. The recent validation of serum amyloid A as a biomarker of influenza virus disease severity offers an additional, objective measure of non-specific, infection-associated inflammation to assist in comparisons between vaccine regimens and was utilised in paper III.

Intranasal delivery volumes

Influenza infection in humans initiates in the upper respiratory tract. In our studies, mice were initially immunised intranasally with soluble construct suspended in a 20μL volume of PBS, with delivery probably restricted to the upper respiratory tract. Mice were then challenged with virus suspended in a 50 μL volume of PBS. When administered intranasally, it should be noted that the infection model likely

represents a primary lower respiratory tract infection. The distinction between these two comparments is important when reviewing the relative contributions of diffferent responding antibody classes, which may confer protection in a compartment-specific manner.

Flow cytometric analysis with MHC Class I and II Tetramers

Flow cytometry is a method used to measure optical and fluorescence characteristics of single cells in suspension. Immune system cells can be characterised by molecules expressed on their surface memmbrane, or internally in their cytoplasm and nucleus. These molecules can be labelled with flurochrome-conjugated antibodies and analysed with a flow cytometer. Flow cytometers essentially direct lasers at specific wavelengths towards a stream of individual cells stained with a combination of fluorochrome-conjugated antibodies. Each fluorochrome has a specific wavelength excitation window. When a laser of the correct wavelength excites a fluorochrome, it emits photons that are detected and converted into a voltage signal, displayed via a graphical user interface as an "event". For example, an event might be the detection of a CD4-labelled lymphocyte in a single cell suspention from lung tissue. Central to many of our experiments, has been the ability to efficiently label and follow vaccine-induced, antigen-specific T cells by flow cytometry using MHC class I or II tetramers. Tetramers consist of four biotinylated human leukocyte antigen (HLA)-peptide epitope complexes bound to streptavidin and conjugated with a fluorochrome. Tetramers bind directly to cognate TCRs with a specificity up to 0.02%; i.e. an M2e MHC class II tetramer is capable of identifying vaccine-induced M2-specific $CD4^+$ T cells, even if their frequency is as low as 1 in 50,000 lymphocytes [175]. Tetramers are an excellent tool for monitoring and characterising memory T cell responses after vaccination.

Pooled- and single-cell RNA-sequencing

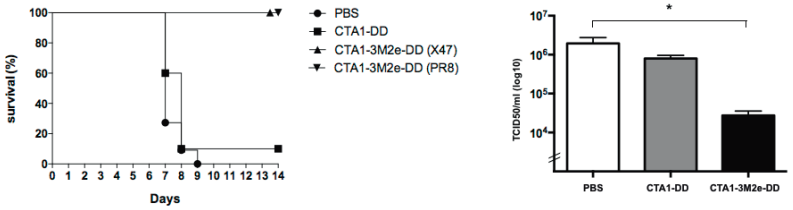
RNA sequencing (RNA-seq) is a genomic approach used to detect and quantify mRNA molecules in a biological sample and is useful for studying cellular responses and processes. For practical reasons, the technique is usually conducted on samples comprising thousands to millions of cells. We employed such a "pooled" approach intitially to evaluate lung-resident, M2e-specific $CD4^+$ T cells, sequencing at a minimum depth of 15 million reads per sample. However, in order understand the

transcriptional diversity within this pool of cells, we embarked on single-cell RNA-seq. A challenge posed by single-cell RNA-Seq is finding the approproiate balance between a large enough number of cells to accurately represent heterogeneity in the population of interest, with sufficient read depth per individual cell, to enable differential gene expression comparisons between individual cells. Whilst there is no clear consensus on the minimum read depth required for unbiased cell type classification, we ensured a minimum depth of 15,000 reads per cell across all experiments [176, 177].

4 KEY FINDINGS

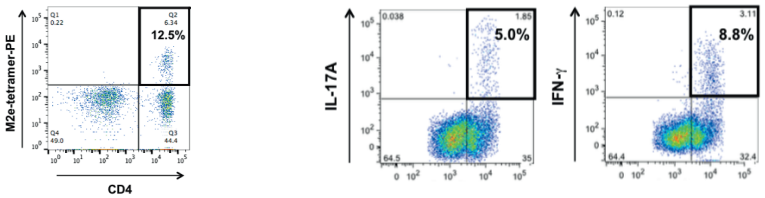
4.1 Paper I

4.1.1 CTA1-3M2e-DD protects against heterologous virus challenge
Fig 1A



This finding confirms that immunisation with the CTA1-3M2e-DD fusion protein results in strong protection from two antigentically different influenza virus strains (left panel), reducing the amount of replicating virus in the lungs (right panel). This serves as an important initial proof of concept that vaccination targeting an influenza surface protein other than the highly variable, immunodominant haemagglutinin can theoretically result in broad protection from severe health outcomes.

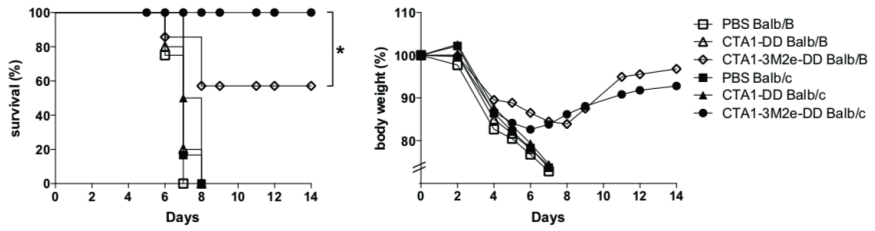
4.1.2 Intranasal immunisation generates M2e-specific CD4$^+$ Th17 cells in lung
Fig 2B, Fig 5A



Studies of M2e-mediated protection largely focus on the role of non-neutralising, serum IgG as the critical factor for protection. There has been little focus on the role M2e-specific CD4$^+$ T cells might play. Here we confirm the presence of M2e-specific CD4$^+$ T cells in lung (left panel), and demonstrate their capacity to respond to M2e peptide by producing inflammatory cytokines (right panel) that may serve to limit virus replication during infection as well as recruit other cells to the site of infection.

4.1.3 *Without M2e-specific CD4$^+$ T cells, protection is diminished*

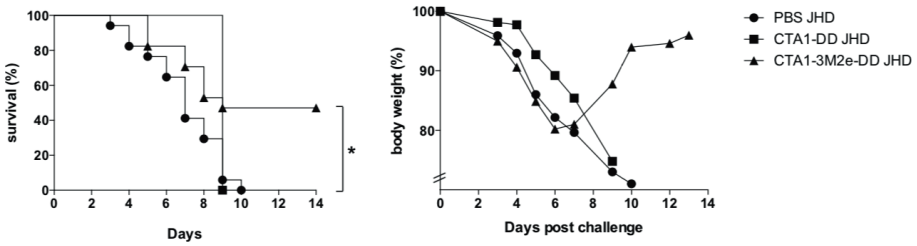
Fig 3A



We used a BALB.B mouse model to demonstrate an important role for CD4$^+$ T cells. Here, immunisation with CTA1-3M2e-DD generates normal antibody responses, but without M2e-specific CD4$^+$ T cells. Reduced protection against infection in BALB.B mice indicates that antibodies alone are not sufficient for full M2e-mediated protection.

4.1.4 *M2e-specific CD4$^+$ T cells protect in the absence of antibody*

Fig 4B

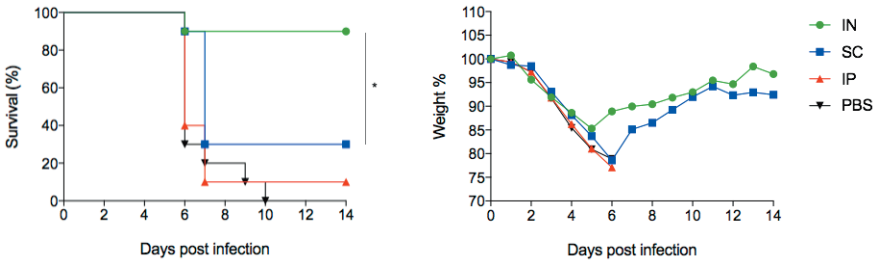


Having confirmed that antibodies against M2e do not work in isolation, we approached the problem from the opposite direction, and asked can M2e-specific CD4$^+$ T cells confer protection in the absence of antibody? The answer was yes. We used JHD mice, which lack B cells and therefore cannot produce anti-M2e antibodies. However, they produce reasonably functional CD4$^+$ T cells that conferred partial protection from infection.

4.2 Paper II

4.2.1 Intranasal administration of CTA1-3M2e-DD is required for protection

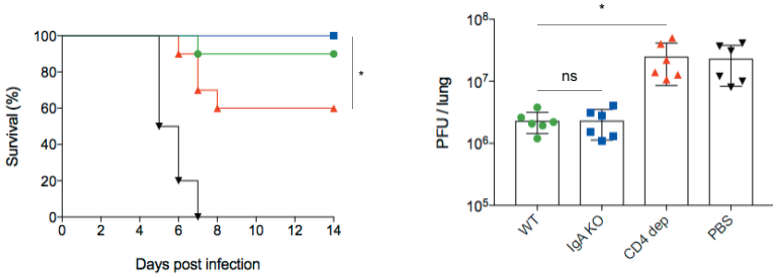
Fig 2A



We hypothesise that intranasal vaccination generates M2e-specific $CD4^+$ T cells that remain in the lung, and that being located at the site of infection is key to their protective function. Surprisingly, we found that when we immunised via other routes, protection was severely affected. This is despite generating similar antibody levels in blood when immunising via these other routes.

4.2.2 Depletion of $CD4^+$ T cells results in impaired viral clearance

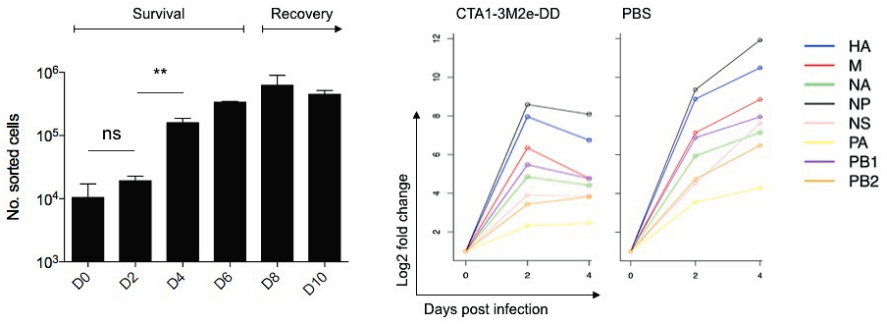
Fig 2C



Given how important the route of vaccine administration was for conferring protection, we tested the hypothesis that $CD4^+$ T cells in lung help to clear virus-infected cells. When immunising via the intranasal route, we generated a special type of antibody that is restricted to mucosal surfaces, IgA. IgA KO mice cannot produce IgA and were protected equally well by CTA1-3M2e-DD. However, elimination of $CD4^+$ T cells from a normally immunised mouse led to reduced survival (left panel) and increased virus replication in the lungs (right panel).

4.2.3 *Rapid expansion of M2e-specific CD4$^+$ T cells triggers viral clearance*

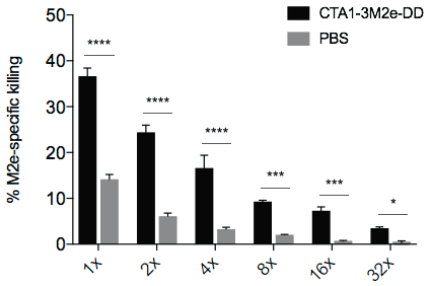
Fig 3B



We used a tetramer label that specifically binds to M2e-specific CD4$^+$ T cells in lung to count the population as it expands during infection. From a resting population of around 10,000 on day 0, M2e-specific cells rapidly divide to reach a peak population closer to 1,000,000 cells 8 days post infection. A statistically significant increase in the number cells between day 2 and day 4 (left panel) correlates well with a reduction in viral genes being expressed in lung that is only observed in immunised mice (right panel).

4.2.4 *M2e-specific CD4$^+$ T cells can directly kill target cells*

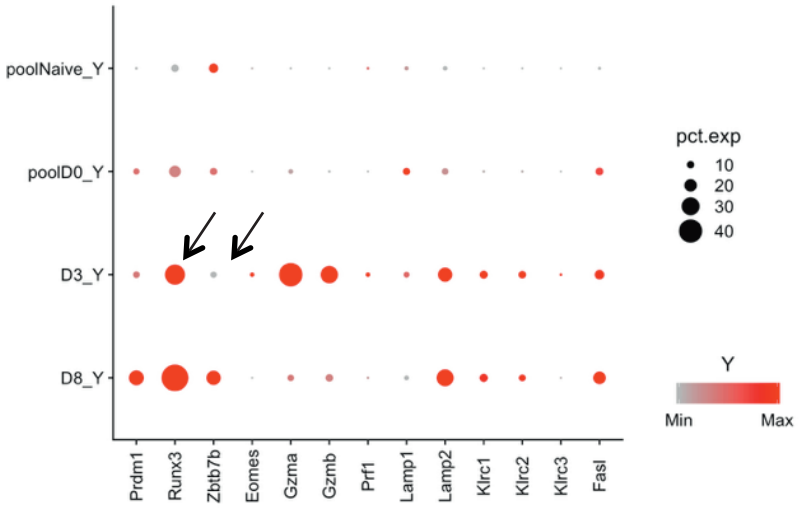
Fig 3E



To test whether M2e-specific CD4$^+$ T cells can kill infected cells presenting viral peptides on their cell surface, we pulsed B cells with M2e peptide and then exposed them to activated CD4$^+$ T cells extracted from the lungs of mice infected 4 days previously. CD4$^+$ T cells taken from the lungs of immunised mice were much better at killing B cells presenting M2e peptide.

4.2.5 *Cytotoxicity is hallmarked by ThPOK (Zbtb7b) downregulation*

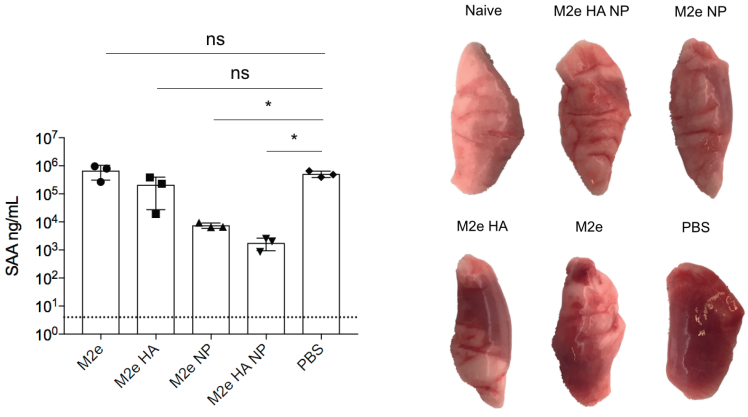
Fig 4C



By monitoring gene expression in M2e-specific $CD4^+$ T cells at different time points during infection, we were able to determine how these cells switch on their cytotoxic functions. ThPOK is an important regulator of $CD4^+$ T cell function. Other studies have shown that downregulation of ThPOK, in combination with upregulation of Runx3, results in the expression of cytotoxic factors such as granzymes (Gzma, Gzmb). We show that these genes are upregulated in the context of a wave of inflammatory signals sent from infected nearby cells, but may also result from direct infection of $CD4^+$ T cells by viruses.

4.3 Paper III

4.3.2 Combining CTA1-DD-vectored influenza epitopes improves protection Fig 3B and 3C



One of the main challenges posed by influenza viruses is their ability to continuously mutate. As a result, vaccine developers strive to incorporate several conserved viral antigens within a single vaccine. But this can sometimes come at a cost as adding a new component, may reduce the effectiveness of existing components. We tested CTA1-3M2e-DD in combination with other candidate vaccines CTA1-2HA-DD and CTA1-2NP-DD, and found that combining these vaccines reduced markers of lung inflammation in blood 4 days post infection (left panel). Indeed, the impact of combining these vaccine formulations was visible to the naked eye (right panel).

5 CONCLUSIONS

1. Immunisation with CTA1-3M2e-DD generates lung-resident, M2e-specific $CD4^+$ Th17 cells, capable of supporting robust mucosal IgA and serum IgG antibody responses.

2. Immunisation with CTA1-3M2e-DD confers long-term homosubtypic and heterosubtypic protection from lethal influenza virus challenge infection.

3. Cxcr6-expressing, M2e-specific memory $CD4^+$ T cells cluster at high density in respiratory sub-epithelium, and contribute to the formation of ectopic iBALT structures.

4. Lung-resident, M2e-specific $CD4^+$ T cells do not require support from lymph node or circulating memory cells to confer protection.

5. M2e-specific $CD4^+$ T cells in lung expand rapidly at the onset of influenza virus infection, exhibiting an early cytotoxic transcriptional signature characterised by termination of Zbtb7b expression and upregulation of Runx3, Gzma and Gzmb, expressed in the context of numerous interferon stimulated genes such as Ifitm3.

6. Viral clearance leads to reduced IFN-mediated signalling and resumed Zbtb7b expression in M2e-specific $CD4^+$ T cells, which adopt a Th17 transcriptional profile hallmarked by increased IL17a and Furin expression.

7. The combination of CTA1-3M2e-DD with CTA1-2NP-DD, a fusion protein including a conserved $CD8^+$ T cell epitope from nucleoprotein, provides significantly stronger anti-influenza protection with reduced inflammation.

6 FUTURE PERSPECTIVES

Experimental work presented in this thesis highlight the potential value of vaccines that generate T cells recognising conserved influenza proteins. Conventional influenza vaccines focus predominanttly on generating B cell responses to immunodominant surface HA. This is a convenient target as antibodies binding to the HA1 domain of HA are neutralising, providing a clear correlate of protection. T cell vaccines do not aim to prevent infection, rather they aim to prevent severe disease. The multifunctional nature of T cell responses—especially $CD4^+$ T cells—make finding a reliable immune correlate much more challenging. We utilised time-series RNA-seq to characterise potential regulatory mechansims that permit M2e-specific $CD4^+$ T cells to become cytotoxic. A key drawback of this approach is the failure to simultaenously capture protein level expression data from sequenced cells. As a result, many of the outputs require additional validation. However, emerging technologies look set to make simultanous characterisation of protein and gene expression foor multiple cell types in parralel, a realistic and affordable possibility in future. What remains to be seen, is whether computational hardware and software advances will be able to keep pace. Should they be able to, integrative "-omic" technologies will enable researchers to evaluate responses to vaccines in a much more global way.

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