ADJUVANTS AND AGE: UNDERSTANDING VACCINE RESPONSE IN OLDER ADULTS

by

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This thesis is dedicated to:

My mother and father, who taught me to value education and cherish knowledge, and who have put up with quite a few impromptu lectures on infectious diseases over the years: I love you, and I could not have done this without you.

My sister, whose humor and unquestioning support have given me strength to finish this degree.

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List of Abbreviations

ACIP: Advisory Committee on Immunization Practices

AIM: Activation induced marker

APC: Antigen presenting cell

aTIV: Adjuvanted trivalent influenza vaccine

BCR: B cell receptor

cT_{FH}: Circulating T follicular helper cells

CSR: Class-switch recombination

DAMP: Danger-associated molecular pattern

DC: Dendritic cell

dLN: Draining lymph node

fDC: Follicular dendritic cell

IFN: Interferon

LN: Lymph node

LPS: Lipopolysaccharide

mDC: Myeloid dendritic cell

MDDC: Monocyte-derived dendritic cell

MHC: Major histocompatibility complex

MPL: Monophosphoryl lipid A, trademarked by GlaxoSmithKline

MPLA: Monophosphoryl lipid A, commercially available

NET: Neutrophil extracellular trap

NF-κB: Nuclear factor kappa light-chain enhancer of activated B cells

NK: Natural killer cells

PAMP: Pathogen-associated molecular pattern

pDC: Plasmacytoid dendritic cell

PRR: Pattern recognition receptor

RZV: Recombinant zoster vaccine

SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2

TCR: T cell receptor

T_{FH}: T follicular helper cells

T_H1: T helper 1

T_H2: T helper 2

TIV: Trivalent influenza vaccine

TLR: Toll-like receptor

T_{REG}: T regulatory cells

VZV: Varicella zoster virus; herpes zoster

Adjuvants and Age: Understanding Vaccine Response in Older Adults

Abstract

by

CARSON L. SMITH

Vaccination forms an immune memory to protect organisms from pathogens. Vaccination remains a critical part of the public health arsenal against infectious diseases. However, with age, the immune system experiences functional declines. This results in both increased susceptibility to disease as well as a decline in vaccine efficacy. To combat this, several vaccines targeted toward older adults use immune-boosting components like adjuvants.

Adjuvants take advantage of the innate immune system to sense potential pathogens and promote effective adaptive responses to vaccines. However, it is not fully clear from mouse models 1) what innate immune responses lead to a protective adaptive response and 2) what a protective adaptive response looks like. In this thesis, we examine two adjuvanted vaccines shown to increase clinical protection in older adults.

In chapter 3 of this thesis, we explore the model provided by the recombinant zoster vaccine (RZV). RZV is highly efficacious in older populations and utilizes the novel adjuvant AS01. We use techniques such as flow cytometry, RNA sequencing, and co-culture with T cells to analyze how AS01 affects human myeloid cells. We show that AS01 activates human myeloid cells, particularly monocytes, towards expression of inflammatory cytokines and costimulatory

molecules. The other vaccine model examined is the adjuvanted trivalent influenza vaccine (aTIV), which has been shown in clinical trials to provide superior protection over unadjuvanted influenza vaccine. In chapter 4 of this thesis, we assess the humoral and cellular immunity induced by aTIV and TIV. We show that aTIV preferentially enhances anti-neuraminidase titers compared to TIV, potentially explaining its superior clinical protection in older populations.

While age-related immune decline is demonstrable and results in increased clinical mortality, growing evidence suggests that aged immune systems can still respond to stimuli under the optimal circumstances. This thesis explores the mechanisms and vaccine outcomes of two vaccines that contain adjuvants and were developed for use in older adults. By better understanding adjuvants that have proven efficacy, we can better understand how adjuvants provide protection in a vulnerable population. This research can help us develop novel adjuvants for vaccines in the ongoing public health battle against infectious diseases.

Chapter 1: The Immune System and Vaccination

1.1 An introduction to the immune system

The immune system is, broadly speaking, a collection of cells, molecules, and structures that detect and eliminate foreign stimuli to protect the organism from disease. In mammals, the immune system is in constant communication with the rest of the organism and is exquisitely managed by a complex network of signals and checkpoints. This network helps the immune system recognize self and target non-self antigens [1–3].

The immune system is divided into two compartments, innate and adaptive immunity. Innate immunity is inborn: all the information needed for these cells to respond to non-self molecules is encoded at the genomic level. Cells belonging to the innate immune system detect universal signals, known as pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), through a series of receptors and sensors known as pathogen-recognition receptors (PRRs) [4]. One class of such PRRs are the Toll-like receptors (TLRs), which detect a wide variety of ligands that are not found in healthy mammalian cells such as unmethylated double-stranded DNA (dsDNA) or the bacterial glycan lipopolysaccharide (LPS)[5]. Innate immune cells are the first line of defense in recognizing and repelling microbial invaders. Cells included in the innate immune system include monocytes, natural killer (NK) cells, neutrophils, and dendritic cells (DCs) [6].

The adaptive immune system is bifurcated into two major components, humoral and cellular immunity. Humoral immunity is composed of antigen-binding molecules called antibodies and the cells that produce them, B cells [7]. In

contrast, the cellular immune compartment consists of cells that either act directly on infected cells or secrete factors that aid in killing. These cells mature in the thymus and are known as T cells. Unlike the cells of the innate immune system, which express PRRs that recognize PAMPs and DAMPS, each cell of the adaptive immune system descends from a unique clone that recognizes one antigen [8].

The adaptive immune system, unlike the innate, requires priming by antigen-presenting cells (APCs) or prior exposure to an antigen for an effective response. B cells require helper T cells known as follicular helper T cells (T_{FH}) in the lymph node (LN) to generate an effective response [9]. T cells require antigens to be presented in the context of an APC on a molecule known as the major histocompatibility complex (MHC). MHC Class I is expressed on all cell types (except for mature erythrocytes) and is a key part of self-recognition, while MHC Class II is expressed only on certain cell types like APCs and B cells [10]. MHC Class I and Class II are highly polymorphic, with several genes and hundreds of alleles for each class[11]. T cells are further subdivided depending on their expression of CD8 and CD4, which interact with MHC Class I and Class II, respectively [12]. Without TCR recognition of MHC: peptide, T cells do not respond to antigens.

These subdivisions between the innate and adaptive immune systems are somewhat artificial. There is extensive crosstalk between the innate and adaptive immune system, for example, and many innate immune cell functions are enhanced by humoral immunity [13]. For example, MHC Class I and II are

constitutively expressed but are strongly upregulated by cytokines such as interferons (IFNs) [10]. Antibodies produced by B cells enhance the engulfment of pathogens by phagocytic cells like monocytes and neutrophils via recognition of the antibody F_C chain [13]. T cells aid in the development and maturation of B cells, and an entire subset of T cells, T helper (T_H) cells, are known for their ability to provide “help” to B cells and other effectors [14]. Additionally, there are some signals recognized by the immune system, such as the expression of MHC Class I, that are expressed by all cells, not just those classically recognized as part of the immune system [15]. However, these subdivisions are useful for conceptualizing the immune system and thus have been maintained over decades.

1.2. The immune system in action against pathogens

The typical immune response begins with an exposure to a substance recognized as non-self. This recognition could occur through a myriad of mechanisms but is most classically initiated through innate recognition of a PAMP or DAMP [5,16]. For example, a virus could enter the body through the lungs and be recognized by the PRRs of a variety of patrolling cells within the lung epithelium. Neutrophils are the usual immediate responders to damage signals, as they make up to 80% of the white blood cells in human blood [6]. Neutrophils may kill the source of an antigen through effector mechanisms such as cytotoxic granule release, reactive oxygen species production, or the production of neutrophil extracellular traps (NETs) made of DNA and protein [6]. Neutrophils and other innate cells such as macrophages may also engulf and

digest viral particles based on detected PAMPs, thus eliminating them and limiting the molecular signal. In cases where the innate cell(s) are unable to immediately eradicate the pathogen, the signals given by PAMP:PRR interactions are prolonged, and gene transcription occurs. Many of the transcribed products downstream of PAMP:PRR interactions are cytokines, molecular signals that can summon or activate other immune cells [17].

As a result of these cytokine signals, as well as chemokines induced in response to damage signals elicited at the site of infection as a result of the pathogen, other innate immune cells such as monocytes or DCs are recruited [18,19]. In an activating cell environment, these cells differentiate into an active form that processes proteins into peptides and displays them on MHC complexes on their surface [6]. In response to signals produced at the site of infection, these activated APCs upregulate CCR7 and migrate to immune structures known as lymph nodes (LN), which are scattered throughout the body [19]. Soluble antigens are also capable of directly entering the lymphatic system and traveling to draining lymph nodes (dLN)[20]. Antigens can also be acquired at the site of infection by professional APCs or be captured by patrolling follicular dendritic cells (fDCs) [19,21].

Once in the LN, the antigen encounters B cells either directly or on the surface of an APC [20]. Small, soluble antigens can infiltrate the LN and encounter B cells directly, while larger antigens usually involve a mediator cell type such as subcapsular CD169+ macrophages or fDCs [21]. Regardless of how the antigen gains access to the LN, it binds the B cell receptor (BCR) and is internalized to

be later re-presented as a complex with MHC Class II. The BCR is a membrane-bound form of the antibody produced by the B cell after a complex series of genetic recombinations known as V(D)J recombination [7]. B cells that have found their cognate antigen then present epitopes on MHC Class II molecules. The MHC:antigen complex is recognized by T cells specific for those epitopes [8,16]. The B cells enter an exchange with T_{FH} cells, in which the activated T_{FH} cell provides cytokine and ligand help to the B cell based on its affinity for the MHC Class II:antigen that the B cell is presenting [7,16]. T_{FH} help, specifically CD40:CD40L interactions, is required for full B cell activation and to promote class-switch recombination (CSR) and somatic hypermutation (SHM) [20,22]. Another key signal is the interaction of inducible T cell costimulator (ICOS) on activated T cells with its ligand, ICOSL, on cognate B cells [6].

At the same time, naive T cells in the T cell zone of the LN recognize antigen presented on DCs. Unlike B cells, T cells are MHC-restricted and must encounter antigens in association with MHC complexes to be activated [20]. T cells compete for antigen binding in the paracortex of the LN [21]. This competition is immense, with an estimated $2.5x10^7$ potential naive T cell receptors (TCRs) per individual[8]. Tells with higher affinities for MHC:antigen complexes ultimately out-compete lower affinity T cells, binding to APCs and receiving activating signals [23]. Naive T cells require costimulation, MHC:peptide, and tertiary signals such as activating cytokines in order to respond to their cognate antigen [16,24]. This combination of signals, particularly the engagement of the TCR by MHC:peptide, results in the activation and clonal proliferation of T cells [23].

Additionally, tertiary signals from APCs play a key role in naive T cell differentiation. Such signals include ligation of the T cell ligands ICOS, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and Ox40 as well as the production of cytokines such as IL-21, IL-12, IL-6, or TGF-β, which can skew T cell development towards a variety of T helper subtypes [24].

As a result of this clonal proliferation, an influx of antibody-producing plasma cells, chemokines, and cytotoxic T cells are released from the LN and follow chemotactic gradients back to the site of infection. CXCL9 and CXCL10 are particularly important ligands for T cells during this process [25,26]. The infection is eliminated by the influx of cells and specialized effector molecules like antibodies. This results in a decrease in antigen concentration [26]. Without this source of ongoing stimulation, T cells and B cells begin to die off in a phase known as contraction [20]. However, some lymphocytes will remain as memory cells, ready to respond and expand upon a second stimulation. This second expansion is more rapid, as the adaptive cells exist already in higher frequencies than before the primary expansion [3]. These memory cells also possess a lower activation threshold, allowing them to respond to antigens with less antigen and minimal costimulation [27]. Further protection is provided by the persistence of long-lived plasma cells, which maintain antibody levels targeted against the antigen in question [7].

1.3. A closer look at the innate immune system

In an immunocompetent individual, a humoral immune response develops in the germinal center of the LN after antigen exposure as a result of complex

interactions between DCs, B cells, and T cells. DCs are the professional APC of the LN and are a highly heterogeneous cell population. They participate in all three signals for lymphocyte activation: the initial TCR:MHC/peptide stimulus, costimulation with CD28 as well as other markers like CD80/CD86, and the requisite third signal for effector T cell function, cytokine instruction. DCs are divided into two main subpopulations: plasmacytoid DCs (pDCs) and myeloid DCs (mDCs). pDCs are typically found in the bloodstream and produce high amounts of IFN-α compared to mDCs; they are also known to express TLR7 and TLR9 [28]. mDCs are specialized towards antigen presentation and can be subdivided based on their expression of CD141 and CD1c, also known as BDCA1 and BDCA3 [29]. In addition to these DC subtypes, CD14+ monocytes can, with specific cytokine signals, differentiate into DCs *in vivo*: this process is often used in *in vitro* models to generate monocyte-derived DCs (MDDCs) for use as antigen-presenting DCs [30].

After antigen exposure, DCs make their way to the LN and proceed to migrate to specialized niches based on the expression of factors such as CXCR3 [25]. Regulation of these markers as well as the cytokines and signaling molecules upregulated on the DC play a key role in T_H differentiation. For example, IL-12 is a key inducer of a subtype of T cells called T_H1 cells, and DCs receive positive feedback from IFN-γ derived from NK cells to promote further IL-12 production [24]. Surface marker expression, such as CD11b found on mDCs, also appears to enhance the proliferation of CD4+ rather than CD8+ T cells in an MHC-dependent manner [31,32]. Other surface markers, such as CXCL9 and CXCL10,

are associated with the development of T_H1 and are upregulated in response to IFN-γ [24]. DC function is thus key to eliciting both cellular and humoral immune responses, and DC expression of surface markers and cytokines helps drive the direction of the adaptive response.

Monocytes are another key innate immune cell population. These cells are circulating phagocytes capable of cytokine production. They recognize foreign antigens based on their PRRs and engulf them through phagocytosis, destroying the antigen by lysosomal fusion with the phagosome [33]. Monocytes and their close tissue-localized relatives, macrophages, are a highly heterogeneous population [34]. Indeed, current theory holds that monocytes infiltrate specific tissues from the blood and specialize into tissue-specific macrophages populations like T cell zone macrophages in the LN, dermal macrophages in the skin, or Kupffer cells in the liver [35–37].

In the periphery, monocytes can be categorized into broad phenotypes that based on their expression of surface markers CD14 and CD16 in humans. CD14+CD16- monocytes are also known as classical monocytes and tend towards inflammatory phenotypes [38]. They are the most common monocyte type found in human blood [39]. In contrast, $CD14^{lo}$CD16+ monocytes are known as non-classical monocytes and typically respond in cases where tissue repair is necessary. They express high levels of CX3CR1, a chemokine receptor for CX3CL1, and display “crawling” behavior along the endothelium, allowing surveillance of the vascular tissue and removal of debris [38,40]. While typically

considered anti-inflammatory, non-classical monocytes can make high levels of TNF-α under certain conditions like the detection of TLR7 ligands [41].

Monocytes are an extraordinarily plastic cell set. Depending on the environmental signals received, they can phagocytose antigens, clear apoptotic cells, present antigens to T cells, or promote wound healing through secretion of factors such as vascular endothelial growth factor (VEGF) [13,33,42–44]. In addition, monocytes can differentiate into both macrophages and monocyte-derived DCs, allowing further tailoring to their environment [45]. Even the classical/non-classical paradigm set out above is more of a representation of two extremes of differentiation rather than distinct subsets [34]. Because of this heterogeneity, monocytes are a key part of an effective innate immune response as well as a bridge with adaptive immunity.

Innate cells also play key roles in the LN. CD169+ macrophages act as antigen traps at the LN sinus. These macrophages have been shown to be capable of directly presenting antigen to B cells [46]. Some evidence also demonstrates that CD169+ macrophages are capable of presenting antigen to naive CD8+ cells, especially in situations where DCs cannot access enough antigen to present properly [47,48]. CD169+ macrophages are particularly important in the case of liposomal adjuvants, where their apoptotic death after inflammasome activation results in an amplified adaptive response [48–50]. Other innate cells such as follicular DCs (fDCs) also play key roles in sampling antigens and presenting them to lymphocytes. By secreting CXCL13, fDCs also play

central roles in maintaining germinal center architecture and guiding lymphocyte migration between the dark and light zones of a B cell follicle [20].

1.4. A closer look at the adaptive immune system

For effective immune memory generation in adults, functional T and B cells must be generated and maintained. These cells are part of resolving initial infection and continue to provide protection against recurrent infection and disease, sometimes for decades [51]. In this section, B cells and T cells will be discussed in more detail, with a particular emphasis on T cells.

After antigen exposure, B cells develop into either antibody-producing plasma cells or long-lived memory B cells [21]. Plasma cells typically have high affinity for antigen and serve as antibody factories. These cells can be identified by their expression of CD38 and CD138 [52]. B cells may also differentiate into memory B cells, which are long-lived and typically have a more diverse repertoire than plasma cells [22]. Upon reactivation, these B cells rapidly begin competing for T_{FH} help, beginning the cycle anew [53]. Thus, B cells can serve as a source of infection prevention in the form of plasma cells as well as a rapid adaptive responder upon reinfection.

B cells continue to evolve throughout the immune response due to competition between V[D]J clones as well as somatic hypermutation (SHM) within clones, ultimately resulting in antibodies with higher affinity for antigen [7]. SHM is a T cell-dependent process in which B cells selectively mutate the heavy and light protein chains that make up their antibodies. Mutated B cells are then able to compete for T cell help in the light zone of the germinal center, allowing

BCR mutants with higher affinities for the antigen to persist [22,53]. Another way that B cells can specialize based on T_{FH} help during this process is class-switch recombination (CSR), which changes the subtype of immunoglobulin (Ig) produced from IgM to a more specialized form [9]. These Ig subtypes, such as IgA, IgG, IgD, and IgE, play specialized roles in different immune compartments. For example, IgE is produced in response to helminth infections, and IgA is typically secreted into mucosal surfaces [6,9]. B cell specialization is thus heavily T-cell dependent.

The T_{FH} that are so key to developing effective humoral immunity are part of a subdivision of T cells known as CD4+ T cells, or T helper (T_H) cells. CD4+ T cells are MHC Class II-restricted and play diverse roles in the coordination of an immune response. Classically, CD4+ T cells are subdivided based on the cytokines they make and the transcription factors driving their differentiation. For example, T_H1 cells are defined by their expression of transcription factor T-bet and production of IFN-γ [24]. Other CD4+ T cell subsets are T_H2, which express GATA-3 and produce IL-4 and IL-5; T_H17, which express RORγT and produce IL-17; and T regulatory cells (T_{REG}), which express FoxP3 as well as inhibitory molecules such as CTLA-4 and IL-10. CD4+ T cells that participate in B cell differentiation (T_{FH}) express transcription factor BCL-6 and produce IL-21 as well as various costimulatory markers [9,24]. T_{FH} play key roles in generating highly specific antibodies via their roles in CSR and SHM.

In humans, T_{FH} are difficult to study due to their location in the LN. However, there are T_{FH}-like cells known as circulating T_{FH} (cT_{FH}) present in the

periphery that can be identified on the basis of CXCR5, a LN homing marker, and PD-1 [54,55]. Like T_{FH}, these cells produce relatively low levels of cytokines and are difficult to identify by intracellular staining for cytokines. Instead, activation-induced marker (AIM) staining can be used to identify antigen-specific cT_{FH} populations. The use of AIM to identify antigen-specific cT_{FH} is well-noted in the literature, and it is believed that increased antigen-specific cT_{FH} are a peripheral reflection of increased germinal center proliferation [55–57]. It is still unclear whether cT_{FH} are merely a measurable reflection of the germinal center response or are themselves a population that interfaces with humoral immunity; however, the increased presence of antigen-specific cT_{FH} following influenza vaccination implies T cell help being given to B cells and a correspondent increase in antibody titer and specificity (see Chapter 4).

CD4+ T cells can also organize CD8+ T cell responses through the generation of cytokines such as IL-2, though their main contribution relates to modulation of antigen presentation and costimulation of DCs for optimal CD8+ response. Indeed, CD4+ T cells are key for CD8+ T cell memory maintenance, and adoptive transfers of CD4+ T cells have been shown to help reverse CD8+ T cell dysfunction in mouse models [58]. CD4+ T cells also marshal adaptive effector cells through cytokine production, such as with macrophage activation through T_H1 IFN-γ or mast cell development prompted by T_H2 IL-4 production [58,59]. CD4+ T cells are thus a heterogeneous population involved in coordinating immune responses on both the adaptive and innate level.

CD8+ T cells are MHC Class I-restricted and recognize antigens that are derived from virally-infected or cancerous cells [60]. They are often referred to as cytotoxic T lymphocytes (CTL) for their ability to directly kill their targets by inducing apoptosis [61]. CD8+ T cells have multiple mechanisms by which they may kill their targets. They secrete lytic granules containing perforin, granzymes, and signaling molecules such as FasL. Perforin oligomerizes to form pores in target cell membranes, through which lytic molecules such as granzymes can pass [62]. Granzymes are able to induce apoptosis after cellular entry via their serine protease activity [62,63]. FasL, meanwhile, signals through the FAS receptor (CD95) on target cells and activates the FAS-associated death domain (FADD). Caspase 8 activation and apoptosis soon follows [63]. These CD8+ T cell functions are typically supported by IFN-γ production [64]. Like CD4+ T cells, CD8+ T cells are critical to control and resolve viral infection.

T cells are generally thought to play key roles in reducing disease severity and duration, reducing hospitalization and mortality. For example, lung resident CD8+ T cells are believed to be central to resolving infection if influenza is contracted [65,66]. Additionally, memory T cells tend to be more cross-reactive than antibodies, increasing protection in case an antigen mutates [67]. The role of T_H cells, particularly antigen-specific T_{FH}, in eliciting humoral responses has also been of interest in recent years. Studies show that T_{FH} responses in mice and cT_{FH} responses in humans increase in proportion to humoral titers against influenza [68,69].

1.5. Vaccination and clinical protection

Vaccines rely on this system of immune memory to provide protection against pathogenic infection. Vaccines provide a controlled initial exposure to pathogenic antigens without the risk of uncontrolled infection. After vaccination, a pool of memory T cells and B cells remain that have experienced the vaccine's antigen and are ready to respond upon natural infection. Additionally, the presence of memory B cells provides protection against acquiring an infection in the first place, as antibody-producing B cells ensure a constant level of serum protection from the cognate antigen [70,71]. Much research has focused on identifying what parts of an immune response are protective against a given disease. As a result, much is known about the roles that various immune cells and mediators play in protecting individuals from infection.

Establishing humoral immunity is one of the major goals of most vaccinations. Humoral immunity provides long-lasting immunity against infection. For example, children administered vaccines such as the measles/mumps/rubella (MMR) vaccine still have detectable titers 20 years later [72,73]. Titers against proteins used by pathogens for entry into host cells are particularly useful for preventing viral infection. Influenza vaccines are widely considered protective if a 1:40 titer against hemagglutinin can be established [74]. Some of this vaccine protection is dependent on the physiological location of antibodies. Vaccination against human papilloma virus (HPV) relies on the translocation of serum antibodies to the cervical mucosa, where high levels of antibodies are able to prevent infection entirely [75]. Pneumococcal vaccines also

rely on localized humoral protection in the form of lung IgA levels [76]. Because of the need for tissue-specific localization, serum antibody titers may not always predict protection against disease.

Cellular immunity also plays a role in vaccine-induced protection. T cells are critical in responding to intracellular bacteria [77], and the role of T cells in responding to viral infection is increasingly recognized [78–80]. The zoster vaccine RZV protects against symptomatic varicella zoster virus (VZV) reactivation via CD4+ T cells, which directly correlate with protection [81]. Skin memory T_H1 cells have been shown to be necessary for protection from poxviruses through their production of IFN-γ and subsequent activation of keratinocyte anti-viral programs [82]. T cells also have roles in limiting the severity of respiratory viruses like influenza and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In older adults, protection against influenza has been linked to granzyme B and IFN-γ/IL-10 ratios [83,84]. CD8+ T cells, while not involved in preventing infection, are key to clearing viruses from the lungs and controlling viral replication. They are therefore crucial in mediating protection from serious disease [78,85]. Lung-resident T cells are also important in cross-strain protection for both influenza and SARS-CoV-2 [79,80].

Vaccination is a world-changing public health intervention, but it often fails to prevent infection on an individual level. Infections acquired after vaccination, or breakthrough infections, can occur because pathogens have sufficiently mutated to be able to evade the memory responses [71,86,87]. However, the immune system also changes as organisms age. These changes affect extant immune memory

as well as the immune system’s ability to respond to vaccines. The next chapter will discuss age-related immune dysfunction and how it affects vaccine efficacy in aging populations.

Chapter 2: Age and Adjuvants

The previous chapter laid out a model of a functional immune system. However, the human immune system experiences many changes with age that move it away from the ideal. Age-associated immune dysfunction is complex and takes many forms. It correlates with but does not necessarily equal chronological age [88,89]. Age-associated changes in the immune system take place across both the adaptive and innate arms and make aged persons more susceptible to infectious diseases such as bacterial pneumonia, herpes zoster, and influenza [90–93]. This has been starkly seen in the recent COVID-19 pandemic, for which age is associated with mortality and ICU admission [94,95].

As the population ages, the need for protective interventions like vaccines increases. As of the 2020 census, over 50 million adults in the US are over the age of 65 [96]. This number is only projected to grow as the large generation born after 1945 transitions to late adulthood: worldwide, adults over 65 are expected to make up 16% of the population by 2050 [97]. In order to develop vaccines that are effective even in this aging population, an understanding of how the immune system functions with age is needed. In this chapter, we will discuss specific immune changes associated with age and how those changes impact the model of immune function laid out in the previous chapter. We will conclude with a discussion of adjuvants as a way to overcome these dysfunctions and generate a protective immune response.

2.1. Age-related immune dysfunction

2.1.1. Adaptive immune dysfunction

Age negatively impacts B cell response, especially to novel pathogens. Naive B cells decrease in frequency and number with age. This phenomenon is at least partially due to a decrease in B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) in serum from older adults [72]. Older adults typically have lower antibody titers than younger adults. However, antibody affinity in older adults is typically similar, potentially due to antigen exposures earlier in an individual's life [72,98–100]. B cells from older adults retain their ability to upregulate costimulatory markers and participate in the germinal center reaction but are deficient in CSR and SHM due to a decreased expression of activation-induced cytidine deaminase (AID) and E47, which mediate these processes [101]. However, mouse models suggest that at least some of the defects seen in aged humoral immunity actually derive from defects in help provided by CD4+ T cells. Mouse studies have shown that CD4+ T cell adoptive transfers from younger mice can rescue germinal center formation in older mice, indicating a role for CD4+ T cell dysfunction in age-associated declines in humoral immunity [68].

The T cell compartment experiences broad changes with age. The key site of T cell maturation, the thymus, shrinks with age. The loss of this developmental niche reduces the production of naive T cells and promotes homeostatic proliferation of naive T cell clones [72]. Both naive CD4+ and naive CD8+ T cells decline in total number and frequency as individuals age, although the decline is much more dramatic for CD8+ T cells [102]. The T cell compartment

also experiences a contraction in TCR diversity, limiting the potential TCR response in favor of antigens already encountered. Reduced TCR diversity is known to result in a reduced immune response, while increased diversity in the TCR repertoire is correlated with disease resistance [8]. In addition to these broad changes, T cells also experience changes specific to their subtype and function.

Like B cells, CD4+ T cells decline in function with age. Aging has been associated with increased expression of CTLA-4, an inhibitory marker that binds costimulatory B7 molecules on APCs and downregulates rather than stimulates TCR activation [103,104]. Elderly CD4+ T cells have been found to have reduced TCR activation, reduced formation of the immunological synapse, reduced production of key cytokines such as IL-2, impaired costimulation for B cells, defective association of the TCR and CD28, and deficits in multiple signaling pathways such as NF-κB and tyrosine phosphorylation [105–108]. Older T cells have differential expression of regulatory factors such as dual phosphatase 4 (DUSP4), impacting their ability to upregulate CD40L, ICOS, and cytokines like IL-21 after stimulation [109]. CD4+ T cells from older donors demonstrate delays in trafficking to germinal centers, negatively impacting the quality of antibody response and resulting in increased vulnerability to viruses [110]. Additionally, CD4+ cT_{FH} proliferation is known to be reduced in aged adults compared to younger controls due in part to increased inflammation associated with age [68,111]. These functional deficits in CD4+ T cells are multi-factorial and contribute significantly to the age-related decline in immune function, or immunosenescence.

CD8+ T cells also experience changes with age, though it is not clear if all of these changes are dysfunctional. For example, aged antigen-specific CD8+ T cells cause more necrotic cell death than cells from younger mice, but this results in faster target lysis, a desirable state of affairs in an older individual with a higher risk of cancer [112]. Other changes, such as the increase in frequency of memory T cells, are also likely an adaptation to a lifetime of antigenic exposure [89,113]. However, some changes do appear to be detrimental, especially to primary immune responses. Older CD8+ T cells are much more prone to terminal differentiation and subsequent apoptosis [48,114]. Naive CD8+ T cells from older individuals have a reduced ability to expand *in vitro* in response to peptide antigens [115,116]. CD8+ T cells from older individuals also express key transcription factors such as T-bet to a lower degree than younger adults [115]. CD8+ T cells from older adults tend to express lower levels of effector markers in response to stimulation [113,115,116]. This combination of reduced frequency, differentiation, and CD8+ T cell functional defects delay viral clearance in the elderly [48].

It is important to remember that adaptive immune defects do not occur in a vacuum. Adoptive transfers of T cells from young to old mice have shown that not all of these age-related defects are immune cell-intrinsic but rather depend on the signaling environment in which they find themselves [117–119]. Immunosenescent changes in adaptive immune cells are amplified or even caused by changes in innate immune cells. In the next section, we will discuss

changes in the innate immune system that can affect downstream adaptive responses.

2.1.2. Innate immune dysfunction

Innate immune cells interact with and affect the adaptive immune system. For B cells, innate immune cells such as fDC provide key survival and differentiation signals as well as antigen presentation [20,21,72]. For T cells, innate immune cells acting as APCs are critical not only for responding to antigens but also for differentiation [24,28]. Expression of tissue-restricted antigen genes (TRA) is critical for preventing autoreactive T cells from developing, and decline in expression of these genes as well as in the expression of TRA-regulator AIRE have been reported in older adults in thymic structures and B cells [2,120]. Changes in innate immune cell function and phenotype contribute to impaired adaptive immune responses in older adults.

DCs with dysfunctional signaling and activation are associated with age. While DC number, morphology, and baseline expression of such markers as CD80/86 and HLA-DR in peripheral blood appear unchanged when compared to young donors, DCs in elderly donors have reduced migratory activity. They also secrete higher TNF-α and IL-6 in response to stimulus, perhaps as a result of the low-level chronic inflammation associated with aging [121]. Additionally, they have delayed late-response Type I IFN signaling in response to viral antigens, limiting the typical positive feedback seen with activation [122]. DCs from older individuals have lower TLR expression and decreased phagocytic ability [123]. Senescent

phenotypes therefore seem to play a role in DC dysfunction and impaired follicular response in aged people.

Monocytes do not change in frequency with age but do change their phenotype. For example, aged monocytes show decreased upregulation of MHC Class II in response to stimuli as well as increased production of TNF-α at baseline and after TLR4 stimulation [123,124]. In general, monocytes from younger donors produce more effector cytokines such as IFN-γ, IL-1β, and IL-6 than their older counterparts [124,125]. RIG-1 signaling, a key PAMP antiviral pathway in monocytes, is reduced in older adults in the context of influenza [126]. However, monocytes are not completely defective with age. Classical monocytes in particular maintain their ability to switch to glycolytic metabolism– a key part of inflammatory activation– in older individuals [127,128]. Classical monocytes also retain their ability to produce IL-6 and TNF-α in aged persons, though overall IL-6 and TNF-α production in response to stimuli decreases with age [125]. Total monocyte frequency is higher in older donors, and the distribution of monocytic subtypes such as classical/non-classical remains similar to younger adults [129].

It is not known whether complete restoration of APC function is necessary for a productive immune response. The fact that vaccines have at least partial efficacy in older populations suggests that, at minimum, a threshold of immune activation is necessary for protective immune memory. It seems likely that a stronger inflammatory response would correlate with a higher degree of APC functional recovery. However, this approach leads to an increased risk of adverse events, which is observed in vaccines like RZV [130]. It is not known if this

threshold can be reached without increased risk of side effects, highlighting the importance of determining relevant pathways of APC activation in older adults for further adjuvant development.

2.1.3. Senescence and Inflammaging

Many of these defects are related to two key T cell states associated with age: senescence or exhaustion. These states are superficially similar, as they both result in decreased T cell proliferation, but they develop from different causes and have different functional roles. Senescence is a phenomenon that culminates in cell-cycle arrest after cell division limits are reached, a status called the Hayflick limit [89,131,132]. Chronic antigen stimulation is believed to contribute to replicative senescence by driving repeated cellular divisions in response to antigen [133,134]. Senescence is not limited to T cells and is actually best studied in fibroblasts [131,132,135].

Senescent T cells can be distinguished functionally by their failure to proliferate in response to antigen in conjunction with their pro-inflammatory cytokine secretion profile [134]. Senescent T cells express CD57 and KLRG-1 on their surface, which contribute to their impaired proliferation capacity. KLRG-1 is used as a marker of cellular differentiation [136]. Functionally, KLRG-1 acts to downregulate T cell responses through its inhibitory ITIM motif [137]. CD57 is a marker of terminal differentiation: cells that express it have reduced proliferation and shorter telomeres [138]. However, CD8+CD57+ T cells have been shown to retain their cytotoxic potential, as they strongly produce granzyme and perforin

and are still capable of cytokine production [138–140]. Senescent T cells are therefore capable of responding to antigens, even if they cannot proliferate.

Senescence is associated with a phenomenon known as inflammaging. Non-lymphoid senescent cells secrete a cytokine profile referred to as senescence-associated secretory phenotype (SASP) that includes IL-6, IL-7, MCP-2, and MIP-3α alongside growth and survival factors [132]. Macrophages are known to be key sources of IL-6 and TNF-α in senescent tissues [141]. Lymphoid cells also can become senescent and secrete additional inflammatory markers such as TNF-α and IFNs alongside SASP factors, particularly IL-6 [142]. This basal inflammation particularly impacts innate immunity, which in turn alters both naive and memory adaptive responses, especially in the T cell compartment [142,143]. Inflammaging is linked to the increase in frailty associated with chronic inflammatory disorders (see section 2.1.5) and can be worsened by ongoing stimulation of the innate immune system [141,142,144]. In fact, some have argued that inflammaging is at the heart of aging itself [145]. Regardless of the exact relationship between inflammaging and immunosenescence, it is clear that any attempt to address immunosenescence will need to account for the impacts that this chronic low-grade inflammation has upon the immune system.

2.1.4. Exhaustion

In contrast to senescent cells, exhausted T cells are derived from constant or repeated antigenic stimulation and display decreased functionality in a progressive manner [131]. Because exhaustion results from chronic antigen stimulation, exhausted T cells are more common in older individuals, though they

are also present in younger individuals in contexts such as cancer or HIV infection [146–148]. Obesity is also linked to increased T cell exhaustion, likely through background inflammatory signals provided by adipose tissue macrophages [148,149]. Exhausted T cells are notable for both their decreased proliferative ability and their lack of inflammatory cytokine production [134]. Typically, the expression of inhibitory marker expression and inefficient cytokine production in exhausted cells is progressive. For example, cells lose the ability to produce TNF-α and IL-2 before they lose the ability to produce IFN-γ [104]. Exhausted T cells can be identified by the surface co-expression of inhibitory markers such as PD-1, LAG-3, or CTLA-4. Programmed cell death receptor 1, or PD-1, however, remains the seminal exhaustion marker [150–152].

PD-1 is expressed briefly during T cell activation, but high expression of PD-1 for longer periods of time is strongly associated with exhaustion [150,153]. PD-1 interacts with its ligand, PD-L1, to attenuate TCR signaling and T cell activation. This regulatory mechanism can serve to prevent hyper-inflammatory immune responses that may cause more damage to the host than the pathogen. PD-1 signaling also can trigger the expression of genes such as BATF, a negative regulator of cytokine production. PD-1 expression has also been shown to limit T cell mobility [131]. However, some T cells such as T_{REG} and T_{FH} constitutively express high PD-1, so PD-1 expression alone cannot be indicative of exhaustion [152]. Antigen persistence alone is insufficient for the long-term upregulation of PD-1, suggesting the need for additional signals or high antigen concentrations for clinical exhaustion [154].

Unlike senescence, exhaustion in T cells can be treated. The most successful example of this process is PD-1 blockade. PD-1 blockade is most studied in CD8+ T cells and NK cells in the context of cancer but has also been shown to be effective in CD4+ T cells [155]. In PD-1 blockade, antibodies are administered that bind to but do not activate PD-1 or PD-L1, preventing the interaction of the receptor and its ligand. In the absence of this inhibitory signal, T cell activation increases [156]. Less differentiated T cells such as T-bet^{HI} or PD-1^{int} cells respond best to PD-1 blockade, possibly due to the fact that they retain baseline proliferative capacity. The partial retention of functionality makes CD4+ T cells an attractive candidate for revitalization, as studies of PD-1 blockade show that reversal of exhaustion is only effective in moderately exhausted T cells [157].

2.1.5. Frailty

Frailty can be defined in various ways but is generally thought of as a complex multi-system reduction in physiological function and recovery that results in increased risk of morbidity and adverse health outcomes [158,159]. Frailty is classically defined as a clinical phenotype in which a person presents with several of the following: unintentional weight loss, lethargy, weak grip strength, low physical activity, and lower walking speed [160]. Other definitions of frailty such as the Frailty Index of Cumulative Deficits, are also used. Regardless of the scale used to score it, frailty has been shown to correlate with a wide variety of events from fall risk to infectious disease mortality [158]. Chronic inflammation and inflammaging is believed to play a role in the progression of frailty [158,161,162].

Sarcopenia, the general muscle wasting that is associated with frailty, is known to be mediated or exacerbated by immune mediators such as TNF-α or IL-1 that are produced during chronic inflammation [161]. However, in part because frailty is defined by clinical outcomes or disease presence, the molecular causes and signs of frailty are ill-defined. It is clear that frailty is a major risk factor for vaccine failure and infectious disease morbidity/mortality [163,164].

Nursing home residents tend to be more frail than independently housed older adults. They are thus more susceptible to infectious disease and less likely to respond well to vaccination [165]. As of 2015, estimated frailty prevalence in nursing homes was over 50%, and the overall frequency of frail individuals is anticipated to increase as the global population ages [165]. Immunosenescence, or the decline in immune function associated with age, is connected with and contributes to frailty phenotypes [162]. Whether frailty is caused or worsened by factors associated with immunosenescence is unclear: however, frailty is generally considered a better indicator of immune decline than chronological age [158]. As a highly frail and aged population, nursing homes can thus be expected (and indeed have long been found) to be increasingly susceptible to infectious disease compared to the community population [166–168].

There are clearly negative impacts of age-associated immune changes on human health, such as an increased risk for cardiovascular disease, type 2 diabetes, cancer, and infectious disease [1], but caution should be used when classifying age-associated changes as “defective.” For example, inflammatory macrophages are more efficient at killing pathogens, but they also require greater

energy expenditure that older cells may not be able to safely maintain [169]. While immunosenescence leads to detrimental responses in the context of infectious disease and immune memory, it is important to remember that these are not the only evolutionary or health pressures faced by aged persons. Immunosenescence may be important to restrain autoimmunity and therefore cannot be considered a universal negative.

2.2. Connecting age and adjuvants

One potential way to protect aged individuals from infectious disease is to amplify innate immune activation during vaccination. The idea is that sufficient inflammatory signaling will overcome adaptive immune deficits and result in a protective immune response. There are, of course, issues with this approach: for one, inflammation does not always correlate with protection and can in fact be detrimental to developing an adaptive response [42,101,170]. For another, as discussed above, the innate immune system has its own defects that make increasing activation difficult [123,169]. However, clinically speaking, the use of adjuvants or high antigen doses does seem to provide better protection in older adults [143,171]. The success of other vaccines such as RZV and SARS-COV-2 mRNA vaccines in older populations raises the question of whether poor vaccine immunogenicity in older adults is truly insurmountable or merely a matter of finding the right vaccine formulation to enhance responses [169].

The first vaccines simply injected killed organisms, such as the cowpox inoculations famously used by Jenner. The standard influenza vaccines still use inactivated virus as the antigen [172]. More recently, recombinant subunit vaccines

have been the focus of development, in which proteins from a pathogen are produced in host organisms to be purified for vaccine use. An example of this vaccine type would be the recombinant zoster vaccine, Shingrix (RZV), which uses glycoprotein E (gE) from engineered Chinese hamster ovary cells [173]. Because of their highly purified form, subunit vaccines require some form of adjuvant to promote effective responses [172,174,175]. Another recently developed vaccine platform uses a recombinant mRNA encased in a liposome to express a viral target protein in host cells. These vaccines are inherently self-adjuvanting and activate PRR signaling through RIG-1 and TLR signaling [176]. This vaccine platform has been popularized by the SARS-CoV-2 vaccines made by Moderna (mRNA-1273, marketed as Spikevax) and Pfizer (BNT162b2, Comirnaty) and shows high initial efficacy even in older adults [177–179].

Adjuvants boost immune responses, making them key to improving vaccine efficacy in older adults. They are ingredient(s) added to a vaccine for the sole purpose of increasing vaccine response [175]. Adjuvants are distinct from additions like emulsifiers or stabilizers that are meant to maintain the shelf life of a vaccine in that an adjuvant directly interacts with the immune system. This is particularly critical in aged adults, who through a combination of factors discussed previously, are less able to mount a protective vaccine response. Various adjuvants have been shown to increase humoral titers, promote SHM and cross-functional antibodies, increase both CD4 and CD8 T cell frequencies, and provide greater clinical protection than unadjuvanted vaccines. In some

cases, particularly for subunit or recombinant vaccines, an adjuvant is necessary to achieve a detectable response at all [172,175].

Despite the frequent inclusion of adjuvants in vaccines, the mechanisms by which they boost immune responses remain unclear. This impedes the ability to develop novel adjuvants, particularly ones that can be directed towards enhancing specific protective responses. As research in vaccine design turns towards recalcitrant diseases for which no effective vaccine exists, such as HIV, malaria, or tuberculosis, the need for rationally designed vaccine adjuvants grows. In the following sections, three commonly used and clinically approved adjuvants and their known mechanisms will be discussed.

2.2.1. Alum

Aluminum compounds, colloquially referred to as alum, are the oldest and most common adjuvants in use in humans [180]. First used in vaccines developed in 1932, this adjuvant was originally believed to act solely as a depot for the slow release of antigen [180,181]. Alum is now known to also amplify immune responses through APC activation by various DAMPs. As an adjuvant, alum seems to preferentially promote T_H2 responses [175,182,183]. The full mechanism for this T_H2 skewing is unknown: however, alum promotes IL-10 production in macrophages and DCs at the site of injection in mice and IL-4 production in human monocytes when cultured with T cells [183,184]. Both alum and uric acid, a signaling molecule associated with alum, lead to the inhibition of the IL-12 subunit IL-12p70 [184]. IL-10 is a known antagonist of the T_H1 skewing cytokine IL-12, and localized production of IL-10 in response to alum may play a role in skewing CD4+ T cells

towards a T_H2 phenotype [24,185,186]. Additionally, IL-33, a T_H2-skewing cytokine, is produced after alum injection into mice [187]. These and other mechanisms are believed to contribute to alum's skew towards humoral and T_H2 responses.

Following vaccination with alum-adjuvanted antigens, neutrophils are the first immune cells summoned to the site of vaccination. Within three hours of vaccination, human neutrophils and their associated NETs can be detected. These NETs play a key role in inducing innate immune responses, as co-injection with DNAse decreases local cell recruitment to dLNs and results in a decreased adaptive response in mice [188–190]. NALP3 inflammasome is also activated by this source of dsDNA, resulting in the cleavage of caspase-1 and the release of IL-1β and IL-18 [188,191].

Alum itself as a particle may also serve as a DAMP, triggering the release of NETs and future immune activation as dsDNA is detected. Charged particles have been shown to cause lysosomal rupture and oxidative stress in innate immune cells, upregulating APC recruitment and activation [192–194]. The dsDNA in NETs as well as DAMPs such as uric acid from necrosing cells can then activate the NALP3 inflammasome [188,191]. The NALP3 inflammasome pathway releases yet more initiators of inflammation [194–196], leading to an inflammatory cascade that summons additional APCs to the site of inflammation and the dLN. The increased number of APCs as well as their activation and upregulation of costimulatory markers ultimately leads to a more productive adaptive response than can be achieved with antigens alone [188,192].

While alum can induce protective antibody responses, it is inefficient at generating T_H1 responses [197]. In fact, the IL-10 generated by alum injection may actively inhibit T_H1 responses [184]. Alum adjuvants do not generate significant CD8+ T cell responses [198,199]. Alum is additionally a weak adjuvant for subunit vaccines [180]. While it serves well enough for humoral-focused vaccines, it quickly became clear that in cases where T cell responses were needed for protection, new adjuvants would have to be developed.

2.2.2. MF59

The adjuvant used in adjuvanted influenza vaccines (aTIV), is an emulsion-based adjuvant composed of squalene and known as MF59. Squalene is a natural triterpene and an intermediate of cholesterol in the human body [200]. It does not activate DCs directly but induces cytokine production and the recruitment of CD11b+ cells to the vaccination site [201,202]. These cells are able to efficiently prime T cells in animal models, and MF59 is associated with increased germinal center formation and subsequent antibody titer increases [203,204]. Influenza-specific cT_{FH} cells have also been shown to proliferate following MF59-adjuvanted vaccination in humans and to correlate specifically with increased production of antibodies [69,205]. However, the impact of age on these responses is not known, as most studies focus on children or healthy adults under the age of 60.

The molecular mechanisms of MF59's adjuvant activity are still incompletely understood, particularly its role in activating cells at the site of injection. It has been shown that MF59, specifically its components Tween80 and

Span85, induces ATP release when injected intramuscularly [206]. This transient ATP presence promotes cell recruitment to the site of vaccination. This ATP also appears to play a role in priming naive T cells, as downstream humoral titers and T cell frequencies are decreased when ATP-consuming apyrase is used at the injection site [206]. Additionally, TLR-linked signaling molecule MyD88 is required for MF59 adjuvant activity, though MF59 does not appear to act through traditional TLR stimulation [201].

MF59 expands T_{FH} in mouse germinal centers, potentially due to increased antigen retention and subsequent enhanced germinal center formation [204,207,208]. After vaccination, MF59 and its accompanying antigen is taken up by neutrophils and monocytes from the site of injection and carried to the dLN [202]. There, they accumulate in sinus and medullary macrophages, resulting in their depletion in association with an upregulation in RIPK3 and caspase-1 [199]. The necrotic cell death of these macrophages seems to be key to CD8+ T cell responses to MF59-adjuvanted vaccines but not to humoral responses, as CD169+ depleted mice have reduced CD8+ responses [199].

A previous study speculated that, on the basis of increased responsiveness to the inclusion of MF59 in studies with younger or naive donors, MF59 acts best to adjuvant naive B cell response, particularly their SHM [209]. Clinical trials have also shown that adjuvanted quadrivalent influenza vaccine provides greater protection in younger cohort brackets [210]. How immunosenescence and the “inflammaging” phenotype associated with cellular senescence impacts adjuvanted vaccine response in older adults is not clear.

There is some evidence to suggest that MF59's impacts may attenuate with age, as H5N1 MF59-adjuvanted clinical trials show stronger antibody generation in children than adults [209,211]. Determining if MF59 remains effective and the mechanisms by which it retains that efficacy are critical to determining appropriate recommendations for clinical deployment of vaccines in older, more susceptible adults.

2.2.3. AS01

AS01 is included in the recombinant subunit vaccine for herpes zoster (RZV), where it induces strong T_H1 expansion and reactogenicity [81]. This relatively new adjuvant is credited with the strong memory response induced by RZV, as VZV gE administered alone has little to no immunogenicity [212]. In humans, prominent and polyfunctional T_H1 expansion has been observed in both RZV and RTS,S/AS01 malaria vaccine Mosquirix [81,213]. This further indicates that AS01 rather than the antigen used is responsible for antigenic effects.

AS01 is believed to induce strong immunogenicity via its activation of multiple immune pathways. The adjuvant is a liposomal formulation of two strong immune stimulators, QS-21 and monophosphoryl lipid A (MPL). QS-21 is a saponin derived from *Quillaja saponaria*, while MPL is a non-toxic ligand for TLR4 derived from LPS from *Salmonella minnesota*. Evidence already points to the critical role of inflammatory crosstalk in generating the superior immune response observed in AS01. Mouse studies utilizing RTS,S/AS01 showed preferential APC recruitment and more antigen-specific T cell generation than RTS,S/MPLA or RTS,S/QS-21[214]. IFN-γ from NK and CD8+ T cells exposed to

IL-18 is known to be critical for the maturation of these APCs in mouse models, implicating multiple key signals from different cell types as necessary for maximal adjuvanticity [49,214]. Mouse studies comparing AS01, MPLA, and QS-21 detected differential gene transcription in the dLN as early as 4 hours. Notably, IFN-related genes were detectable in AS01-treated, but not QS-21 or MPL-treated, mice. Many of the genes upregulated by AS01 were key crosstalk points between pathways triggered by MPL or QS-21, suggesting synergistic effects were responsible for the strong CD4+ T cell response observed post vaccination [214].

AS01 contains MPL, a ligand for key bacterial sensor TLR4. TLR4's most well-known and potent ligand, LPS, is a component of many bacterial cell walls. TLR4 sensing triggers Type I IFN production as well as additional intracellular signaling that connects to the activation of the NF-κB pathway. This transcription factor is at the nexus of a variety of cellular events, including those related to cell proliferation, apoptosis, and inflammation. Among its many transcriptional targets are genes for cytokines such as TNF-α as well as pro-IL-1β. NF-κB is normally regulated by IκB, which binds it and prevents nuclear translocation. The degradation of IκB results in nuclear translocation and the transcription of NF-κB targets, among which are inflammasome components. The inflammasome is a catalytic cellular complex and is believed to require two signals to activate: a preliminary signal that activates NF-κB and leads to transcription of the NLRP3 inflammasome and the production of pro-IL-1β as well as a secondary signal that catalytically activates the inflammasome composed of NLRP3 and caspase-1 [215–217]. Type I IFNs are sensed by the membrane receptor IFNAR and activate

caspases, triggering further inflammatory signaling events. Notably, like most TLRs and PRRs, TLR4 signaling activates IRAK, which acts to degrade regulatory molecules limiting NF-κB activity and serves as a key link to other signaling pathways [218].

In addition to TLR4 activation by MPL, QS-21 has been shown to induce inflammation. It does so through the NLRP3 inflammasome and the production of IL-1β and IL-18 [219]. Additional signals for inflammasome activation come from cellular stress caused by the co-localization of QS-21 to the lysosome [220]. The inflammasome is "licensed" by the transcription factor NF-κB, which is necessary to transcribe the components of the inflammasome. Inflammasome activation triggers a cascade that results in the cleavage of IL-1β and IL-18 to their active forms. TLR4 activation intersects with inflammasome signaling both at the level of NF-κB activation via degradation of IκB as well as activation of the inflammasome via NLRP3 stabilization [215–217].

While much is known about AS01's components and how they activate immune cells, less is known about how AS01 is able to overcome immunosenescence in older adults for a protective cellular response. Nearly all mechanistic studies for AS01 take place in mice and ignore age entirely [49,212,214,220,221]. Mechanistic studies also rely MDDC even when they do use human data, despite evidence that MDDC are not phenotypically nor functionally the same as mDC [30,220,222–225]. Further, it is unclear if AS01 induces signals that promote a T_H1 phenotype or if that skew is caused by factors inherent to the aged immune system.

As more is understood about the immunomodulatory mechanisms of adjuvants, next generation adjuvants with more targeted effects can be developed. In this thesis, we explore mechanisms by which adjuvanted vaccines provide protection to older adults using two models: adjuvanted recombinant zoster vaccine (RZV) and adjuvanted trivalent influenza vaccine (aTIV). By exploring how these clinically available vaccines provide protection, we hope to both understand the basis for effective vaccine protection in older adults as well as provide screening measures for the development of new adjuvants.

Chapter 3: Adjuvant AS01 Activates Human Monocytes for Costimulation and Cytokine Production

3.1. Author Contribution Statement

Carson Smith contributed to the conceptualization, formal analysis, investigation, original drafting, review, editing, and visualization of this work, as defined by the CRediT (Contributor Roles Taxonomy) protocol available via Elsevier.

3.2. Abstract

Background

The adjuvanted recombinant zoster vaccine (RZV) is highly effective even in adults over 80 years old. The high efficacy of RZV is attributed to its highly reactogenic adjuvant, AS01, but limited studies have been done on AS01's activation of human immune cells.

Methods

We used flow cytometry and RNA Sequencing (RNAseq) to analyze the impacts of AS01 on human primary cells. The ability of AS01-treated antigen-presenting cells (APC) to costimulate human T cells using an in vitro assay was also tested.

Results

We found that AS01 activated monocytes to a greater extent than any other cell population, including dendritic cells. Both classical and non-classical monocytes demonstrated this activation, and monocytes were able to provide greater costimulation to T cells after AS01 incubation. RNASeq showed that TNF and IL1R pathways were highly upregulated in response to AS01 exposure, even in older adults.

Conclusions

AS01 strongly activates human monocytes, enabling them to provide enhanced costimulation to T cells and inducing cytokines that mediate systemic inflammation. Understanding AS01's impacts on human cells opens possibilities to further address the reduced vaccine response associated with aging.

3.3. Introduction

Vaccination remains the most effective public health strategy for reducing disease mortality and morbidity. However, older adults display a reduced vaccine response compared to younger counterparts, which is exacerbated by any underlying conditions [164]. Reduced vaccine response is a consequence of immunosenescence, a natural, multi-factorial decline in immune function. An incomplete understanding of the complex immunologic factors driving the development of immunosenescence obstructs our ability to optimize vaccine response in older populations.

Despite this lack of understanding, one vaccine has been found to be highly protective in older adults, even in the oldest cohorts studied. Administration of two doses of the recombinant zoster vaccine (RZV, Shingrix) provides 70-90% protection from zoster, even in adults over 85 [226–228]. In stark contrast, the previous zoster vaccine formulation, live attenuated zoster vaccine (Zostavax), was only 20% effective in populations over 80 [229,230]. Herpes zoster is a common and exceptionally painful disease caused by the reactivation of latent herpesvirus (varicella zoster virus) and can be very debilitating in individuals who go on to develop post-herpetic neuralgia (PHN) which can persist for months to years after the resolution of lesions [226]. Zoster reactivation is highly associated with age, immunodeficiencies and frailty, all factors associated with immunosenescence [231]. That RZV can provide protection regardless of these conditions is nothing short of ground-breaking.

RZV is composed of the novel adjuvant AS01 and varicella zoster (VZV) glycoprotein E (gE). AS01 is highly reactogenic and believed to be the primary source of RZV's efficacy [232,233]. AS01 itself is a liposomal formulation of QS-21, a fractionation of *Q. saponin* tree bark, and MPL, a TLR4 agonist derived from *S. minnesota* lipopolysaccharide (LPS) [234]. RZV provides outstanding long-term protection through the induction of a protective T cell memory response [81]; however, the specific mechanism(s) by which the adjuvant AS01 contributes to the development of this protection remain(s) unknown.

Mechanisms of action for AS01 have been studied in mice, but very limited data on the mechanism of action in humans is available. Mouse models have identified key responding immune cell populations, such as subcapsular CD169+ monocytes in the lymph node, that are critical for the full breadth of AS01-adjuvanted vaccine response [49,214]. Vaccines containing AS01 are well-known for their systemic reactogenicity, suggesting a role for immune cells in the periphery as well as in the lymph node [233,235]. However, while serum analysis and systemic inflammation has been studied in clinical trials, little has been reported on AS01's impacts on circulating human cells [214,233,236]. Addressing this knowledge gap will not only provide important insights into mechanisms that could reverse age-related decreases in vaccine response, but will also inform adjuvant development strategies to allow for maximizing response and minimizing toxicity

In this study, we use primary human cells to study AS01's activation profile and find that monocytes strongly respond to AS01 in vitro. We perform

RNA sequencing on isolated CD14+ cells and show key pathways that are activated in both old and young donors such as TNF-α and IL1R signaling. We propose that AS01 works well in older populations in part by activating monocytes to serve as antigen-presenting cells (APCs) to primarily VZV-specific memory T cells, present due to the extremely high rate of prior VZV infection in RZV recipients. Our study provides insight into a highly AS01-responsive immune cell population in humans, signature of adjuvant response in this immune cell subset, and is the first published transcriptomic profile of the direct effects of AS01 on human cells.

3.4. Materials and methods

Subject recruitment

Healthy community donors gave informed consent under University Hospitals Cleveland Medical Center institutional review board approved protocol. Both male and female donors were included in all assays. Throughout this paper, “sex” is used in the sense of biological assignment at birth.

PBMC collection and isolation

Venous blood was processed to PBMC using Ficoll gradient density centrifugation. Freshly isolated cells were used for experiments in order to maintain the viability of the myeloid compartment, particularly the DCs.

Flow cytometry

For all assays, PBMC from healthy donors were incubated for 18 hrs at 37°C with various ligands or unstimulated as a control. When cells were stained intracellularly, brefeldin A (BFA; Sigma, B7651; 1 µg/mL) was used during culture to block cytokine release. After incubation, cells were stained at 4°C with appropriate panel (see **Supplemental Table 3.1**), fixed with 1% paraformaldehyde, and run on a BD Symphony or Fortessa flow cytometer depending on the panel. For **Figure 3.1**, PBMC were incubated 1:100 AS01 (the adjuvant component vial from Shingrix vaccine, GlaxoSmithKline) or LPS (400 ng/mL; Invivogen, tlrl-smlps) with 1:100 Fluzone High Dose vaccine (Sanofi-Pasteur, 2019-2020, Swiftwater, PA) in X-Vivo 15 media (Lonza, 04-418Q). For **Figure 3.2**, PBMC were stimulated with 1:100 AS01, 250 ng/mL MPLA-SM Vaccigrade (Invivogen, vac-mpla), or 5 µg/mL QS-21 in liposomal formulation

(formulated according to [237]; S-21 from Desert King, lot L021219PP18-1) in X-Vivo 15 media. Adjuvants were titrated in preliminary experiments on human PBMC for a dose that induced a detectable IL-6 induction without cytotoxicity. In the case of liposomal QS-21, titrations were determined on the basis of minimal cytotoxicity and detectable surface marker upregulation. For **Figure 3.3**, PBMC were incubated 1:100 AS01 or LPS (100 ng/mL) in X-Vivo 15 media.

RNASeq

Donors were selected among consented younger (<35 years old) or older (≥65 years old) adults The median age of donors under 35 was 28.5 years old (n=8), while the median age of the donors >65 was 70 years old (n=8). The minimum and maximum ages were 25 and 82, respectively. PBMC were incubated for 10 hours at 37°C in X-Vivo 15 media with 1:100 AS01. Untreated cells were used as a control. Timepoints were determined using upregulation of *ICAM-1, HLADR,* and *CD86*, as assessed by qPCR (**Supplemental Figure 3.1**) After stimulation, cells were stained with a sorting panel using the markers in **Supplemental Table 3.1**. Monocytes were sorted directly into lysis buffer RLT from Qiagen kit used for purification (Live, Singlet, CD3/CD19-, CD14+) using a BD FACS ARIA-SORP. RNA was purified using Qiagen's RNAeasy Micro kit (74004) and stored at -80°C before library preparation and sequencing. RNA quality, specifically purity and lack of degradation, was checked using Bioanalyzer.

RNASeq analysis was performed as described in detail previously [238]. Briefly, fastq files were filtered out if they had an average phred quality score <30 or a length <36 before alignment to Homo Sapiens NCBI reference genome GRCh38. These count files were imported into R and were assessed for

quality control, normalized and analyzed using an in-house pipeline utilizing the limma-trend method for differential gene expression testing and regression modeling and the GSVA library for gene set sample enrichment [239,240].

We performed a network analysis of the AS01-stimulated vs. unstimulated monocytes to visualize the protein-protein interaction (PPI) network. Upregulated genes were selected using a threshold value of Log2 Fold Change > 1.00, and differentially expressed genes (DEGs) were detected with the adjusted p-value > 0.25. Next, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for selected DEGs was used to generate the PPI network (**Figure 3.6C**). We identified a zero-order interaction schema and selected the STRING interactome (confidence score = 900) database to determine protein hub interactions for these genes.

Costimulatory assay

Monocytes were isolated from PBMC using human CD14+ positive selection kit (Biolegend, 480024), and mDC were isolated using CD1c (BDCA-1) dendritic cell positive isolation kit (Miltenyi Biotec, 130-119-475). Sorts were checked for purity using flow cytometry (median purity 80% for CD14; 57% for mDC). Monocytes (20,000/well) or mDC (10,000/well) were incubated in 96-well round-bottomed plates in triplicate in X-Vivo 15 media along with 1:100 AS01, 250 ng/mL MPLA, or 5 µg/mL liposomal QS-21, as well as an unstimulated control. After 18 hours, cells were incubated with SEB (0.1 µg/mL) for 1 hour before washing. Monocytes and mDC were fixed using 1% paraformaldehyde. CD3+ cells from a control donor that serve as a readout of costimulatory ability

were added to fixed monocytes at a concentration of 150,000 cells/well and incubated for 3 days. Supernatants were harvested and tested for IFN-γ production using an ELISA kit (Biolegend, 430115). The same donor's CD3+ T cells were used for all costimulatory experiments. T cells were isolated with human CD3 T cell isolation kit (Biolegend, 480131) and cryopreserved in 10% DMSO + FBS until use.

3.5. Results

AS01 preferentially activates innate myeloid cells in human PBMC

To determine what cell populations were directly activated by AS01, peripheral blood mononuclear cells (PBMC) were stimulated for 18 hours with either adjuvant AS01 or a LPS+influenza vaccine as a positive control. Influenza vaccine was included in the positive control to provide viral ssRNA (TLR7) and antigenic stimulus alongside the TLR4 ligand LPS. Cells were then analyzed by flow cytometry to assess the frequency and lineage of cells responding to AS01, as indicated by expression of the cytokines IFN-γ, IL-1β, and TNF-α. The gating strategy is shown in **Supplemental Figure 3.2.** AS01 strongly induced IL-1β and TNF-α production in CD14+ monocytes and to a lesser extent, in mDC. TNF-α and IL-1β were not significantly produced in response to AS01 in T cells, B cells, pDC, and NK cells (**Figure 3.2**). Additionally, AS01 did not induce IFN-γ in either T cells or NK cells (**Figure 3.2**)

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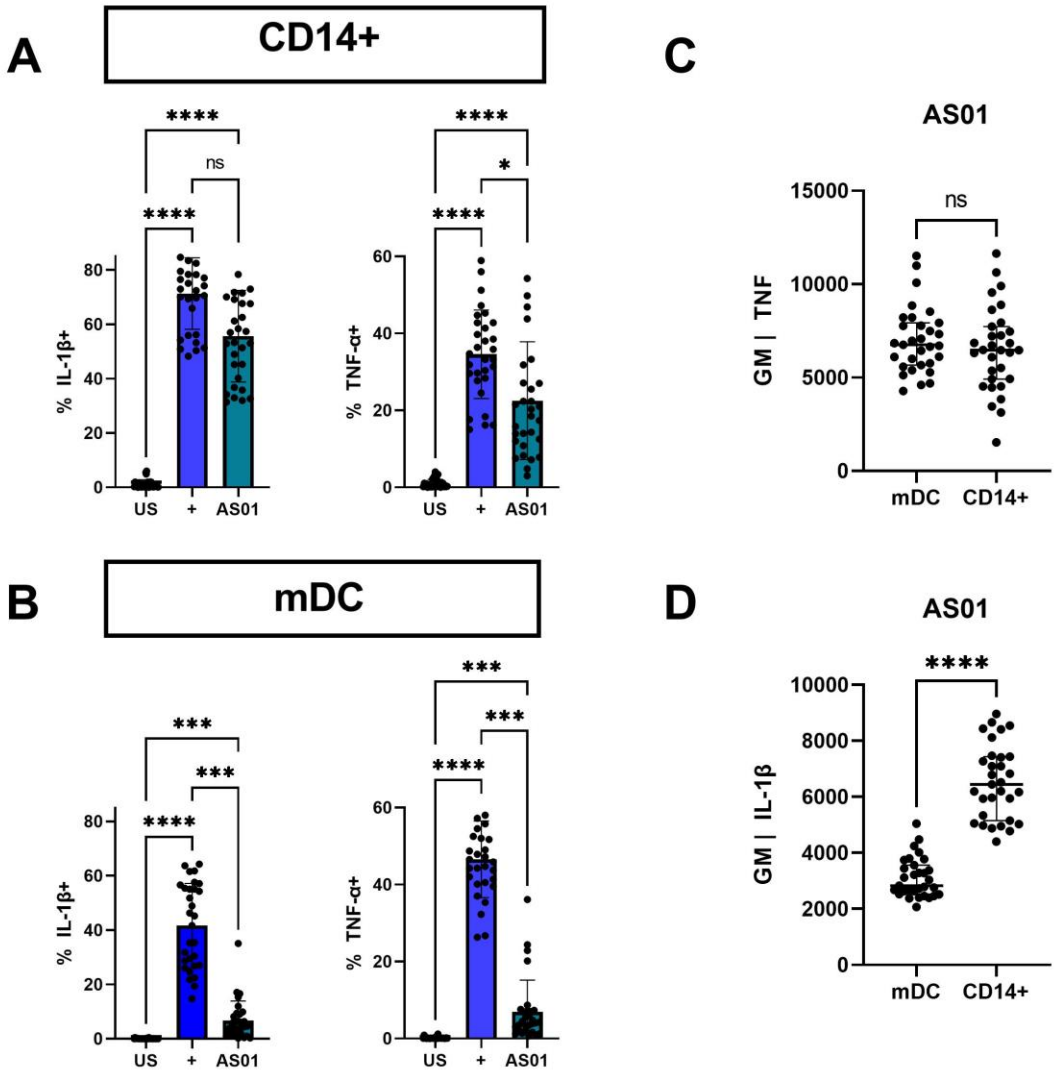


Figure 3.1. Myeloid cell responses to AS01 stimulation of PBMC. Peripheral blood mononuclear cells (PBMC) were either unstimulated (US), treated with positive control (+, 1 µg/mL LPS + 1:100 dilution of ssRNA containing influenza vaccine), or stimulated with AS01 (1:100 dilution) for 18 hours. After stimulation, monocytes (**A**) and mDCs (**B**) were assessed for production of inflammatory cytokines (n=31) using flow cytometry. Comparison between conditions was performed using a Friedman test followed by Dunn's multiple comparison test in Prism. Geometric mean (GM) was compared between TNF-α+ (**C**) and IL-1β+ (**D**) cells as a measure of the amount of cytokine being produced by indicated cell types. MFI comparisons were performed using Wilcoxon test. Asterisks correlate to the degree of significance (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$). Donors ranged from 22-82 years old, with a median age of 55 years old.

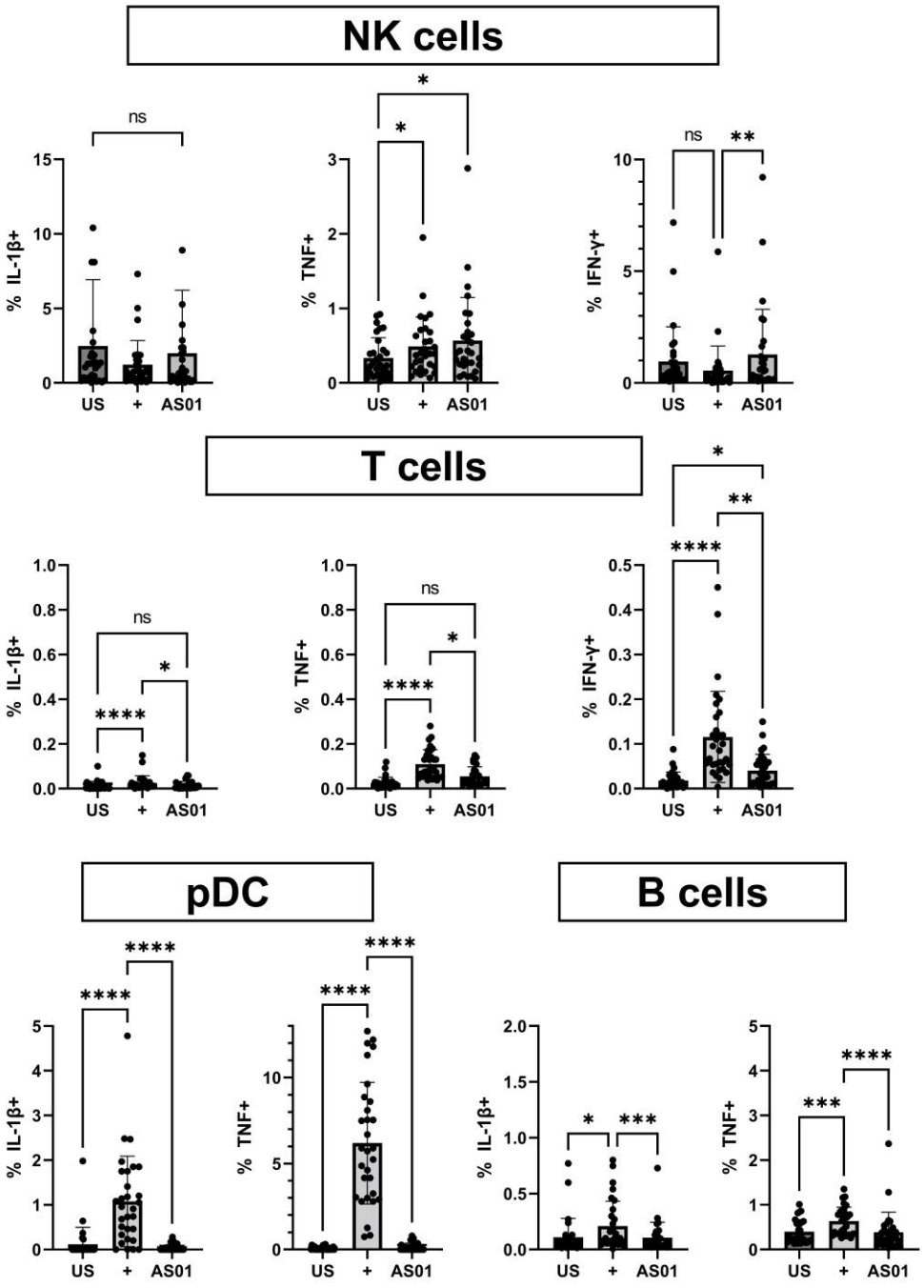


Figure 3.2. Cytokine expression in response to AS01 in PBMC. As in **Figure 1**, after 18 hours of stimulation with media alone (US), a positive control ((+, 1 µg/mL LPS + 1:100 dilution of ssRNA containing influenza vaccine), or AS01 (1:100 dilution), cytokine production in pDC, B cells, NK cells, and T cells was examined. Data for mDC and CD14+ is presented in Figure 1. Statistical analysis was performed using the Friedman test. Asterisks correlate to the degree of significance (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$). Donors ranged from 22-82 years old, with a median age of 55 years old.

In order to better understand AS01's activation of myeloid cells, we used flow cytometry to assess the expression of costimulatory markers on mDCs and monocytes (gating in **Supplemental Figure 3.3**). AS01 contains two components known to synergistically stimulate the immune system in mice [13]. In order to examine the effects of each component of AS01, we stimulated with AS01 and individually with its components, the TLR4 ligand monophosphoryl lipid A (MPLA), and liposomal QS-21. Treated monocytes displayed upregulation of HLA-DR, CD86, CD11c, and CD54 in response to AS01. Surprisingly, MPLA treatment did not result in the upregulation of any marker besides CD11c in monocytes, while QS-21 had similar effects as AS01 on surface marker expression (**Figure 3.3A**). In contrast, mDC did not significantly upregulate any marker in response to AS01 or MPLA, although QS-21 was able to induce a small but significant degree of HLA-DR, CD86, and CD11c upregulation in comparison to unstimulated mDC (**Figure 3.3B**).

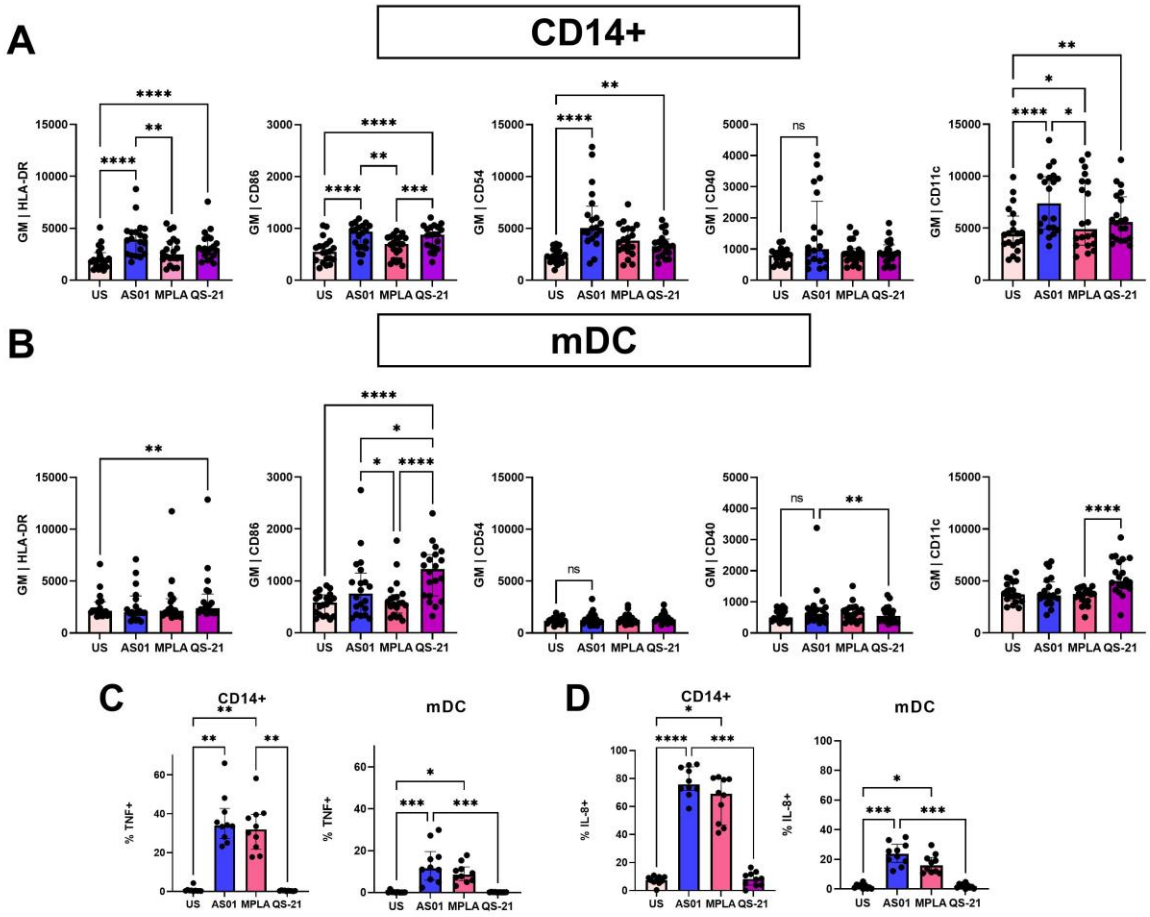


Figure 3.3. AS01 assessment of costimulatory marker upregulation in monocytes and mDC. Peripheral blood mononuclear cells (PBMC) were either unstimulated (US) or stimulated for 18 hours with AS01 (1:100 dilution), MPLA (250 ng/mL), or liposomal QS-21 (5 µg/mL) before assessment by flow cytometry (n=20). Surface marker geometric mean (GM) is reported for **A**) monocytes and **B**) mDC. **C**) PBMC were stimulated as above and stained for intracellular cytokine expression. Frequency of TNF-α+ cells were compared in CD14+ cells and mDC (n=10). **D**) IL-8+ frequencies after stimulation were compared between CD14+ and mDC (n=10). IL-8 and TNF-α expression in monocytes and mDC was compared between incubation conditions (n=10). Analysis in C and D was performed using the Friedman test followed by Dunn's multiple comparisons test. Asterisks correlate to the degree of significance (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$). Donors in surface analysis ranged from 26-74 years old (median 40.5 years old), while donors in cytokine analysis ranged from 29-59 years old, with a median age of 44.5)

Flow cytometry was also used to assess cytokine production in response to AS01 and its components. Intracellular flow cytometric analysis of monocytes

and mDC shows that both cell types were activated in response to AS01 (gating strategy in **Supplemental Figure 3.4**). When IL-8 and TNF-α production were examined via flow cytometry, both cytokines were strongly induced by AS01 and MPLA, despite the lack of costimulatory marker upregulation in response to MPLA. In contrast, QS-21 did not induce cytokine production in either population. The proportion of monocytes producing IL-8 and TNF-α was higher than in mDC (**Figure 3.3C and 3.3D).**

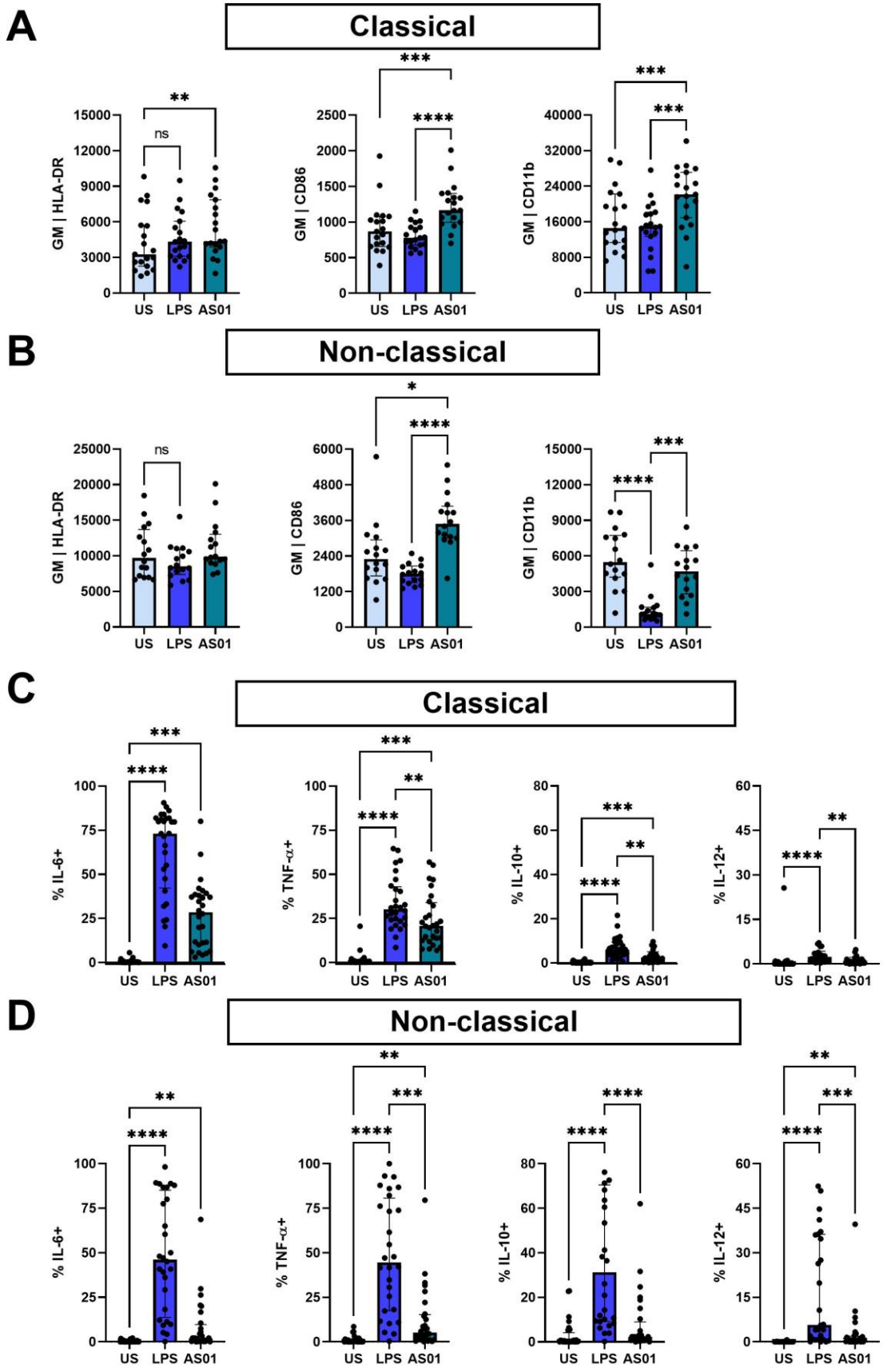

A
Classical
GM | HLA-DR
GM | CD86
GM | CD11b
ns
US LPS AS01
B
Non-classical
GM | HLA-DR
GM | CD86
GM | CD11b
ns
US LPS AS01
C
Classical
% IL-6+
% TNF-α+
% IL-10+
% IL-12+
US LPS AS01
D
Non-classical
% IL-6+
% TNF-α+
% IL-10+
% IL-12+
US LPS AS01

Figure 3.4. AS01 activation of both classical and non-classical monocytes. PBMC were stimulated for 18 hours with either media (US), LPS alone (100 ng/mL), or AS01 (1:100 dilution) and stained for surface marker or intracellular cytokine expression. Flow cytometry was used to examine surface marker expression after activation with AS01 or LPS on **A)** classical ($CD14^{++}$) and **B)** non-classical ($CD14^{lo}CD16+$) monocytes from the same donor (n=19). Geometric mean (GM) values for the respective marker are reported on the Y axis of each graph. **C.** Intracellular staining was performed on PBMC after 18 hours of stimulation, as above (n=27). IL-6, TNF-α, IL-10, and IL-12 expression was examined in **C)** classical and **D)** non-classical monocytes. Figure analysis was performed using nonparametric ANOVA (Friedman test followed by Dunn's multiple comparison test). Asterisks correlate to the degree of significance (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$). For A and B, donor age ranged from 26-72 years old, with a median age of 51. For C and D, donors ranged from 22-68 years old, with a median age of 47.

AS01 activates both classical and non-classical monocytes towards inflammatory phenotypes

Because our initial panel showed that AS01 had the greatest effect on monocytes, we sought to interrogate the monocytic response to AS01 in greater detail. We examined the adjuvant's effects on classical (CD14++CD16-) and non-classical (CD14-CD16+) monocyte subsets using flow cytometry (gating strategy in **Supplemental Figure 3.5**). As anticipated, classical monocytes showed higher frequencies of cells expressing inflammatory cytokines than non-classical monocytes ($P < 0.0001$ for IL-6; $P = 0.0002$ for TNF-α; IL-10 and IL-12 non-significant by Wilcoxon test). Additionally, HLA-DR and CD11b upregulation on classical monocytes exceeded that of non-classical monocytes. However, both non-classical and classical monocytes responded to AS01 (**Figure 3.4A**).

At 18 hours, non-classical monocytes responded to AS01 by significantly upregulating CD86, despite reports of lower TLR4 expression on non-classical compared to classical monocytes (**Figure 3.4B**) [39,41]. As AS01 contains a TLR4

agonist, minimal response was expected out of this population. In contrast, classical monocytes upregulate HLA-DR, CD86, and CD11b in response to AS01.

Flow cytometry was used to examine cytokine expression in monocyte subsets. IL-6 and TNF-α were selected due to their inflammatory phenotype and presence in supernatants from PBMC incubated with AS01 (not shown). IL-10 and IL-12 are cytokines that regulate T_H1 development that are reported to be produced in human cells in response to MPLA [241]. IL-10 and IL-12 were thus investigated in light of their potential role in RZV's noted skewing towards a T_H1 phenotype [81]. Classical monocytes are the primary producers of inflammatory cytokines IL-6 and TNF-α after AS01 exposure but poorly produce IL-10 and IL-12 (**Figure 3.4C**). However, non-classical monocytes generate significant frequencies of IL-10+ and IL-12+ cells in response to LPS and, to a lesser degree, AS01 (**Figure 3.4D**).

Age correlates with some, but not all, molecules induced by AS01

We examined age correlations within our surface marker and cytokine expression findings. Age correlated with some, but not all, outcomes examined in response to AS01 in **Figure 3.1** and **Figure 3.3**. Baseline expression of HLA-DR, CD54 and CD86 did not correlate with age in monocytes, although baseline expression of CD11c did increase with age (**Figure 3.5A**). In mDC, no baseline surface marker correlated with age (P = 0.95, 0.47, 0.31, and 0.21 for HLA-DR, CD86, CD54, and CD11c, respectively**)**. Age did not correlate with TNF-α produced in response to AS01 (**Figure 3.5B,** data also reported in **Figure 3.1)**

but did negatively correlate with IL-1β in monocytes. Regarding costimulatory markers, age did not correlate with HLA-DR or CD54 upregulation but did significantly correlate with upregulation of CD86 and CD11c in response to AS01 (**Figure 3.5C,** data also reported in **Figure 3.3).** No correlations were found between age and surface marker upregulation in mDC (not shown). IL-6, TNF-α, IL-10, and IL-12 frequencies in both classical and non-classical monocytes do not show a correlation with age after AS01 treatment (P = 0.38, 0.64, 0.19, and 0.19, respectively).

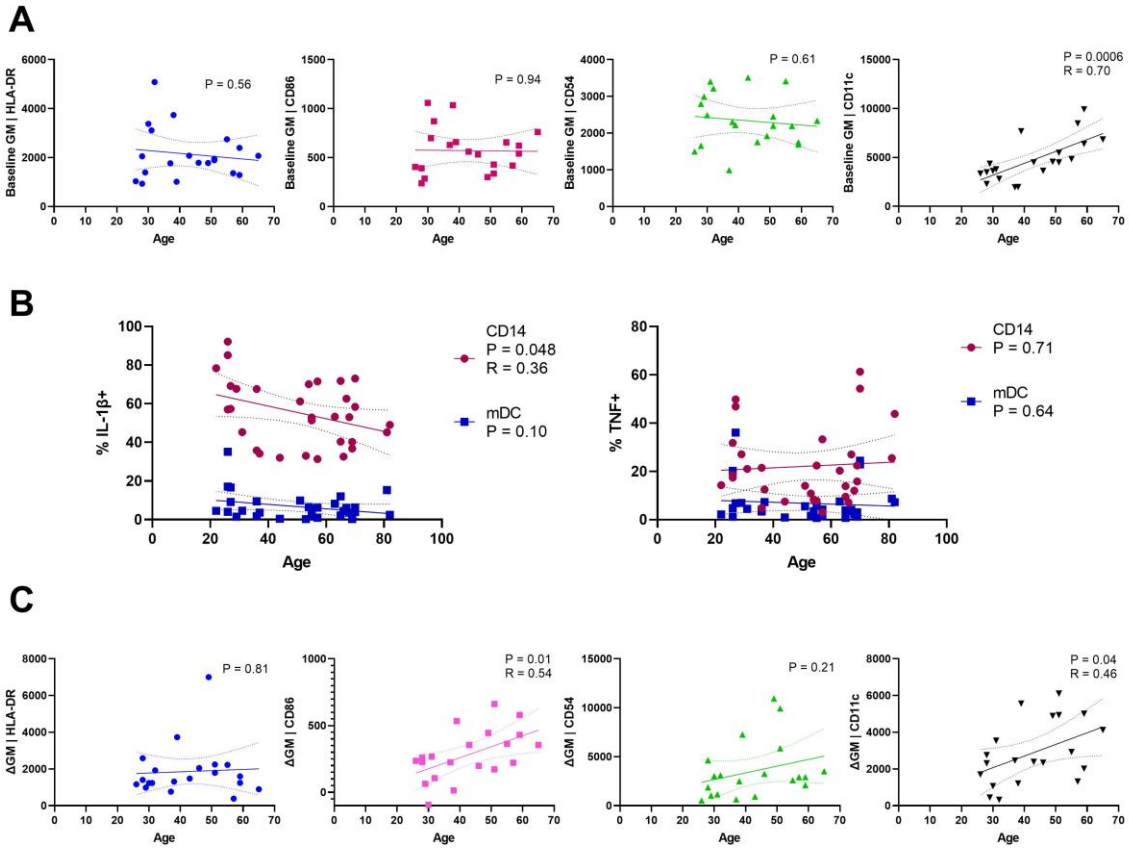


Figure 3.5. Impact of age on activation status of monocytes and mDC in response to AS01. A. Baseline expression of costimulatory molecules HLA-DR, CD86, CD54, and CD11c in CD14+ monocytes is shown, graphed against donor age in years. The unstimulated condition at 18 hours was considered the baseline expression for each cell compartment indicated. The fluorescence of each respective marker is reported as geometric mean (GM). Data shown is from **Figure 3.3**. **B**. Net frequency (AS01 - US) of cytokine expression was calculated for each donor in the AS01 condition in the cell type shown (pink= CD14+; blue= mDC; n=31). Primary data is shown in **Figure 1**. **C.** The net change in surface markers on CD14+ cells was graphed against the age of the donor (AS01 - US). Correlations between age and net molecule upregulation were performed using simple linear regression in Prism (n=20).

Transcriptomic analysis shows minimal difference in monocytic response between young and old donors

In order to better understand AS01's activation of monocytes, we performed RNA sequencing on monocytes purified by flow cytometry after 10 hours of stimulation. The gating strategy used to purify monocytes is reported in **Supplemental Figure 3.6**. RNA sequencing showed a massive upregulation of inflammatory genes after AS01 treatment in both young (<35) and older (>65) donors (**Figure 3.6A**). As expected of an adjuvant containing a TLR4 ligand, TLR4 signaling pathways were significantly upregulated. Differentially expressed pathway (DEP) and gene set enrichment (GSEA) analysis revealed upregulation in key inflammatory pathways like TNF and IL1R signaling (**Figure 3.6B**). This is confirmed by our data showing large induction of TNF-α and IL-1β in myeloid cells by flow cytometry after AS01 treatment (**Figure 3.1**). Neither Type I or Type II interferon genes showed significant differences in expression after AS01 treatment (**Figure 3.6A**)

Network analysis of genes upregulated by AS01 exposure identified several major hubs (**Figure 3.6C**). These hubs included major transcriptional regulators such as JUN, MAPK8, KRAS, and NFKBIA. Additional hubs of interest were HIF-1α and CTNNB1. In addition, a mRNA regulation hub branched off from CTNNB1 and involved heterogenous nuclear ribonucleoprotein (hnRNP) expression such as HNRNPA2B1 as well as DDX5, a helicase implicated in IFN signaling [242]. These secondary hubs connected to the network hub centered around transcription factor Jun (**Figure 3.6C**).

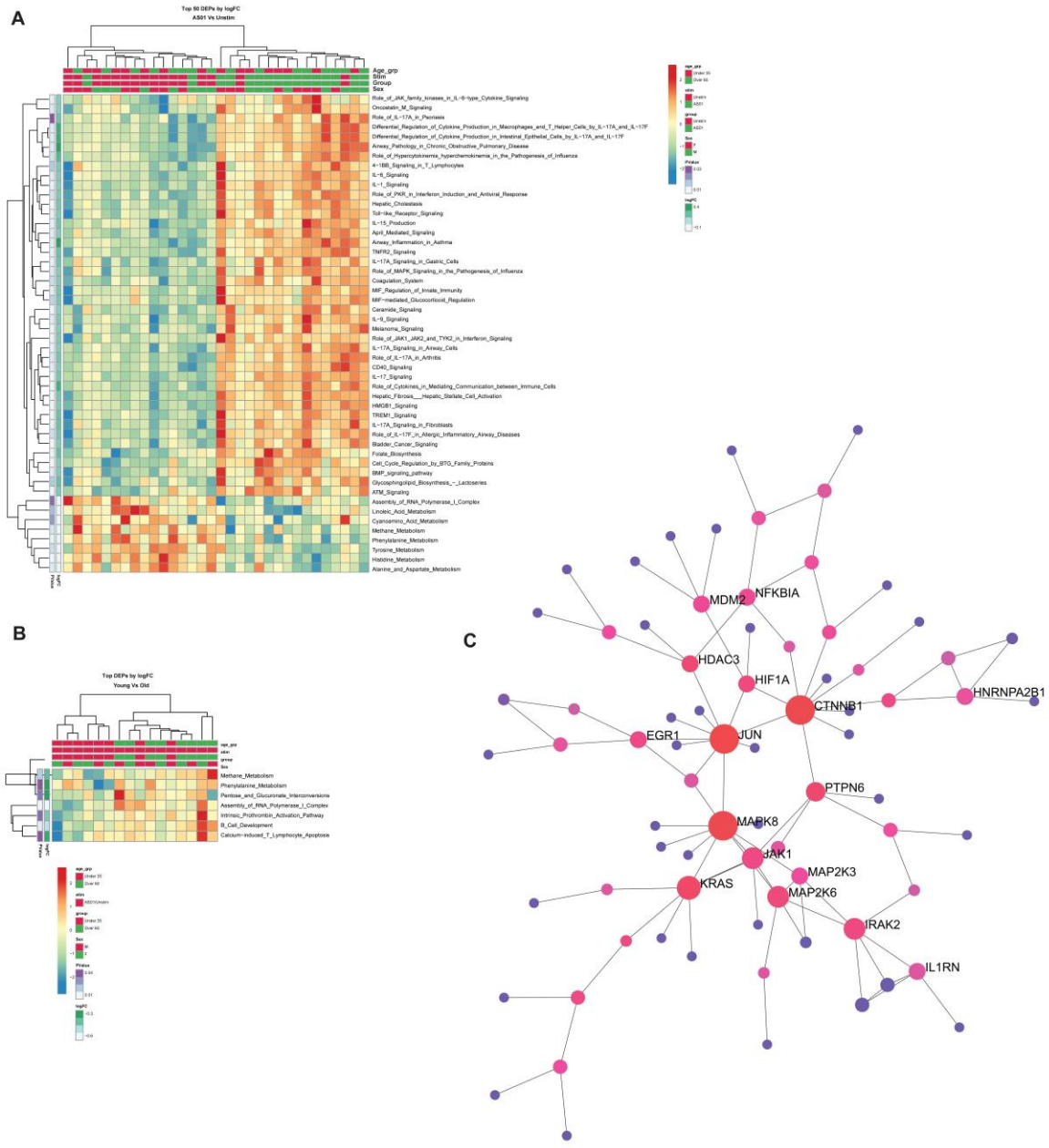


Figure 3.6. RNA sequencing shows pathways upregulated by AS01 stimulation in monocytes. RNA was isolated and sequenced from purified human monocytes after 10 hours of stimulation with AS01. Analysis was then performed as described in the methods section. **A)** Pathway analysis shows the top differentially expressed pathways (DEP) between unstimulated (US) and AS01-stimulated (AS01) conditions in all donors. The heatmap is clustered by two-way (gene:donor) hierarchical clustering, and their stimulation condition and age group is shown in the legend rows above the graph. P values and log fold

change (logFC) between US and AS01 conditions are shown on the left-hand side of the axis. **B)** Gene transcripts expressed in response to AS01 were normalized by z-score to US expression in each donor. Contrasts were then performed to probe for DEP between young versus old donors. **C)** Simplified network analysis was performed using K means clustering, a data visualization metric that groups data points based on how similar they are to the mean of a cluster. The model determines clusters by degree of "betweenness" rather than by random and is described in detail in the methods section. Warmer colors are associated with greater "betweenness," or higher connectivity within the colors, while cooler colors are associated with less.

In order to assess potential effects of age on adjuvant response, comparisons were performed between older and younger donors' response to AS01. Differentially regulated pathways between young and old donors were largely metabolic in nature such as Methane_Metabolism and Pentose_and_Glucuronate_Interconversions (**Figure 3.6B**). Pathways including TNFa_via_NFkB and IL1R_pathway did not show differential expression in old compared to young donors, though both are highly upregulated in response to AS01. Notably, cytokine pathways that were significantly upregulated in analysis of US versus AS01 conditions did not show significant differences in expression between older (>65) and younger (<35) donors (**Supplemental Table 3.2 and 3.3**).

AS01 influences expression of costimulatory markers on monocytes to provide better help to T cells

Based on **Figure 3.3**, AS01 induces costimulatory molecule upregulation. In order to assess how AS01-induced molecule expression affects APC costimulation of T cells, a functional costimulation assay was performed. Monocytes and mDC were isolated and incubated with AS01, its component parts, or media alone. After 18 hours, APCs were treated with a low dose of

superantigen SEB to force the TCR:MHC interaction and fixed with paraformaldehyde. The APCs were then incubated with heterologous CD3+ cells. An IFN-γ readout was used to quantify the magnitude of T cell activation induced by the AS01-treated APCs. Since the APCs were fixed, the degree of costimulatory help that the APCs were able to provide was dependent on their surface expression of costimulatory molecules at time of fixation rather than their cytokine production. This assay is not antigen-specific but provides a general look at how the T cell interacts with surface markers expressed at the time of APC fixation.

Monocytes displayed increased costimulatory ability after treatment with AS01, as evidenced by higher IFN-γ production by T cells after incubation **(Figure 3.7A).** Monocytes treated with MPLA and QS-21 provided similar amounts of costimulation to T cells as untreated monocytes. In contrast to monocytes, the ability of mDC to stimulate IFN-γ production in response to SEB did not vary from unstimulated mDC with AS01 treatment (**Figure 3.7B**). Despite data showing that QS-21 positively impacts costimulatory marker expression, neither CD14+ or mDCs treated with QS-21 were able to augment IFN-γ expression compared to unstimulated APCs. Both mDC and monocytes required the presence of SEB to induce IFN-γ from T cells.

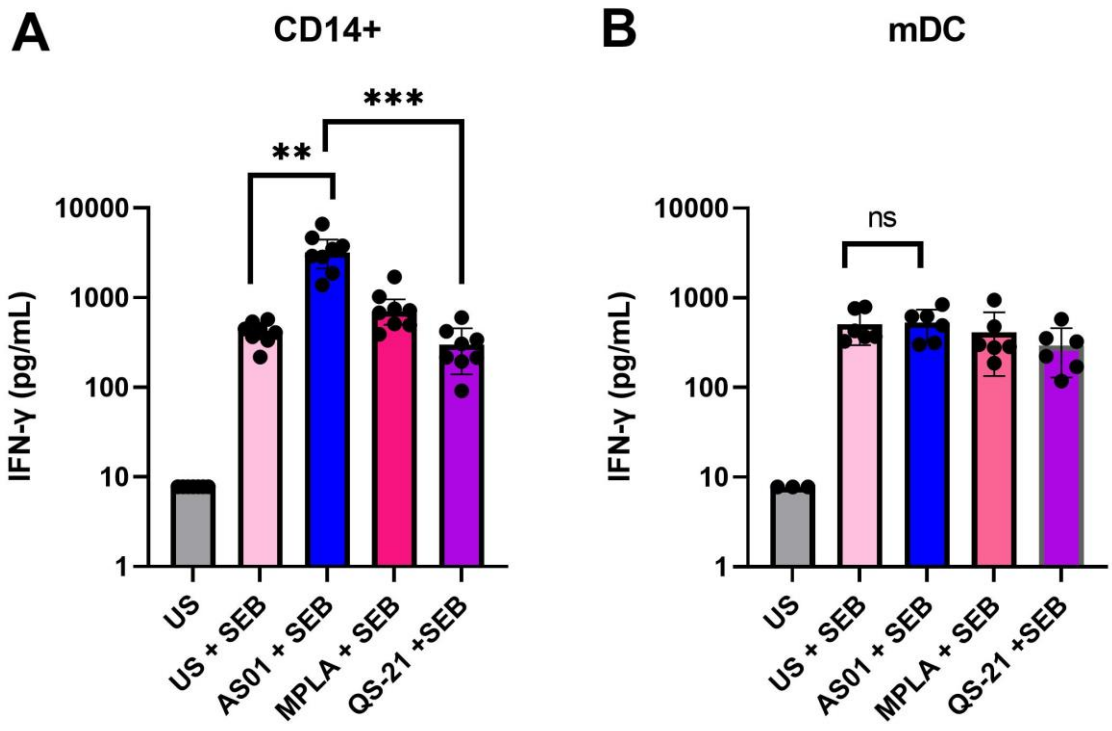


Figure 3.7. Induction of IFN-γ after incubation of T cells with adjuvant-treated antigen-presenting cells (APC). APCs were incubated with media alone (US), AS01 (1:100 dilution), TLR4 agonist MPLA (250 ng/mL), or liposomal saponin QS-21 (5 µg/mL) for 18 hours followed by SEB (0.1 µg/mL) for 1 hour. After SEB incubation, APCs were fixed and incubated with T cells for 72 hours. Where APC number permitted, a negative control of untreated APCs without SEB was included. IFN-γ production was measured by ELISA and reported in pg/mL. Assays used **A)** CD14+ cells or **B)** myeloid dendritic cells (mDCs) as APCs. Comparison between groups was performed using the Friedman test followed by Dunn's multiple comparisons test. The negative control without SEB was excluded from the statistical analysis, as gaps in the dataset meant that group did not meet the conditions of a Friedman test. Asterisks correlate to the degree of significance (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$). All donors used for this assay were under 60 years old due to the high volume of blood needed to assay mDCs.

3.6. Discussion

Our results suggest that AS01 has direct stimulatory effects on monocytes and mDC. Monocytes upregulate costimulatory markers and make cytokines in high frequency after stimulation with AS01. Such molecules enhance T cell responses through direct presentation to the TCR (HLA-DR), costimulation of the T cell (CD86, CD40), or increased expression of adhesion molecules (CD54). Peripheral mDC show limited upregulation of costimulatory markers in response to AS01 (**Figure 3.3**). Cytokine production data shows that mDC respond to AS01 and produce inflammatory cytokines, though in lower frequencies than monocytes. The limitation in costimulatory marker upregulation suggests limited mDC maturation at 18 hours. In contrast, CD14+ cells upregulate both the production of cytokines as well as costimulatory molecules in response to AS01 (**Figure 3.1, 3.3**). It has been reported previously that AS01 has synergistic effects on costimulation markers and cytokines in comparison to its component parts [214]. We show that QS-21 drives surface costimulatory marker upregulation and MPLA drives cytokine expression in human primary cells (**Figure 3.3**). However, in contrast with published literature, we found that AS01 did not promote costimulation marker upregulation on mDC [49,220]. We attribute this difference to our use of primary mDC rather than monocyte-derived DC as well as intrinsic differences in mouse and human DC (reviewed in [243]).

When we examined CD14+ subpopulations in particular, we found that classical (CD14++CD16-) monocytes seemed to be the primary source of surface marker upregulation (**Figure 3.4A**). Classical monocytes also expressed

inflammatory cytokines IL-6 and TNF-α at higher frequencies than non-classical (**Figure 3.4B**). We conclude that classical monocytes are the primary responders to AS01 in human PBMC, upregulating markers associated with costimulation and cell adhesion as part of their activation profile at 18 hours. Non-classical monocytes were able to upregulate costimulatory marker CD86 expression but made only limited amounts of cytokine in response to AS01, showing a great deal of donor-specific variability. Interestingly, while classical monocytes produced cytokines in response to both LPS and AS01, non-classical monocytes had significantly lower cytokine production in the AS01 compared to LPS condition (**Figure 3.4C** and **3.4D**).

To gain a broader picture of the AS01-induced monocytic response, RNASeq was performed on purified monocytes exposed to AS01. Transcriptomic analysis showed a strong induction of key inflammatory pathways such as TNF and IL1 signaling, likely providing a superior environment for T cell activation. These inflammatory pathways were upregulated even in the oldest of donors (**Figure 3.6A**). More intriguingly, pathway analysis of older donors compared to younger donors showed no significant differences in cytokine signaling pathways despite a plethora of evidence that age impacts monocyte cytokine secretion, which may suggest that AS01 is able to overcome age-related downregulation of cytokine signaling (**Figure 3.6B**) [124,128,244]. Instead, age affected AS01's upregulation of metabolic pathways, in accordance with reports that aged monocytes have metabolic differences from younger monocytes [124,127]. While significant downregulation in pathways affecting B and T cell functionality was

seen in older donors, those pathways were driven by relatively few genes. The intensity of gene induction does not, at our sample size of 8 donors per age group, show statistically significant differences in inflammatory genes between young and older donors. The pathway analysis of this RNASeq data supports AS01's ability to induce inflammatory responses even in older adults.

Network analysis showed that several transcription factors associated with inflammation were highly upregulated in response to AS01 exposure (**Figure 3.6C**). Many of the major hubs identified in this analysis are known to be associated with TLR4 signaling. Three of the major hubs identified by our network analysis– NFKBIA, Map2k3, and JUN– were also identified as upregulated in whole blood RNA sequencing from mice post vaccination [214]. Another hub centered around HIF-1α, a transcription factor involved in adapting to hypoxic conditions such as those found during inflammation [245]. HIF-1α upregulation is also a key part of inflammatory macrophage polarization through its promotion of glycolysis [246]. Intriguingly, HIF-1α is also a major mediator of trained immunity in monocytes [247]. Trained immunity, or the functional reprogramming of innate immune cells based on external stimuli, is mediated by epigenetic and metabolic changes and is hallmarked by increased TNF-α, IL-1β, and IL-6 production in response to stimuli [247,248]. Others have found that this trained immunity phenotype can be induced in older adults and is linked to improved monocyte function [244]. Further exploration is required to see if AS01 is acting to promote vaccine efficacy by inducing prolonged monocyte activation via epigenetic modification.

Network analysis also shows transcriptional differences for genes associated with mRNA splicing and regulation, such as the hnRNP family and DDX5 (**Figure 3.6C**). Both DDX5 and hnRNPs regulate mRNA stability via methylation of 6-N adenine (m6A), which stabilizes mRNA and increases the number of times a transcript can be used for translation [249,250]. Together, our data shows that AS01 exposure in human monocytes leads to a robust and multifaceted upregulation of immune transcriptional pathways.

Other upregulated hubs are not transcription factors but nevertheless are known to be key mediators of immune activation. MAPK8 is a mitogen-activated protein kinase associated with TLR4 signaling [251]. In our network analysis, MAPK8 is upstream from JAK1 and IRAK2 activation, showing how it links to TLR-induced activation (**Figure 3.6C**). Likewise, we show upregulation of CTNNB1, or β-catenin, which is a key mediator of Wnt signaling and a positive regulator of apoptosis and autophagy [252]. However, we do not see signs of apoptosis in our samples at 18 hours, nor are mediators of apoptosis such as TRAIL or the CARD family upregulated in RNA sequencing data at 10 hours. In fact, the only caspase upregulated is CASP1, involved in the inflammasome. In contrast, several genes involved in the initiation and maturation of autophagy such as GSK, ATG4, and ATG16 are significantly upregulated. Autophagy is known to impact adaptive immunity by increasing antigen presentation on MHC Class II molecules [253]. These hubs are likely contributing to monocytic activation.

A number of these hubs are being driven by younger donors. For example, CTNNB1, MAPK8, KRAS, HIF-1α, and NFKBIA are all significantly

upregulated in donors under 35, but not donors over 60 years old. In contrast, JUN, PTPN6, IRAK2 and JAK1 are upregulated in both older and younger donors. PTPN6 is a negative regulator of MyD88-mediated Syk activation [254]. Fas, an apoptotic effector, is upregulated in older, but not younger, monocytes, as is GSK-3β, a key signal mediator in apoptosis and autophagy [252].

When we looked in our data for age-related changes in cytokine production, we saw that production of cytokines such as TNF-α and IL-10 did not correlate with age. While IL-1β+ frequency did decrease with age, positivity for IL-1β following AS01 treatment remained quite high (**Figure 3.5B**). Likewise, upregulation of other costimulatory markers such as CD86 or CD11c in response to AS01 actually increased with donor age (**Figure 3.5C**). We conclude that despite age-associated decline in immune function, monocytes were still capable of producing inflammatory and costimulatory signals in response to AS01.

In a functional assay, AS01 was able to induce superior T cell costimulation by CD14+ APCs compared to mDC, which are typically considered the more potent APC. We attribute this difference to costimulatory molecule upregulation induced in CD14+. While QS-21 is also able to induce the expression of costimulatory molecules in APCs (**Figure 3.3**), it typically did so to a lesser extent to AS01. Costimulation promotes T cell effector functions, even in secondary responses [255]. Our results indicate that AS01 is able to promote costimulation from monocytes within 18 hours, showing functional impacts of the costimulatory marker upregulation induced by AS01 (**Figure 3.1, Figure 3.3**). While DCs are typically thought of as superior APCs in the context of naive T

cells, monocytes are known to provide direct antigen presentation to memory CD4+ T cells [256–258] and may be acting in the context of AS01 to boost crucial memory CD4+ T cell responses to VZV. Nearly all persons receiving RZV have prior exposure to VZV due to childhood exposure, providing an antigen-experienced pool of T cells for RZV to amplify.

However, vaccines that contain AS01 likely induce protection through more than the adjuvanticity of AS01 alone. In our data, AS01 itself does not induce Type II IFN signaling, which has been shown to be key in vaccine protection previously [214,259]. In conjunction with our observation that AS01 alone does not induce IFN-γ production in NK cells or T cells (**Figure 3.2**), our data supports that AS01-induced IFN-γ signaling seems to be antigen-dependent in AS01-containing vaccines [259].

Age-associated sterile inflammation, or inflammaging, is linked to poor immune outcomes (reviewed in [143]). Increased baseline expression of inflammatory cytokines by senescent cells leads to decreased ability to mount immune responses in response to antigenic stimulus [121,122,260–263]. However, a distinction must be made between baseline inflammation and inflammation that is induced by a pathogenic stimulus. Local and systemic inflammatory responses after vaccination are clearly linked to immunogenicity in several vaccine contexts [259,264–266]. While some molecules show decreased upregulation with donor age, such as IL-1β, others such as TNF-α do not correlate negatively with age in our dataset. Additionally, upregulation of surface molecules associated with costimulation such as CD86 and CD11c actually increase with donor age (**Figure**

3.5). While the IFN response of myeloid cells are known to be defective in older adults [122,124], we show in this paper that AS01's cytokine signature involves inflammatory cytokines that are associated with an aging phenotype such as TNF-α and IL-6, which are strongly produced even in older donors. While more investigation is needed into how AS01 acts in a vaccine setting in humans, our data paired with clinical outcomes of RZV suggest that the strong myeloid activation induced by AS01 results in a broadly protective response despite advanced donor age. In this paper, we show a potential mechanism for this efficacy by showing that AS01 increases the costimulatory capacity of monocytes in addition to upregulation of inflammatory cytokines.

The clinical success of RZV demonstrates that establishing protective and lasting immune responses is possible even in older adults. In this study, we seek to characterize how AS01 impacts human peripheral blood cells. We show by flow cytometry that monocytes, rather than mDC, are a key responding population in both young and old adults. RNA sequencing in monocytes shows that key functional pathways are upregulated in response to AS01, even in older adults. We demonstrate that costimulatory marker upregulation connects to APC functionality using a T cell costimulation assay, where AS01 promotes IFN-γ production in T cells co-cultured with AS01-treated monocytes. We demonstrate that monocytes are a major responding population after AS01 exposure and are capable of providing help to T cells, suggesting a role for them in the strong efficacy profile of RZV.

Limitations

One weakness of this approach is that we were unable to commercially acquire the MPL formulation that is actually used in GSK's AS01 formulation, which is a 3-O-deacylated derivative of MPLA derived from *S. minnesota.* The additional base hydrolysis step added to MPL results in a mixture of tetra-, penta-, or hexacylated products that lack additional acyl chain(s) at the 3' position [267]. We attempted to adjust for this by using a commercially available MPLA formulation that displayed similar agonist activity in human cell lines as vaccine adjuvants containing MPL [268]. MPLA is still only representative of the MPL used in AS01's formulation, though no vaccine containing only MPL has yet to be released on the market.

3.7. Supplemental

Supplemental Table 3.1. Antibody panels.
Unless otherwise noted, all antibodies are mouse-derived. All antibodies listed are raised against human targets.

AS01 stimulation				
Target	Fluorophore	Species/Clone	Vendor	Cat. No.
LIVE/Dead Fixable Aqua Dead Stain	BV510	--	Invitrogen	L34957
CD11c	APC	3.9	Biolegend	301614
CD123	PE/Cy7	6H6	Biolegend	306010
CD14	APC/Cy7	HCD14	Biolegend	325620
CD19	BUV395	SJ25C1	BD Biosciences	563549
CD3	BUV395	UCHT1	BD Biosciences	563546
CD56	PE	5.1H11	Biolegend	362508
HLA-DR	BV785	L243	Bolegend	307642
IFN-γ	AF700	B27	Biolegend	506516
IL-1β	FITC	JK1B-1	Biolegend	508206
TNF-α	BV650	Mab11	Biolegend	502838
Contributions of AS01 components to myeloid activation: surface upregulation				
Target	Fluorophore	Species/Clone	Vendor	Cat. No.
LIVE/Dead Fixable Aqua Dead Stain	BV510	--	Invitrogen	L34957
CD11c	APC	3.9	Biolegend	301614
CD123	PE/Cy7	6H6	Biolegend	306010
CD14	APC/Cy7	HCD14	Biolegend	325620
CD19	BUV395	SJ25C1	BD Biosciences	563549
CD1c	PE	L161	Biolegend	331506
CD3	BUV395	UCHT1	BD Biosciences	563546
CD40	PE/Dazzle	SC3	Biolegend	334342
CD54	FITC	HCD54	Biolegend	322720
CD86	Pacific Blue	IT2.2	Biolegend	305423
HLA-DR	AF700	L243	Biolegend	307626

Contributions of AS01 components to myeloid activation: cytokine upregulation

Target	Fluorophore	Species/Clone	Vendor	Cat. No.
LIVE/Dead Fixable Aqua Dead Stain	BV510	--	Invitrogen	L34957
CD11c	FITC	3.9	eBioscience	11-0116-73
CD123	PE/Cy7	6H6	Biolegend	306010
CD14	BV421	M5E2	Biolegend	301828
CD19	BUV395	SJ25C1	BD Biosciences	563549
CD3	APC/Cy7	UCHT1	Biolegend	300426
HLA-DR	AF700	L243	Biolegend	307626
IL-8	APC	E8N1	Biolegend	511410
TNF-α	PE	Mab11	eBioscience	12-7349-12

Activation of monocyte subtypes: surface marker panel

Target	Fluorophore	Species/Clone	Vendor	Cat. No.
LIVE/Dead Fixable Aqua Dead Stain	BV510	--	Invitrogen	L34957
CD115	PE/Dazzle 594	Rat-4D21E4	Biolegend	347320
CD11b	PE/Cy7	LM2	Biolegend	393104
CD14	FITC	6103	Invitrogen	11-0149-42
CD16	BV785	B73.1	Biolegend	360734
CD19	BUV395	SJ25C1	BD Biosciences	563549
CD3	BUV395	UCHT1	BD Biosciences	563546
CD86	Pacific Blue	IT2.2	Biolegend	305423
HLA-DR	AF700	L243	Biolegend	307626

Activation of monocyte subtypes: cytokine marker panel				
Target	Fluorophore	Species/Clone	Vendor	Cat. No.
LIVE/Dead Fixable Aqua Dead Stain	BV510	--	Invitrogen	L34957
CD14	FITC	6103	Invitrogen	11-0149-42
CD16	BV785	B73.1	Biolegend	360734
CD19	BUV395	SJ25C1	BD Biosciences	563549
CD3	BUV395	UCHT1	BD Biosciences	563546
CD56	BV421	HCD56	Biolegend	318326
HLA-DR	AF700	L243	Biolegend	307626
IL-10	BV711	Rat JES3-9D7	BD Biosciences	564050
IL-12/IL-23 p40	PE	C11.5	Biolegend	501807
IL-6	APC	Rat MQ2-13A5	Biolegend	501112
TNF-α	BV650	Mab11	Biolegend	502938
RNA sequencing: sort panel				
Target	Fluorophore	Species/Clone	Vendor	Cat. No.
LIVE/Dead Fixable Aqua Dead Stain	BV510	--	Invitrogen	L34957
CD11c	APC	3.9	Biolegend	301614
CD123	PE/Cy7	6H6	Biolegend	306010
CD14	FITC	6103	Invitrogen	11-0149-42
CD19	BUV395	SJ25C1	BD Biosciences	563549
CD3	BUV395	UCHT1	BD Biosciences	563546
CD56	PE	5.1H11	Biolegend	362508
CD86	PE/Dazzle 594	IT2.2	Biolegend	305434
HLA-DR	AF700	L243	Biolegend	307626
Sort Check: APC Fixation Sort				
Target	Fluorophore	Species/Clone	Vendor	Cat. No.
LIVE/Dead Fixable Aqua Dead Stain	BV510	--	Invitrogen	L34957
CD11c	APC	3.9	Biolegend	301614
CD123	PE/Cy7	6H6	Biolegend	306010
CD14	FITC	6103	Invitrogen	11-0149-42
CD19	BUV395	SJ25C1	BD Biosciences	563549
CD3	BUV395	UCHT1	BD Biosciences	563546
HLA-DR	AF700	L243	Biolegend	307626

Supplemental Table 3.2. Differentially expressed pathways between AS01 and US condition. RNA sequencing analysis was performed as described for **Figure 3.6** before differential expression between AS01 and unstimulated (US) condition were compared. Top pathways were filtered by p value ≤ 0.05.

AS01 vs US: Custom Ingenuity Analysis DEP

	AS01 vs US Regulation	AS01 vs US P.Value	AS01 vs US Adj P.Value	AS01 vs US logFC
IL-17_Signaling	Up	0.000109	0.0176	0.209
Erythropoietin_Signa...	Up	0.000279	0.0176	0.155
Cholecystokinin_Gast...	Up	0.00033	0.0176	0.146
Histidine_Metabolism	Dn	0.000345	0.0176	-0.17
Hepatic_Cholestasis	Up	0.000415	0.0176	0.202
Bladder_Cancer_Signa...	Up	0.000482	0.0176	0.176
Role_of_Cytokines_in...	Up	0.000509	0.0176	0.364
Role_of_Macrophages_...	Up	0.000528	0.0176	0.134
Hepatic_Fibrosis___H...	Up	0.000555	0.0176	0.206
GNRH_Signaling	Up	0.000573	0.0176	0.147
Ceramide_Signaling	Up	0.000601	0.0176	0.16
Pancreatic_Adenocarc...	Up	0.000653	0.0176	0.155
CXCR4_Signaling	Up	0.000684	0.0176	0.124
Galpha12_13_Signalin...	Up	0.000714	0.0176	0.14
Cell_Cycle_Regulatio...	Up	0.000743	0.0176	0.171
ATM_Signaling	Up	0.00076	0.0176	0.187
Renal_Cell_Carcinoma...	Up	0.000864	0.0182	0.114
TREM1_Signaling	Up	0.000938	0.0182	0.242
Molecular_Mechanisms...	Up	0.000943	0.0182	0.104
Melanoma_Signaling	Up	0.000982	0.0182	0.162
Role_of_JAK1_JAK2_an...	Up	0.00105	0.0182	0.18
HMGB1_Signaling	Up	0.00109	0.0182	0.178
HIF1alpha_Signaling	Up	0.0012	0.0182	0.133
TNFR1_Signaling	Up	0.00122	0.0182	0.155
Renin-Angiotensin_Si...	Up	0.00124	0.0182	0.135
Folate_Biosynthesis	Up	0.00128	0.0182	0.265
IL-1_Signaling	Up	0.00179	0.0209	0.166
p38_MAPK_Signaling	Up	0.00189	0.0209	0.121
IL-17A_Signaling_in_...	Up	0.00192	0.0209	0.225
Toll-like_Receptor_S...	Up	0.00193	0.0209	0.18
PPARalpha_RXRalpha_A...	Up	0.00194	0.0209	0.101
Glioblastoma_Multifo...	Up	0.002	0.0209	0.107
Phenylalanine_Metabo...	Dn	0.00201	0.0209	-0.158
IL-17A_Signaling_in_...	Up	0.00206	0.0209	0.174
Colorectal_Cancer_Me...	Up	0.00216	0.0209	0.131

Relaxin_Signaling	Up	0.00217	0.0209	0.152
IL-15_Production	Up	0.0022	0.0209	0.23
IL-2_Signaling	Up	0.00226	0.0209	0.105
Prostate_Cancer_Sign...	Up	0.0023	0.0209	0.119
IL-17A_Signaling_in_...	Up	0.00231	0.0209	0.238
Role_of_JAK_family_k...	Up	0.00231	0.0209	0.166
TNFR2_Signaling	Up	0.0024	0.0211	0.247
PI3K_AKT_Signaling	Up	0.00244	0.0211	0.1
HGF_Signaling	Up	0.00252	0.0212	0.115
MIF_Regulation_of_In...	Up	0.00263	0.0216	0.207
Endothelin-1_Signali...	Up	0.00268	0.0216	0.0912
BMP_signaling_pathwa...	Up	0.00288	0.0228	0.168
Acute_Phase_Response...	Up	0.00306	0.023	0.146
Role_of_Osteoblasts_...	Up	0.00309	0.023	0.123
LPS-stimulated_MAPK_...	Up	0.0031	0.023	0.135
Airway_Inflammation_...	Up	0.00321	0.0231	0.482
Airway_Pathology_in_...	Up	0.00324	0.0231	0.483
IL-9_Signaling	Up	0.00349	0.0244	0.191
April_Mediated_Signa...	Up	0.00362	0.0246	0.171
Role_of_MAPK_Signali...	Up	0.00365	0.0246	0.171
Alanine_and_Aspartat...	Dn	0.00376	0.0249	-0.166
CD40_Signaling	Up	0.00394	0.0254	0.17
Differential_Regulat...	Up	0.00401	0.0254	0.402
Oncostatin_M_Signali...	Up	0.0041	0.0254	0.16
Differential_Regulat...	Up	0.00412	0.0254	0.398
IL-6_Signaling	Up	0.00419	0.0254	0.17
B_Cell_Activating_Fa...	Up	0.00436	0.0254	0.156
CD27_Signaling_in_Ly...	Up	0.00437	0.0254	0.156
Type_II_Diabetes_Mel...	Up	0.00438	0.0254	0.106
ILK_Signaling	Up	0.00461	0.0257	0.106
Role_of_IL-17A_in_Ar...	Up	0.00462	0.0257	0.201
Insulin_Receptor_Sig...	Up	0.0047	0.0257	0.0772
Melanocyte_Developme...	Up	0.00471	0.0257	0.106
NF-kappaB_Signaling	Up	0.00486	0.026	0.106
Aminoacyl-tRNA_Biosy...	Dn	0.00491	0.026	-0.13
RANK_Signaling_in_Os...	Up	0.00515	0.0266	0.119
PXR_RXR_Activation	Up	0.00527	0.0266	0.155
p53_Signaling	Up	0.00528	0.0266	0.108
IL-8_Signaling	Up	0.00553	0.0266	0.128
Ubiquinone_Biosynthe...	Dn	0.00556	0.0266	-0.105
Glucocorticoid_Recep...	Up	0.00557	0.0266	0.102
Role_of_PI3K_AKT_Sig...	Up	0.00558	0.0266	0.155
TGF-beta_Signaling	Up	0.0056	0.0266	0.111
Methane_Metabolism	Dn	0.00588	0.0273	-0.162
Role_of_NANOG_in_Mam...	Up	0.00588	0.0273	0.094

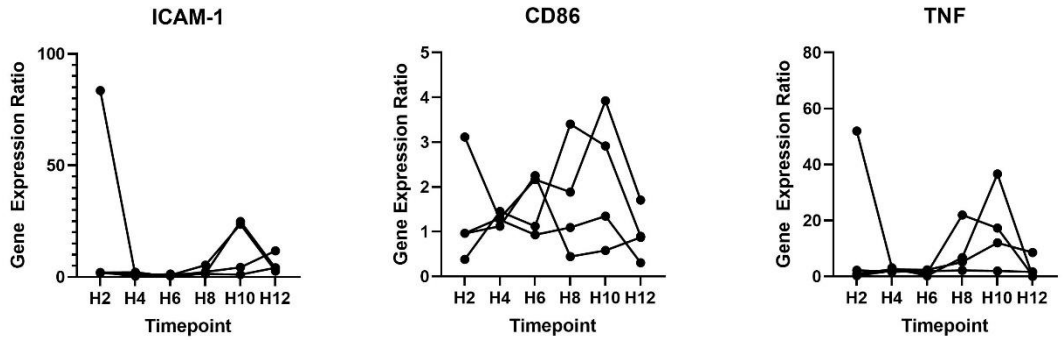
Role_of_PKR_in_Inter...	Up	0.00614	0.0279	0.179
IL-12_Signaling_and_...	Up	0.0062	0.0279	0.127
Nicotinate_and_Nicot...	Up	0.00632	0.0279	0.119
Role_of_Tissue_Facto...	Up	0.00632	0.0279	0.121
Role_of_Hypercytokin...	Up	0.00649	0.028	0.321
Lymphotoxin_beta_Rec...	Up	0.00651	0.028	0.139
Cardiac_Hypertrophy_...	Up	0.00657	0.028	0.0823
Role_of_IL-17F_in_Al...	Up	0.00679	0.0286	0.212
Neurotrophin_TRK_Sig...	Up	0.00714	0.0298	0.103
Coagulation_System	Up	0.00784	0.032	0.173
CDK5_Signaling	Up	0.00786	0.032	0.123
Tyrosine_Metabolism	Dn	0.00801	0.0323	-0.169
Production_of_Nitric...	Up	0.00851	0.034	0.0962
Thrombin_Signaling	Up	0.00876	0.0346	0.085
Chondroitin_Sulfate_...	Up	0.00902	0.0352	0.114
Small_Cell_Lung_Canc...	Up	0.00929	0.0359	0.121
Glioma_Invasiveness_...	Up	0.00965	0.0369	0.138
14-3-3-mediated_Sign...	Up	0.00989	0.0371	0.0884
PPAR_Signaling	Up	0.00989	0.0371	0.117
Death_Receptor_Signa...	Up	0.0102	0.038	0.137
IL-10_Signaling	Up	0.0103	0.038	0.135
Role_of_CHK_Proteins...	Up	0.0105	0.0383	0.114
MIF-mediated_Glucoco...	Up	0.0108	0.0388	0.204
Glutamate_Metabolism	Dn	0.0113	0.0403	-0.135
Citrate_Cycle	Dn	0.0114	0.0403	-0.154
Induction_of_Apoptos...	Up	0.0115	0.0403	0.156
Angiopoietin_Signali...	Up	0.0117	0.0405	0.13
Chronic_Myeloid_Leuk...	Up	0.0128	0.0439	0.0982
Atherosclerosis_Sign...	Up	0.0129	0.0439	0.156
Glycosphingolipid_Bi...	Up	0.0131	0.0439	0.255
Lysine_Degradation	Dn	0.0131	0.0439	-0.136
Role_of_Pattern_Reco...	Up	0.0133	0.0441	0.137
P2Y_Purigenic_Recept...	Up	0.0134	0.0441	0.0947
4-1BB_Signaling_in_T...	Up	0.0137	0.0444	0.183
Synaptic_Long_Term_D...	Up	0.0138	0.0444	0.0933
Pentose_and_Glucuron...	Dn	0.0141	0.0452	-0.115
Neuregulin_Signaling	Up	0.015	0.0474	0.0671
Sphingosine-1-phosph...	Up	0.0151	0.0474	0.0964
SAPK_JNK_Signaling	Up	0.0156	0.0487	0.0878
LPS_IL-1_Mediated_In...	Up	0.0162	0.0499	0.109
Germ_Cell-Sertoli_Ce...	Up	0.0163	0.0499	0.0807
Linoleic_Acid_Metabo...	Dn	0.0167	0.0505	-0.168
Cell_Cycle:_G2_M_DNA...	Up	0.0168	0.0505	0.0706
Notch_Signaling	Up	0.017	0.0508	0.127
Estrogen-Dependent_B...	Up	0.0174	0.0513	0.117

Type_I_Diabetes_Mell...	Up	0.0174	0.0513	0.0992
Ovarian_Cancer_Signa...	Up	0.0176	0.0513	0.104
Extrinsic_Prothrombi...	Up	0.0182	0.0528	0.15
Aryl_Hydrocarbon_Rec...	Up	0.0201	0.0577	0.0767
Endometrial_Cancer_S...	Up	0.0212	0.0599	0.0934
TWEAK_Signaling	Up	0.0212	0.0599	0.14
LXR_RXR_Activation	Up	0.0219	0.0617	0.148
IGF-1_Signaling	Up	0.0224	0.0622	0.0693
Pentose_Phosphate_Pa...	Dn	0.0225	0.0622	-0.131
IL-22_Signaling	Up	0.0239	0.0658	0.116
Docosahexaenoic_Acid...	Up	0.0244	0.0666	0.101
Cyanoamino_Acid_Meta...	Dn	0.0246	0.0667	-0.189
Melatonin_Signaling	Up	0.0249	0.0669	0.0748
Communication_betwee...	Up	0.0255	0.0681	0.123
IL-3_Signaling	Up	0.0257	0.0681	0.0715
Regulation_of_IL-2_E...	Up	0.0264	0.0695	0.0917
Apoptosis_Signaling	Up	0.0282	0.0736	0.0954
Nucleotide_Excision_...	Dn	0.0291	0.0755	-0.126
ERK5_Signaling	Up	0.0295	0.0759	0.0884
Xenobiotic_Metabolis...	Up	0.0306	0.0783	0.0801
Pyruvate_Metabolism	Dn	0.032	0.0809	-0.115
Role_of_JAK1_and_JAK...	Up	0.0321	0.0809	0.0922
FGF_Signaling	Up	0.0323	0.0811	0.09
IL-15_Signaling	Up	0.0343	0.0854	0.0951
Assembly_of_RNA_Poly...	Dn	0.0348	0.0862	-0.179
Neuropathic_Pain_Sig...	Up	0.0353	0.0868	0.0882
Cyclins_and_Cell_Cyc...	Up	0.0362	0.0882	0.0863
O-Glycan_Biosynthesi...	Up	0.0372	0.0903	0.143
Retinoic_acid_Mediat...	Up	0.0386	0.093	0.0662
Role_of_IL-17A_in_Ps...	Up	0.0392	0.0938	0.225
Semaphorin_Signaling...	Up	0.0413	0.0983	0.085
Dendritic_Cell_Matur...	Up	0.044	0.103	0.0778
PDGF_Signaling	Up	0.044	0.103	0.0715
Wnt_beta-catenin_Sig...	Up	0.0443	0.103	0.086
Aldosterone_Signalin...	Up	0.0461	0.107	0.0589
Inhibition_of_Angiog...	Up	0.0491	0.113	0.0794
Role_of_RIG1-like_Re...	Up	0.0499	0.114	0.123

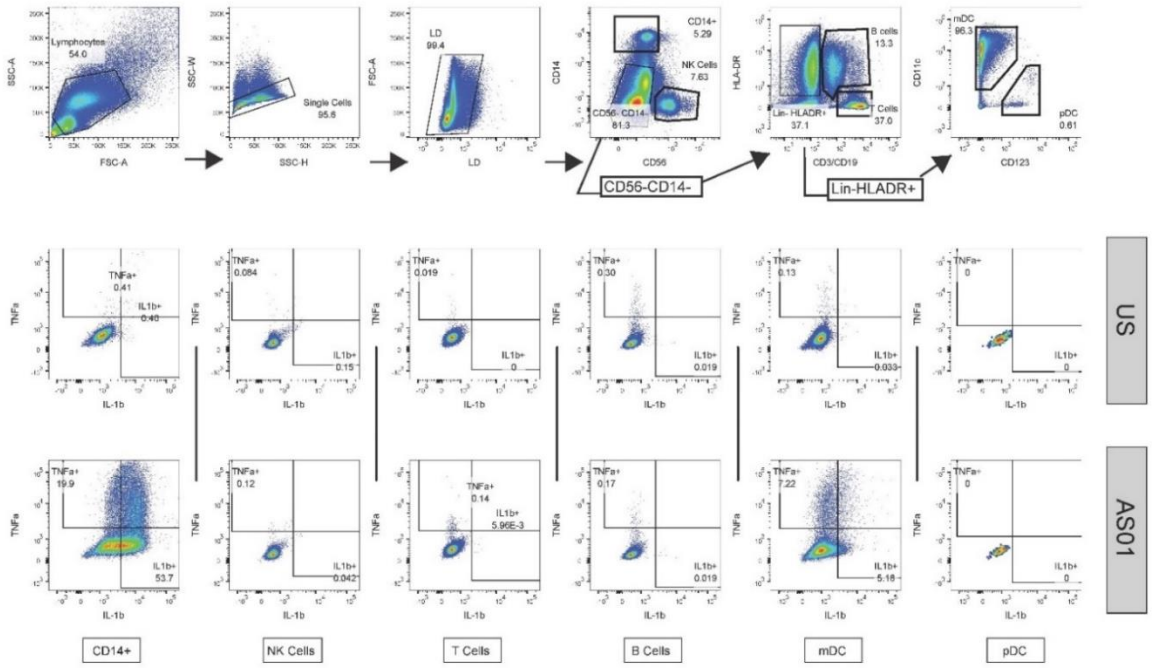
Supplemental Table 3.3. Differentially expressed pathway (DEP) analysis of old versus young donors stimulated with AS01. RNA sequencing analysis was performed as described for **Figure 3.6**. Top pathways were filtered by p value ≤ 0.05. Comparisons between young and old AS01 response were performed after normalizing each donor's gene expression in the unstimulated condition.

Young versus Old Baselined DEP Custom Ingenuity Pathway Analysis				
Pathway	**Young Vs Old Regulation**	**Young vs Old P Value**	**Young vs Old Adj. P value**	**Young vs Old logFC**
Assembly_of_RNA_Polymerase_1_Complex	Dn	0.0073	0.795	-0.618
B_Cell_Development	Dn	0.00983	0.795	-0.517
Calcium-induced_T_Lymphocyte_Apoptosis	Dn	0.0431	0.795	-0.301
Intrinsic_Prothrombin_Activation_Pathway	Dn	0.0387	0.795	-0.417
Methane_Metabolism	Dn	0.0187	0.795	-0.362
Pentose_and_Glucuronate_Interconversions	Dn	0.0368	0.795	-0.265
Phenylalanine_Metabolism	Dn	0.043	0.795	-0.262

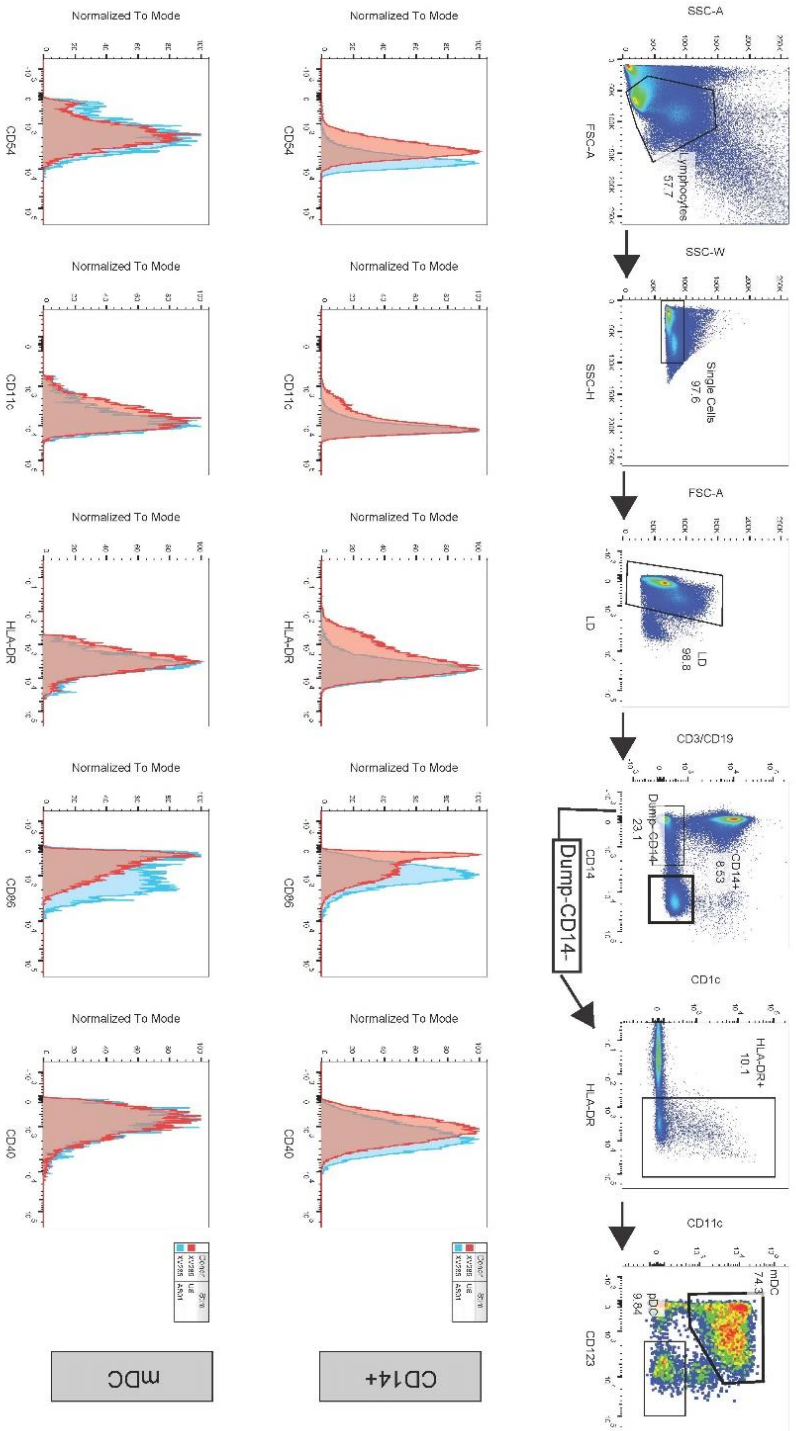
Supplemental Figure 3.1. Timepoint selection for RNAseq. qPCR was performed on RNA isolated from CD14+ cells after selected timepoints of incubation with AS01. Primers were designed using the Mass Gen Primer Bank (PrimerBank (harvard.edu)) and ordered through Thermofisher. qPCR focused on 3 genes known to be upregulated in response to AS01 in monocytes. PBMC was stimulated (or not) with 1:100 AS01. At 2, 4, 6, 8, 12, and 18 hours, CD14+ cells were purified from PBMC using CD14 positive selection kit (Biolegend, 480024). CD14+ cells were lysed in RLT buffer and processed to RNA using Qiagen's RNAeasy Micro kit (74004). RNA was quantified using Nanodrop and frozen at -20*. qPCR was performed using iQ SYBR Green Supermix (Bio-rad, 1708882) according to manufacturer instructions on StepOne Plus (Applied Biosystems, serial number 272003145). Gene expression ratio was calculated using Pfaffel test compared to unstimulated monocytes from the same timepoint. Actin B (ACTB) was used as a housekeeping gene for normalization. Timepoints for RNAseq were selected based on peak expression of CD86, HLA-DR, and CD54, which had been identified as targets that increased by 18 hours via flow cytometry.



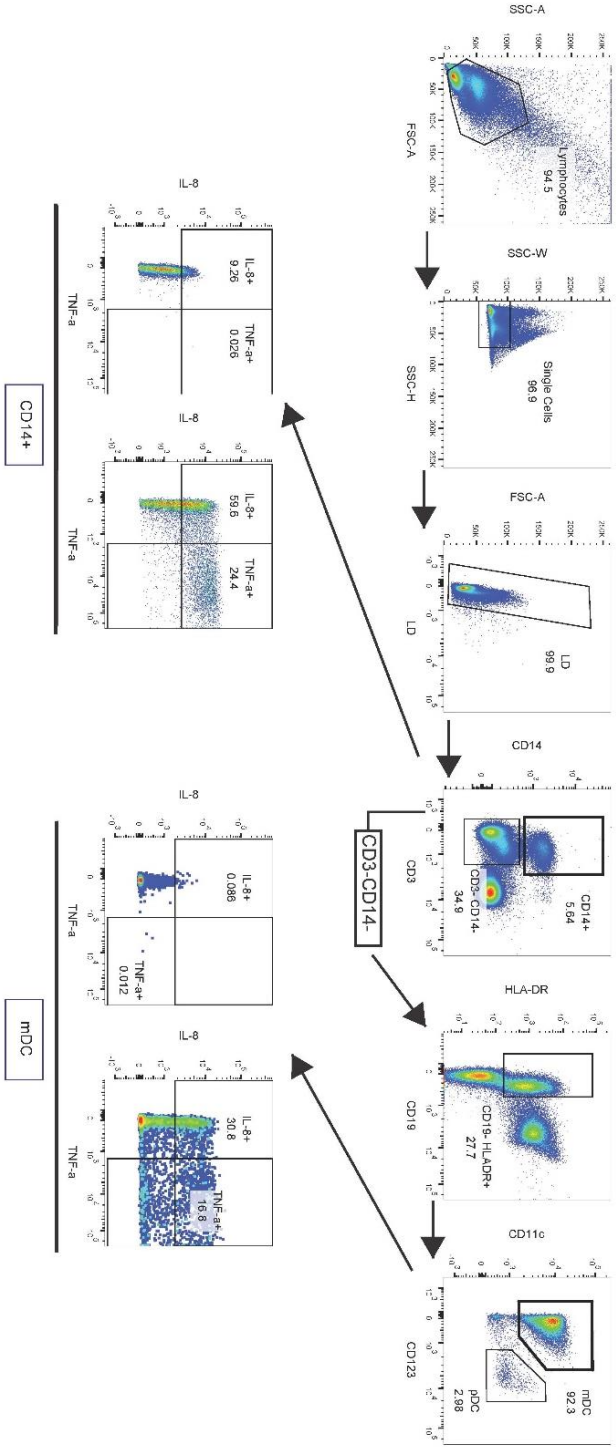
Supplemental Figure 3.2. Gating layout for initial examination of effects of AS01 on human PBMC. PBMC were stimulated with AS01 or a positive control, LPS + 1:100 killed flu vaccine, for 18 hours. BFA was added to the cells to prevent release of cytokines during the assay. After stimulation, cells were run on a flow cytometer and gated according to the following strategy. Data is reported in Figure 3.1 (mDC and monocytes) or Figure 3.2 (NK cells, T cells, pDC, or B cells).



Supplemental Figure 3.3. Gating layout for surface marker analysis of mDC and CD14+ response to AS01 and its component parts. PBMC were gated according to the following strategy after 18 hours of stimulation with AS01, MPLA, or QS-21.

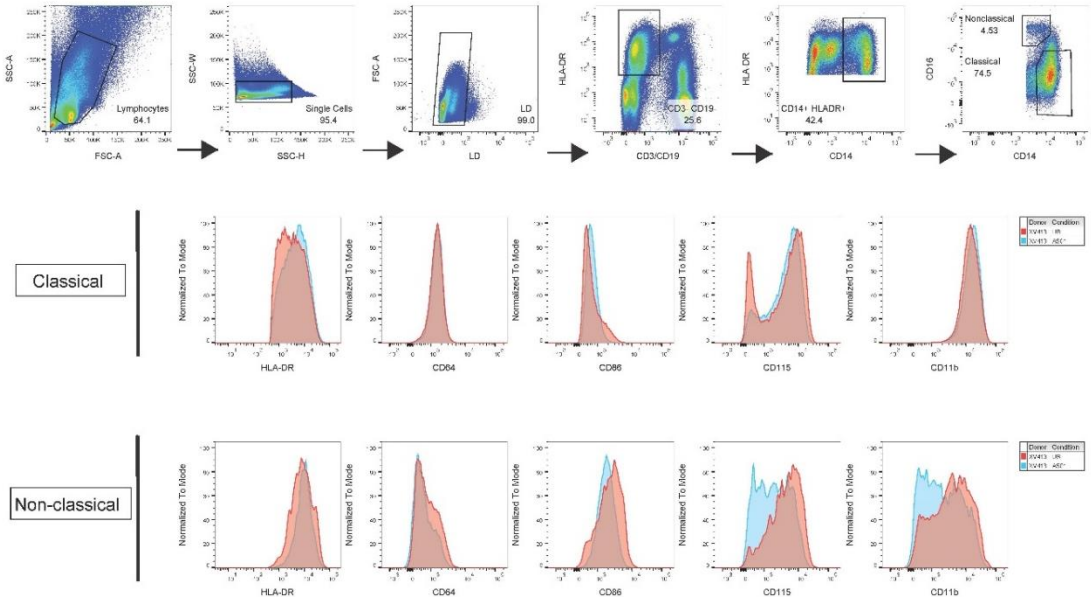


Supplemental Figure 3.4. Gating layout for cytokine analysis of mDC and CD14+ response to AS01 and its component parts. PBMC were gated according to the following strategy after 18 hours of stimulation with AS01, MPLA, or QS-21. BFA was added to the cells during incubation to retain cytokines produced.

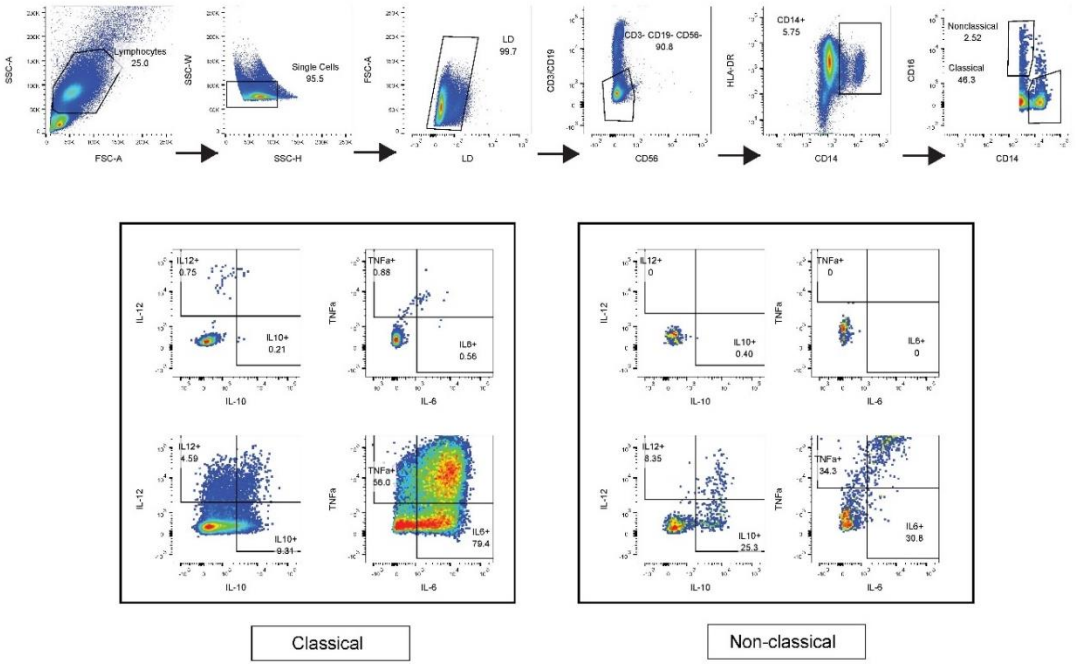


Supplemental Figure 3.5. Gating strategies for monocyte subsets. Example gating for **A**) surface marker upregulation in classical or non-classical monocytes and **B**) cytokine production by monocyte subsets.

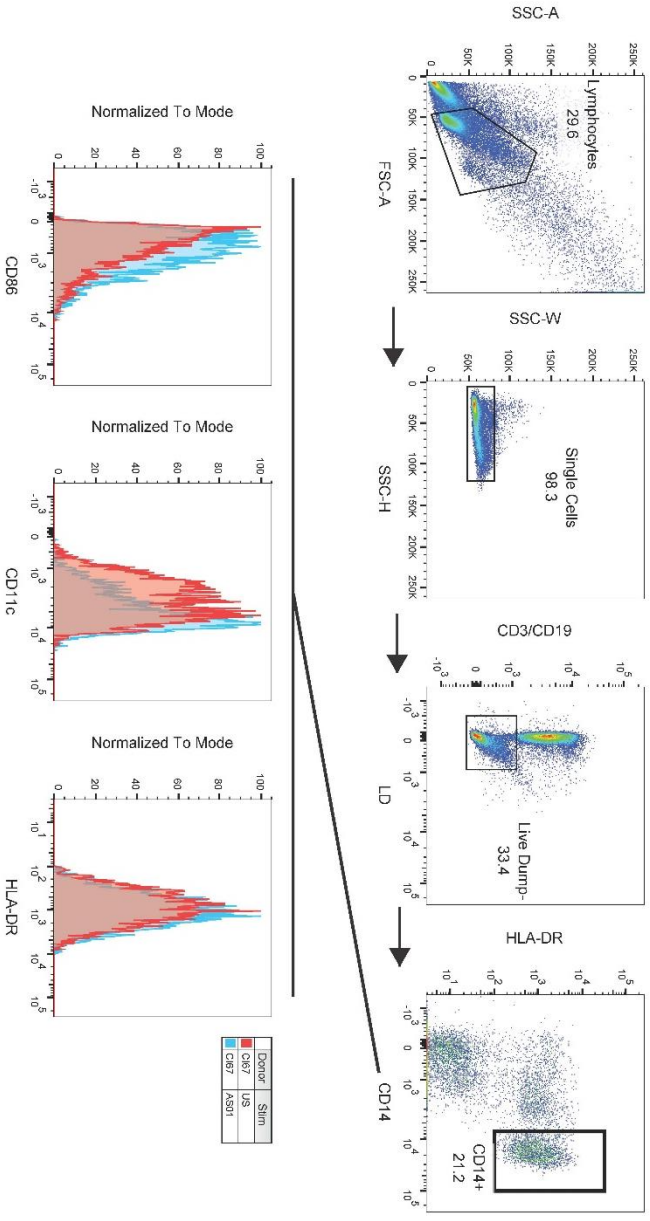
A



B



Supplemental Figure 3.6. Gating strategy used for RNAsequencing sort. Monocytes were sorted from human PBMC according to the following gating strategy after 10 hours with or without AS01 stimulation.



Chapter 4: Humoral and Cellular Immunity Induced by Adjuvanted and Standard Trivalent Influenza Vaccine in Older Nursing Home Resident

4.1. Author Contribution Statement

Carson Smith contributed to the formal analysis, investigation, original drafting, review, editing, and visualization of this work, as defined by the CRediT (Contributor Roles Taxonomy) protocol available via Elsevier.

4.2. Abstract

Background

Despite wide use of adjuvanted influenza vaccine in nursing home residents (NHR), little immunogenicity data exist for this population.

Methods

We collected blood from NHR (n=85) living in nursing homes participating in a cluster randomized clinical trial comparing MF59-adjuvanted trivalent inactivated influenza vaccine (aTIV) with non-adjuvanted vaccine (TIV) (parent trial, NCT02882100). NHR received either vaccine during the 2016-2017 influenza season. We assessed cellular and humoral immunity using flow cytometry and hemagglutinin inhibition (HAI), anti-neuraminidase (ELLA), and microneutralization assays.

Results

Both vaccines were similarly immunogenic and induced antigen-specific antibodies and T cells, but aTIV specifically induced significantly larger D28 titers against A/H3N2 neuraminidase than TIV.

Conclusions

NHRs respond immunologically to TIV and aTIV. From these data, the larger aTIV-induced anti-neuraminidase response at D28 may help explain the increased clinical protection observed in the parent clinical trial for aTIV over TIV in NHR during the A/H3N2-dominant 2016-2017 influenza season. Additionally, a decline back to pre-vaccination titers at 6 months after vaccination emphasizes the importance of annual vaccination against influenza.

4.3. Introduction

Influenza ranks among the most common causes of respiratory illness, making it a target for vaccine campaigns that aim to limit its associated morbidity and mortality. Estimates attribute over 100,000 hospitalizations annually to influenza in the US alone, most of which occur in older adults [269,270]. Phenomenon such as antigenic drift and shift undermine the effectiveness of annual vaccination campaigns within and across influenza seasons. Additionally, vaccine effectiveness declines with increasing age, comorbidities, and frailty, a particularly prevalent combination of vulnerabilities for nursing home residents (NHR). NHR generally also risk more opportunities for disease transmission due to communal living [165,271]. One strategy developed to improve vaccine response includes the addition of an adjuvant to the influenza vaccine formulation.

In a 2016-2017 cluster randomized clinical trial, we found reduced NHR hospitalization in facilities that offered adjuvanted trivalent inactivated vaccine (aTIV) as a care standard compared to those offering unadjuvanted trivalent inactivated vaccine (TIV) [272]. Immunogenicity studies conducted in community-dwelling older adults consistently report increased antibody titers to aTIV compared to non-adjuvanted vaccine [273,274]. Here, we focus on the immunogenicity of aTIV and TIV in NHRs who are at higher risk for serious infection. Clinical efficacy studies that also examine immunogenicity have typically limited their observations to HAI titer. Despite the increased susceptibility of both aged individuals and those living communally, NHRs'

immunological response to influenza vaccination remains understudied, especially compared to healthier populations.

In this study, we sought to understand the immunological basis of the differential clinical protection that TIV and aTIV provided during the 2016-2017 influenza season, as described in our parent cluster-randomized clinical trial in the NHR population (NCT02882100) [272]. We examine not only HAI titers but also investigate differences in anti-neuraminidase (anti-NA) titers and cellular immunity to address gaps in data on aTIV's relative impact on immunity compared to TIV in NHR. We found that humoral immunity induced by these vaccines differed only in anti-NA titers, specifically against A/H3N2. We found no differences in cellular immunity between the two vaccine groups. As others have found, we show that immune responses expanded after vaccination and contracted by D180 post vaccination [56,275].

4.4. Materials and methods

Study design/recruitment

Persons that were aged 65 years and older and residents of a Medicare-certified long-term care facilities in the metro areas of Denver, Colorado, or Cleveland, Ohio, were recruited for this study. These facilities were cluster-randomized as part of a large, pragmatic clinical trial (NCT02882100) with each building as a whole randomized to provide aTIV or standard-of-care TIV for all residents over age 65 who were going to receive an influenza vaccine [272]. We obtained informed consent directly from NHR or their legally authorized representative as approved by the New England IRB (NEIRB) for this immunogenicity sub-study.

Sample processing.

We sampled blood from subjects 0-14 days prior to vaccination to determine a baseline for their influenza immunity and again at seven (D7) and 28 (D28) days after vaccination. Blood was also collected from a subset of donors approximately 6 months (D180) after vaccination. Blood samples underwent peripheral blood mononuclear cell (PBMC) purification using Ficoll gradient centrifugation and were cryopreserved in fetal bovine serum (FBS) containing 10% DMSO in liquid nitrogen. We also separated serum from blood and stored serum samples at -80°C.

Assays

HAI. Hemagglutination inhibition assays (HAI) were performed according to established protocols using hemagglutinin from the 2016-2017 influenza

season and turkey red blood cells (Lampire Biological Laboratories, Coopersburg, Pennsylvania) [276]. Strain-matched inactivated virus for A/H1N1 (A/California/07/2009 NYMC X-179A), A/H3N2 (A/Hong Kong/4801/2014), and influenza B (B/Brisbane/60/2008) were provided by CSL Seqirus.

Anti-neuraminidase titers. The anti-neuraminidase enzyme-linked lectin assay (ELLA), was performed based on established protocols [277]. Briefly, plates were coated with fetuin (Sigma, F3385; 25 µg/mL) before addition of heat-treated human sera. Active neuraminidase for A/H1N1 or A/H3N2 was added to plate and incubated at 37°C for 16 hours. For A/H1N1, neuraminidase with exact strain match was supplied by CSL Seqirus (A/California/07/2009 NYMC X-179A; sequence accession GQ214336). For A/H3N2 neuraminidase, a strain with 95.7% homology (A/Babol/36/2005 (A/H3N2)) was used (Antibodies-online Inc. Limerick, PA; sequence accession ACN50232). Plates were then washed and incubated with peanut agglutinin horseradish peroxidase (Sigma, L7759; 1:500) for 2 hrs at RT before washing and visualization with citrate buffer and o-phenylenediamine dihydrochloride (OPD) tablet (Sigma; P8287). Reaction was stopped with 0.5 M sulfuric acid before optical density was read at 490 nm.

Microneutralization. Microneutralization assays (MN) were performed according to established protocols using live A/H1N1 and A/H3N2 strains that were vaccine strain-matched, supplied by CSL Seqirus [276].

Flow cytometry. For flow cytometry analysis of vaccine-specific T cell frequency, PBMC were thawed and stimulated in X-VIVO 15 media (Lonza, Cat 04-418Q) overnight using pooled A/H1N1 and A/H3N2 antigen (10 µg/mL as

quantified by HA; CSL Seqirus) and anti-CD28/CD49d costimulation (eBiosciences, #16-0289; Biolegend, #304310). These were the exact antigens used in the production of the vaccines. After 2 hours, brefeldin A (BFA; Sigma, B7651; 5 μg/mL) was added to the stimulation and cells were returned to incubator. After 20 hours of stimulation, cells were stained using eBioscience Foxp3/Transcription Factor Staining Buffer set (Invitrogen; 00-5523) and LIVE/Dead Fixable Aqua Dead Stain (Invitrogen L34957); anti-CD3 BUV395 (BD Biosciences, 563546), anti-CD4 APC/Cy7 (Biolegend, 300518), anti-CD8 BV786 (BD, 563823), anti-CD45RA PE-Texas Red (Invitrogen, MHCD45RA17), anti-CCR7 PE/Cy7 (Biolegend, 353226), anti-granzyme B PE/Cy5.5 (Invitrogen, GRB18), anti-perforin-1 PE (Cell Sciences, CDM247), anti-CD107a FITC(Biolegend, 328606), anti-IL-2 APC (Biolegend, 500311), anti-IFN-γ AF700 (Biolegend, 506516), and anti-TNF-α Pacific Blue (Biolegend, 502920). Cells were fixed after staining with 1% paraformaldehyde. Flow cytometry was performed on BD Fortessa and analyzed using FlowJo.

For flow cytometry evaluation of cT_{FH} activation, cells were stimulated as above and anti-CD40 (Beckman-Coulter MAB89, Catalog #IM1374; 2.5 μg/mL) was added with anti-CD28/CD49d costimulation. After 20 hours, cells were stained with LIVE/Dead Fixable Aqua Dead Stain (Invitrogen L34957), anti-CD3 BUV395 (BD Biosciences, 563546), anti-CD4 BUV805 (BD, 612887), anti-CD8 BV786 (BD, 563823), anti-CCR7 BV605 (Biolegend, 353224), anti-CD45RA APC-H7 (BD, 560674), anti-CD297 BV421 (Biolegend, 329920), anti-CXCR5 PE/Dazzle 594 (Biolegend,356928), anti-CD278 (ICOS) FITC (Biolegend,

313506), anti-CD154 (CD40L) PE (Biolegend, 310806), anti-CD274 (PD-L1) APC (Biolegend, 329708), and anti-CD134 (OX40) PE/Cy7 (Biolegend, 350012).

Analysis

Humoral immunity. We defined seroconversion as a four-fold rise in titer and seroprotection as an absolute HAI titer above 40. We calculated the geometric mean fold change from baseline to D28 and its 95% confidence interval within both vaccine groups. We summarized baseline comparability in humoral endpoints between vaccine groups, calculating the standard mean difference (SMD) between the vaccine groups for each log-transformed assay at baseline [125]. Assays with SMD <0.1 at baseline were considered as comparable within this subset of the cluster randomized cohort and were analyzed at day 28 without adjustment using t-test of log-transformed assays to compare geometric mean titer ratio of the two vaccines against a null value of 1. For assays with baseline SMD ≥0.1, analyses of day 28 and D180 values were adjusted for baseline levels. These adjusted analyses implemented an ANCOVA/regression model predicting log assay at D28 or D180 using [log assay at day 0 + vaccine]. Pearson correlations by vaccine and assay were calculated for log-transformed baseline assays and D28 fold change.

Cellular immunity. Cytokine immune endpoints were compared using mixed modeling analysis given the repeated measures within subjects and missing data over time. Square-root transformed values were estimated as a function of vaccine arm, time (baseline, D7, and D180), and their interaction. The interaction of vaccine arm and time was the effect of interest. Detected model

effects were further assessed with post-hoc Tukey-adjusted contrasts. Analysis of humoral endpoints and cellular immune endpoints was performed in R version 4.1.3 with mixed models estimated using functions in the nlme package.

T cell polyfunctionality. The analysis used SPICE and Monte Carlo generated permutations to compare timepoints. Cells that singly expressed granzyme B or perforin as well as co-expressing only perforin and granzyme were removed from analysis due to our assay's inability to distinguish whether these cells were vaccine antigen-specific or constitutively expressing perforin and granzyme [278].

All P-values are presented as unadjusted unless otherwise indicated in this exploratory analysis to understand mechanistic differences between vaccine groups with significant observed outcomes in NHR.

4.5. Results

We evaluated 88 nursing home residents with half over age 80, over two-thirds women, and nearly a quarter non-white (**Table 4.1**).

Cohort Demographics		
All (n=85)	TIV (n=42)	aTIV (n=43)
Age: Mean +/- SD	83 +/- 10	80 +/- 9
Age: Median (IQR)	83 (76, 91)	79 (74, 86)
Age: Range	61-103	65-97
Race & Ethnicity: White, non-Hispanic	32 (76%)	33 (77%)
Race & Ethnicity: Black, non-Hispanic	10 (24%)	10 (23%)
Race & Ethnicity: Hispanic	1	0
Sex: Male	13 (31%)	10 (23%)
Sex: Female	29 (69%)	33 (77%)

Table 4.1. **Demographics of recipients of trivalent inactivated vaccine (TIV) and adjuvanted trivalent inactivated vaccine (aTIV).**

<u>Humoral Immunity</u>

For all assays, aTIV and TIV titers rose from baseline titers to significantly elevated D28 titers within a vaccine group. Additionally, anti-NA titers against A/H1N1 decreased significantly from D28 to D180. In contrast, A/H3N2 anti-NA titers did not differ significantly from D28 to D180. For A/H1N1, HAI and MN

decreased significantly from D28 to D180 after vaccination. For A/H3N2, D28 to D180 HAI and MN titers were not statistically different in either vaccine (**Table 4.2; Figure 4.1**).

Vaccine Group			aTIV		TIV	
	Strain		H1N1	H3N2	H1N1	H3N2
BL to D28	Anti-NA	GMFC	2.73	1.32	1.68	1.23
		p value	1.00E-05	0.01	2.00E-04	0.04
	HAI	GMFC	3.55	6.34	3.52	7.08
		p value	1.00E-07	2.00E-07	2.00E-07	1.00E-08
	MN	GMFC	3.82	5.55	4.88	11.7
		p value	3.00E-07	1.00E-08	1.00E-07	1.00E-07
D28 to D180	Anti-NA	GMFC	0.72	1.28	0.72	0.92
		p value	0.04	0.27	0.002	0.54
	HAI	GMFC	0.4	0.44	0.5	0.67
		p value	5.00E-04	0.06	4.00E-04	0.08
	MN	GMFC	0.35	0.7	0.43	0.66
		p value	2.00E-03	0.24	1.00E-04	0.12

Table 4.2. Statistics for humoral titer changes within a vaccine group between timepoints. For standard (TIV) and adjuvanted (aTIV) influenza vaccines, geometric mean fold changes (GMFC) were calculated and statistical tests comparing GMFC to 1 within a group were performed using t-tests on log-transformed fold changes. Anti-neuraminidase (NA) immunity was assessed using ELLA directed against strain-specific (NA), while anti-hemagglutinin (HA) immunity was assessed using HA inhibition (HAI) and microneutralization (MN) assays. Analysis was performed using GMFC of the titers reported at baseline (BL), D28, or D180 post vaccination. Data is shown for comparisons between BL and D28 as well as D28 and D180. P-values are presented without adjustment.

In addition to changes over time, titer changes between vaccine groups were also compared. Anti-NA A/H3N2 titers were statistically incomparable at BL (SMD >0.1). After adjusting for differences in baseline titer, anti-NA A/H3N2 titers were statistically different between aTIV and TIV subjects for A/H3N2 at D28 post vaccination (adjusted P [P*]=0.013). Anti-NA A/H3N2 titers remained statistically

different between vaccine groups even at D180 post vaccination when adjusting for baseline (p*=0.02) (**Figure 4.1A**). MN and HAI titers do not show differences between vaccine groups (**Figure 4.1B and 4.1C**).

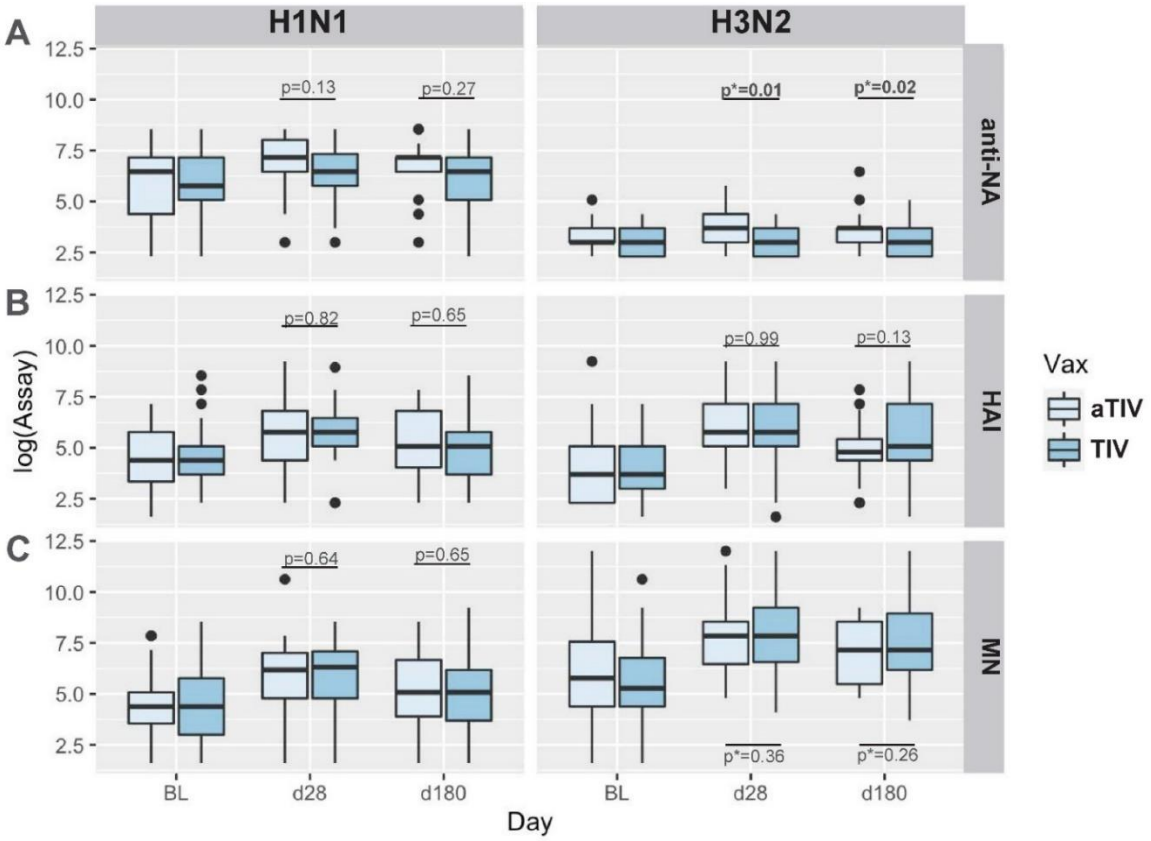


Figure 4.1. Humoral responses of donors after receiving aTIV or TIV at baseline, D28, and D180 post vaccination. Humoral immunity was assessed. Anti-neuraminidase (NA) titers were assessed using an ELLA assay directed against NA specific to the strain listed, while titers against hemagglutinin (HA) were assessed using hemagglutinin inhibition (HAI) and microneutralization (MN) assays. The standard mean difference (SMD) of baseline titers was used to determine if vaccine groups had comparable assay titers at baseline (BL). The model-estimated vaccine group p-values are marked with (P*). Assays with SMD < 0.1 were considered comparable at BL and values from D28 and D180 were compared between vaccine groups using a t-test on log-transformed assays and marked with a (P). A) Anti-NA results at BL, D28, and D180 from serum collected from vaccine recipients in the adjuvanted (aTIV) and standard dose (TIV) arms of the study against A/H1N1 or A/H3N2 antigens. P values are bolded when differences between the responses at a given timepoint are significantly different between vaccines. B) HAI titers at BL, D28, and D180 for aTIV and TIV donors, respectively. C) MN titers against A/H3N2 were not comparable at BL between vaccine groups and comparisons between later timepoints were therefore adjusted for baseline values (P*).

Around half of all donors were considered seroconverted at baseline based on HAI (**Table 4.3**). Seroconversion rates were higher with A/H3N2 than for A/H1N1. Both vaccine groups had high baseline seroprotection by HAI, yet seroprotection still increased after vaccination (**Table 4.3**). Interestingly, increases in influenza B seroprotection were limited at D28 (TIV= 62%, aTIV= 72%) compared to the more robust responses to influenza A strains (seroprotection >85% for both aTIV and TIV) (**Suppl. Fig. 4.1**). For HAI and MN assays, high baseline titers to influenza A strains correlated negatively with fold change (Pearson r = 0.4-0.5; $p < 0.05$; **Suppl. Fig. 4.2**). Vaccine groups had similar seroprotective titers.

Cellular immunity

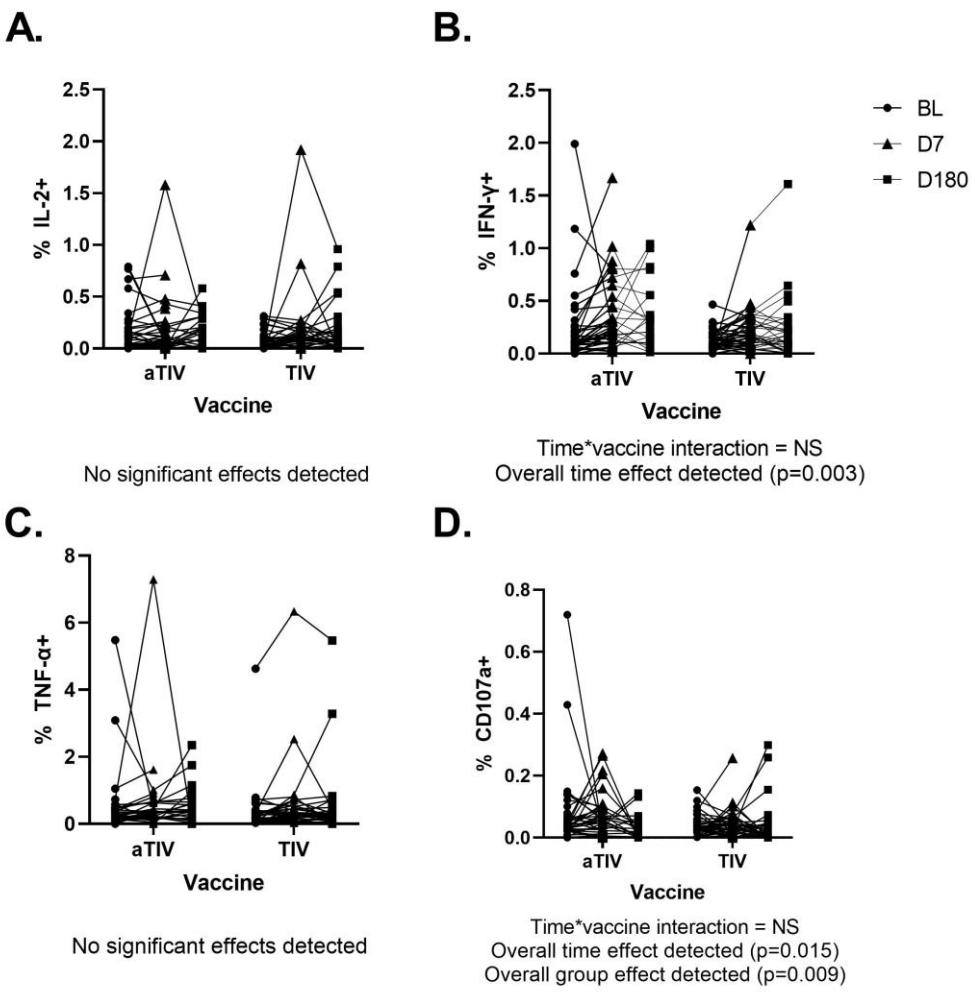
Total CD4+ and CD8+ T Cell Response

Flow cytometry was used to collect data on vaccine-induced cell-mediated immunity, particularly cytokine production and expression of cytotoxic molecules (gating strategy available in **Suppl. Fig. 4.3**). Mixed effects modeling did not detect differences between aTIV and TIV cellular response to virus over time as an interaction effect. There were therefore no significant differences between TIV and aTIV in cytokine-producing cells detected post-vaccination when cells were stimulated with pooled A/H1N1 and A/H3N2 antigens. However, statistically significant changes from baseline to D7 could be detected across groups, specifically in CD4+ T cells expressing IFN-γ and CD107a (**Figure 4.2**). No cytokines in CD8+ T cells showed significant increases from baseline to post vaccination timepoints in either vaccine group (**Suppl. Fig 4.4**). Notably, many

donors have high pre-existing cellular responses against influenza before vaccination**.**

Group	Percentage of (%):	A/H1N1	A/H3N2
TIV	Seroprotection BL	76	62
	Seroprotection D28	95	88
	Seroconversion	45	64
	Seroprotection or conversion	95	86
	HAI GMT D28	301.2	407.1
aTIV	Seroprotection BL	76	76
	Seroprotection D28	91	87
	Seroconversion	42	51
	Seroprotection or conversion	93	89
	HAI GMT D28	323.9	405.9
	Seroconversion p-value	0.924	0.315

Table 4.3. Baseline (BL) and D28 comparison of humoral measures. Seroprotection (HAI titer of at least 1:40) values were assessed at baseline (BL) and at day 28 post vaccination (D28). Seroconversion, or four-fold rise in titer by D28 post vaccination, was also assessed for each strain of influenza included in standard dose (TIV) and adjuvanted (aTIV) influenza vaccine. Values reported are for hemagglutinin inhibition assay (HAI), the standard assay used for evaluating vaccine response to influenza. P values were calculated using Chi-squared test to compare seroconversion rates between groups at D28.

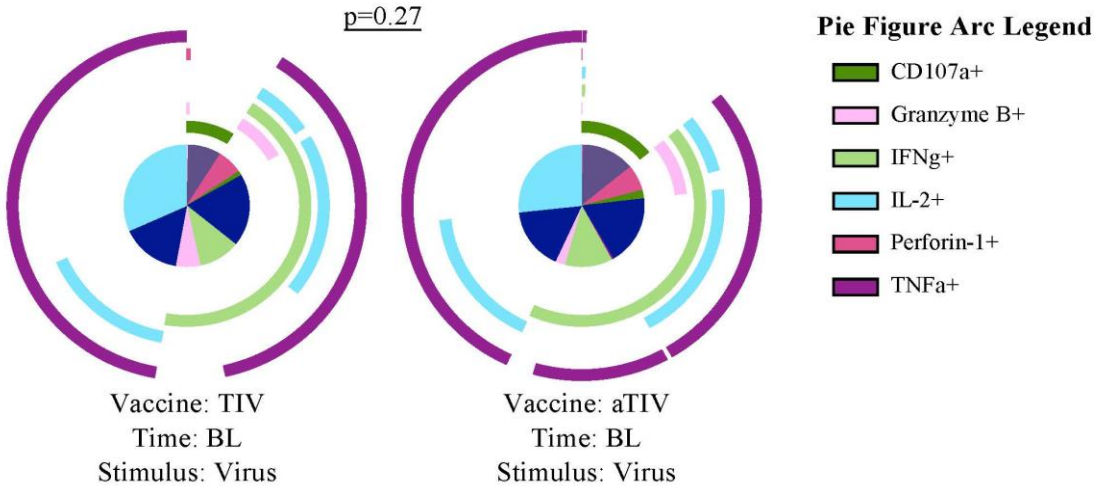


Contrast	IFN-γ p value	CD107a p value
BL vs D7	**0.0001**	0.67
BL vs D180	**0.003**	**0.04**
D7 vs D180	0.97	**0.004**

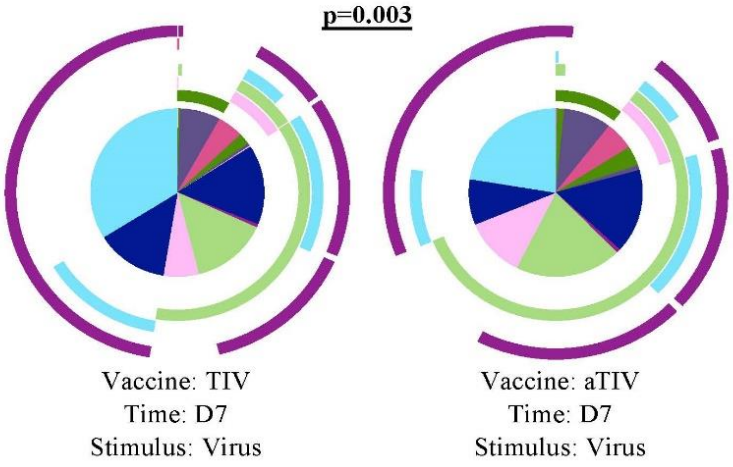
Figure 4.2. CD4+ T cell cytokine and cytotoxic molecule response before and after vaccination for each vaccine group. Flow cytometry was performed on PBMC stimulated overnight with pooled A/H1N1 and A/H3N2 antigen at baseline (BL, n=81), D7 (n=75) and D180 (n=50) after vaccination. Cells were analyzed at each timepoint for expression of A) IL-2, B) IFN-γ, C, TNF-α, and D) CD107a, normalized to effector molecule expression in unstimulated cells at that timepoint. The interaction of vaccine arm and time was the effect of interest in the mixed-effects model used. If the model detected effects, they were further assessed with post-hoc Tukey-adjusted contrasts to determine which levels of the effect variable differed from each other. Effects are reported below the graphical representation of each datapoint. Differences between vaccines after vaccination are indicated as vaccine*time, while differences within a vaccine group are referred to as time interactions. Comparisons between timepoints, when significant, are reported in the table below the figure.

We analyzed CD4+ memory (as defined by CD45RA and CCR7; see **Suppl. Fig. 4.3**) T cells for changes in polyfunctionality using SPICE, a program designed to reduce complex multi-factorial cytometry data into a graphical representation [279]. SPICE arranges Boolean-gated cytometry data into a matrix that is normalized to the unstimulated condition. Changes in the co-expression of markers over time can then be analyzed, and differences in marker co-expression between conditions can be compared. While there were no statistically significant differences in baseline polyfunctionality between vaccine arms (p=0.27), analysis of polyfunctional categories showed differences between aTIV and TIV at D7 (p=0.003) (**Figure 4.3**). The overall frequency of cells expressing more than 2 effector molecules does not appear to change: rather, differences between groups at D7 are driven by the types of molecules being co-expressed (**Figure 4.3B**). No statistically significant differences were observed in overall polyfunctionality at baseline, D7, or D180 within either vaccine arm (**Suppl. Fig. 4.5**). We did not perform CD8+ T cell polyfunctionality analysis due to the low overall number of responding CD8+ T cells in most of our donors, since SPICE is vulnerable to artifact introduced by a low number of responding cells.

A.


p=0.27
Pie Figure Arc Legend
CD107a+
Granzyme B+
IFNg+
IL-2+
Perforin-1+
TNFa+
Vaccine: TIV
Time: BL
Stimulus: Virus
Vaccine: aTIV
Time: BL
Stimulus: Virus

B


p=0.003
Vaccine: TIV
Time: D7
Stimulus: Virus
Vaccine: aTIV
Time: D7
Stimulus: Virus

C.

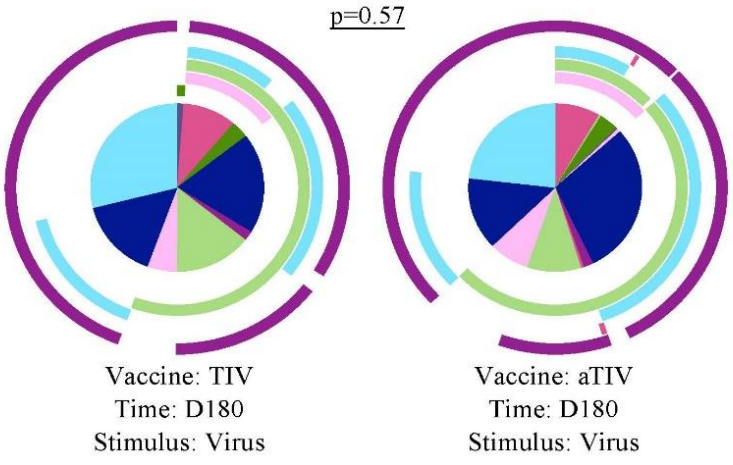

p=0.57
Vaccine: TIV
Time: D180
Stimulus: Virus
Vaccine: aTIV
Time: D180
Stimulus: Virus

Figure 4.3. Differences in polyfunctional responses generated after vaccination between aTIV and TIV. Polyfunctionality was analyzed using SPICE for both standard dose (TIV) and adjuvanted (aTIV) influenza vaccine at A) baseline (BL), B) D7, and C) D180 post vaccination. Polyfunctionality pies show co-expression of effector molecules in CD4+ memory T cells when cells were stimulated with influenza A antigens. The rings indicate the proportion of responding cells to the cytokines indicated in the legend. Rings overlap to represent cells that are expressing two or more molecules. Pie slices indicate the various combinations of effector molecules. The listed p values are for Monte Carlo-based permutation tests examining the difference between pies at the timepoints shown (α=0.05),

cT_{FH} responses

In the subset of donors with sufficient PBMC remaining after initial cytokine analysis, cT_{FH} response to pooled A/H1N1 and A/H3N2 antigens was examined (gating strategy in **Suppl. Fig. 4.6;** see Methods for more information). These peripheral cells share features of the T_{FH} found in the LN germinal center and are believed to reflect germinal center responses. In this study, we found that donors that received aTIV, but not TIV, experienced an increase in Ox40+ICOS+ and Ox40+PDL1+ cT_{FH} from baseline to D7 (**Figure 4.4**). However, SMD values ≥ 0.1 indicated baseline cT_{FH} levels were not comparable between aTIV and TIV in this subset. After adjusting for baseline levels in regression models, as described in methods, there was no difference detected in the increase in D7 antigen-specific cT_{FH} between the vaccines.

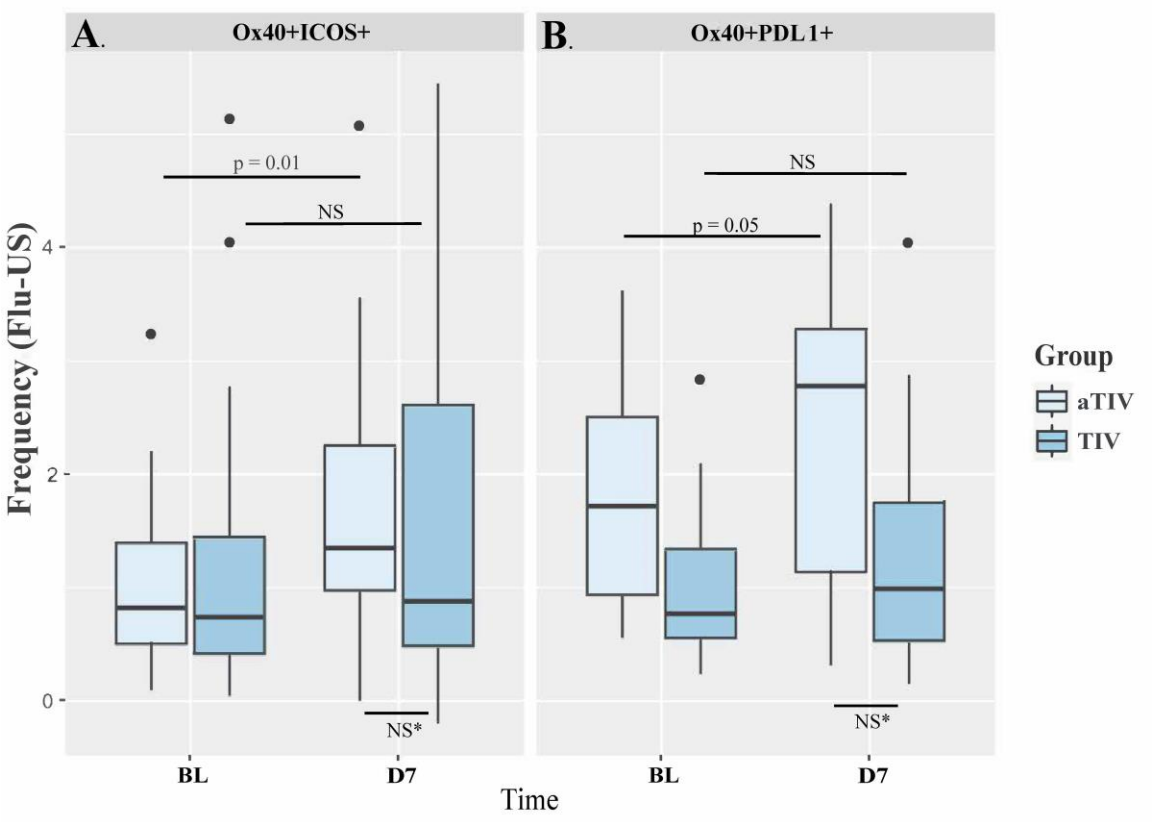


Figure 4.4. Circulating T follicular helper (cTFH) response 7 days after vaccination in aTIV and TIV subjects. Flow cytometry was used to analyze cT_{FH} response to vaccination for both cT_{FH} (aTIV= 13; TIV= 15) against pooled A/H1N1 and A/H3N2. Box-and-whisker plots are constructed as described for Figure 1. Cells were gated on PD-1+CXCR5+ memory CD4+ T cells and examined for A) activation state (Ox40+ICOS+) and B) co-expression of activation induced markers (AIM) Ox40 and PD-L1. Values reported are normalized by subtracting background unstimulated frequencies from antigen-stimulated data (Flu-US). Significance on the graph was calculated by Mann-Whitney U test for paired comparisons between timepoints. For comparisons between groups, BL differences were calculated using standard mean difference (SMD) scores as indicated in methods.

4.6. Discussion

As of 2022, the ACIP recommends the use of enhanced influenza vaccines, including adjuvanted vaccines, over standard dose vaccines in adults 65 or older [280]. Several investigators have reported that adjuvanted influenza vaccine offers greater clinical protection to older adults compared with standard TIV [272,273,281]. Yet while we accept antibody titer as assessed by HAI as a proxy for clinical protection, our present study does not demonstrate the HAI titer differences we anticipated based on the parent cluster randomized clinical trial. In that trial, we found reduced hospitalization and reduction in suspected influenza facility outbreaks in NHR was associated with aTIV, indicating better clinical protection in this vaccine despite the lack of significant difference in HAI titer reported here [272,282]. While both vaccines elicit titer increases in HAI and MN assays from baseline to D28, only the ELLA assay assessing antibodies against A/H3N2 NA showed differences between vaccine groups, and only after statistically adjusting for baseline differences between groups (**Fig. 4.1A)**. This differential response can be detected even at 180 days post vaccination, suggesting aTIV elicits long-term differences in anti-NA humoral immunity compared to TIV.

Anti-NA immunity is a known correlate of protection from severe disease in influenza, as demonstrated in a controlled inoculation trial [283]. One clinical trial comparing high dose vaccines with TIV found that anti-NA immunity, particularly anti-N2 ELLA, appeared to complement HAI titers as a correlate of protection [284]. These studies suggest that anti-NA antibodies may play an important role in

reducing the risk of hospitalization [283]. Additionally, anti-NA immunity is cross-protective in years in which strain mismatch occurs between the vaccine and circulating strains, in part due to lower substitution mutation rates in the gene for NA and the existence of fewer NA subtypes than HA [285]. Immunity against NA protein, particularly for A/H3N2, is especially important to evaluate for vaccines in the elderly population, for whom vaccines are notoriously less efficient at eliciting immunity against A/H3N2 [286,287]. Despite the known role of anti-NA immunity in protecting aged populations, comparative studies between vaccines tend to focus on anti-HA immunity and potentially miss key differences between vaccines.

We report low overall increases in seroprotection and seroconversion, but donors had high rates of baseline seroprotection, particularly against A/H1N1. Based on lower rates of seroconversion and seroprotection after vaccination, both vaccines were less effective at eliciting humoral immunity as measured by HAI against influenza B compared to influenza A strains (**Supplemental Figure 4.1; Table 4.3**). Pre-existing humoral immunity negatively correlates with HAI titer fold change after vaccination [288,289], suggesting that low fold change may be related to these subjects' high levels of pre-existing immunity. In this present study, we show this negative correlation for HAI and the NA-targeted ELLA assay as well as the functional MN assay (**Suppl. Fig 4.2A-C)**. Donors are slightly more likely to seroconvert against A/H3N2, perhaps as a result of lower initial titers and repeated previous exposures to pandemic-strain A/H1N1 during earlier years through vaccination or natural infection. Seasonal influenza vaccination rates in Ohio nursing homes have been around 70% for over 15 years [290], and repeated

influenza vaccination negatively impacts influenza vaccine response in subsequent years [291–293]. While vaccine history was not collected on donors enrolled in this study, high rates of vaccination in the sample population may account for the limited increase in humoral titers seen in both vaccine groups.

Cellular responses were similar between aTIV and TIV. TIV produced a modest but significant increase in IFN-γ-producing CD4+ T cells at D7, while aTIV did not (**Fig. 4.2B)**. However, modeling showed that aTIV and TIV cellular responses at D7 did not differ significantly between vaccines. Both TIV and aTIV are inefficient at boosting influenza-specific memory T cell frequencies in NHR, similar to a report comparing vaccine responses in older adults from the 2011-2012 influenza season [294]. Existing influenza vaccines have long been noted to be relatively inefficient at inducing memory T cell responses (reviewed in [295]), despite data that indicates T cells are particularly important in resolving influenza infection in older adults [65–67,296,297]. It is therefore unsurprising that both aTIV and TIV are only able to weakly boost frequencies of cytokine-producing CD4+ T cells. Like TIV, aTIV has limited efficacy at inducing influenza-specific T cell responses. This might be expected for a vaccine that is thought to work primarily by enhancing germinal center maturation based on animal studies of the mechanism of adjuvant MF59. MF59 has been found to induce higher antibody titers, induce greater germinal center formation, and promote antibody affinity maturation compared to standard dose or even alum adjuvant formulations [203,208,209]. These mechanisms of MF59's efficacy appear to be focused on

amplifying humoral responses and therefore are not necessarily advantageous in promoting cellular immune responses.

Polyfunctional T cells are protective in a variety of disease states or vaccine models [298–300]. When CD4+ memory T cells were analyzed for changes in polyfunctionality, aTIV induced a differential polyfunctional response than TIV (**Fig. 4.3B**). In particular, aTIV seemed to promote the co-expression of IFN-γ compared to TIV in antigen-specific cells. IFN-γ producing cells have been shown to contribute to protection against influenza in older adults [84,296]. However, there was no significant change in polyfunctional categories within a vaccine arm across timepoints (**Suppl. Fig. 4.5**): the differences between vaccine arms appear to be driven by different directions of change rather than by magnitude (**Fig. 4.3**). Influenza vaccination is a repeated antigen exposure, and additional polyfunctionality gains from vaccination may be limited in the study population of older adults, such as ours, who were on average over 80 years old and in a highly vaccinated nursing home population.

Antigen-specific cT_{FH}, defined as CD4+ memory PD1+CXCR5+ T cells, typically do not produce detectable levels of cytokines measured by routine intracellular flow cytometry methods. These cells can be identified by upregulation of AIM like Ox40 and PD-L1 in lieu of cytokine production [57,301]. In influenza vaccination, activated cT_{FH} correlate with post-vaccination titers [69,302]. These cells are reduced in frequency following vaccination in elderly adults compared to younger adults, partially due to an increased age-associated inflammatory profile that limits cT_{FH} differentiation [54,111]. In our study, donors that

received aTIV showed a statistically significant increase in antigen-specific Ox40+PDL1+ cT_{FH} at D7, but vaccine arms did not differ in cT_{FH} responses at D7 after adjustment for baseline differences (**Fig. 4.4**).

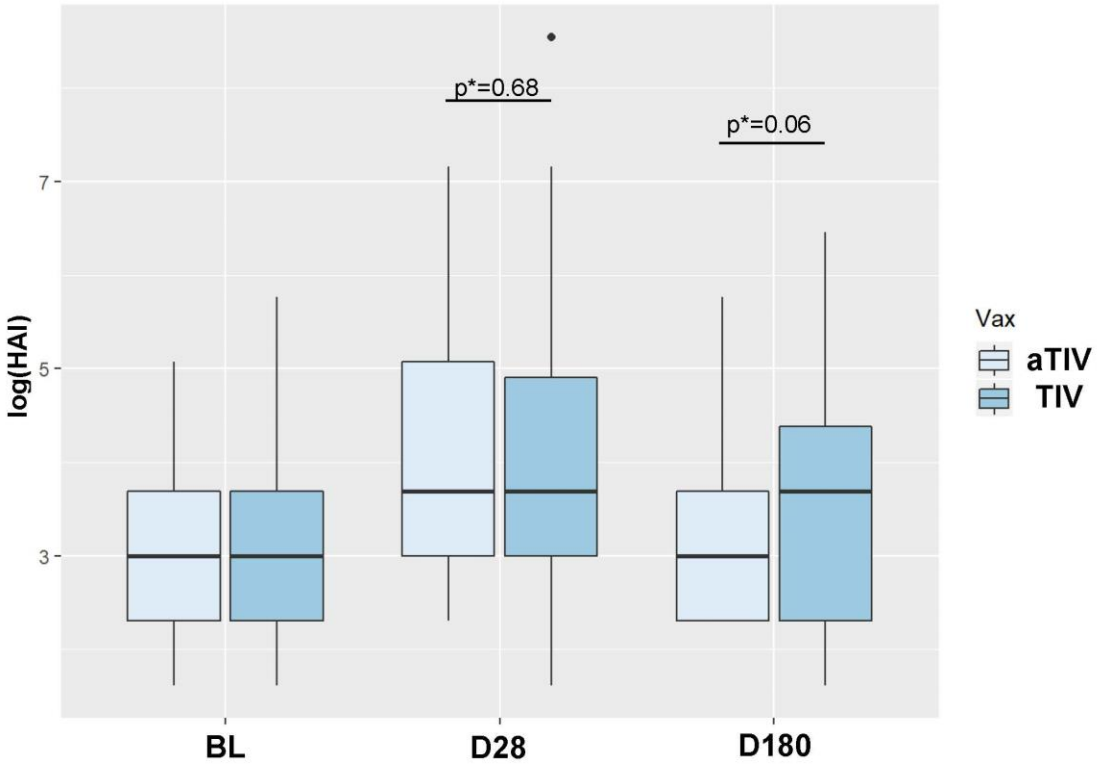
One caveat to our study is that it was only powered to detect medium-to-large effect sizes in the populations studied [303]. We may not have detected smaller vaccine-induced effect sizes in various assays that could still prove clinically relevant between vaccine groups. Our limited power also impacted our ability to correlate factors such as antibody titer and cT_{FH}. Additionally, we only assayed T cell responses at D7 and D180. Our study cannot rule out differences between the groups in T cell response that fall outside of those timepoints.

Overall, we report limited *in vitro* differences between the immune responses elicited by the two vaccines. While both vaccines show transient humoral and cellular immunity increases after vaccination, the major difference between vaccines in NHR was the higher anti-NA neutralization titers against A/H3N2 in aTIV recipients. These findings are noteworthy in light of the published clinical arm of this study [272]. In that study, we found that aTIV was more effective than TIV in preventing all-cause, pneumonia, and influenza hospitalization of NHR. Another subanalysis of the parent study showed that aTIV was associated with a reduction of suspected influenza outbreaks in the facilities in which it was used, another measure of increased clinical protection if not also less efficient transmission [282]. This present study contributes to available clinical data for the 2016-2017 influenza season by showing that the primary immunological difference between aTIV and TIV in this aged NHR population was anti-NA

humoral immunity for A/H3N2. This finding could help explain the increased protection found in our multi-site study in 2016-2017. While NHR receiving aTIV do not respond to the degree has been reported for younger adults, they have increases in immune outcomes that may prove clinically relevant compared to subjects that receive TIV. Our study confirms that aTIV is able to boost cT_{FH} levels and humoral immunity even in NHR and supports the ACIP's recent decision replacing standard dose influenza vaccines in older adults with enhanced vaccines [280].

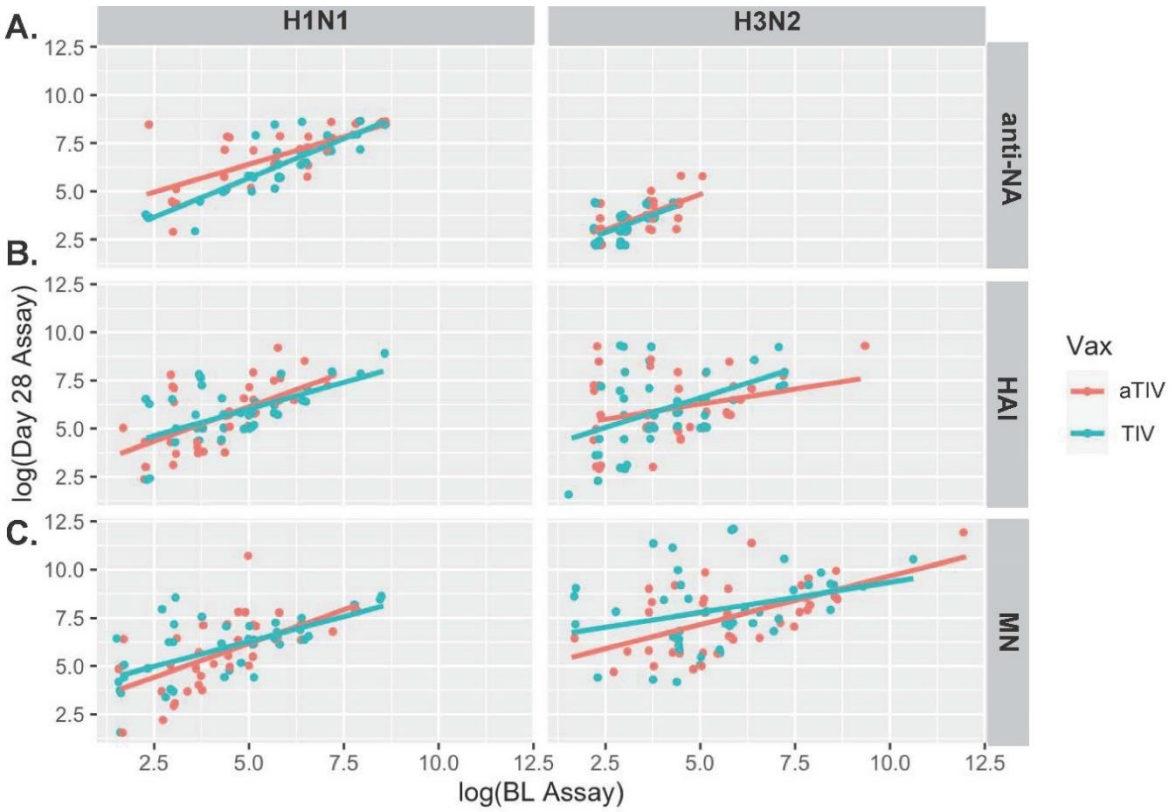
4.7. Supplemental

Supplemental Figure 4.1. Influenza B humoral responses were evaluated by HAI. Humoral responses were evaluated against influenza B/Brisbane/60/2008 using hemagglutinin inhibition (HAI) assay at baseline (BL), D28, and D180 post-vaccination. Statistical tests were performed for differences between standard dose (TIV) and adjuvanted (aTIV) vaccines. Standard mean deviation (SMD) showed differences between vaccines at BL, so p values comparing vaccine groups at D28 and D180 were adjusted for BL (p*). B) Statistical data on seroprotection and seroconversion at baseline as assessed by HAI.



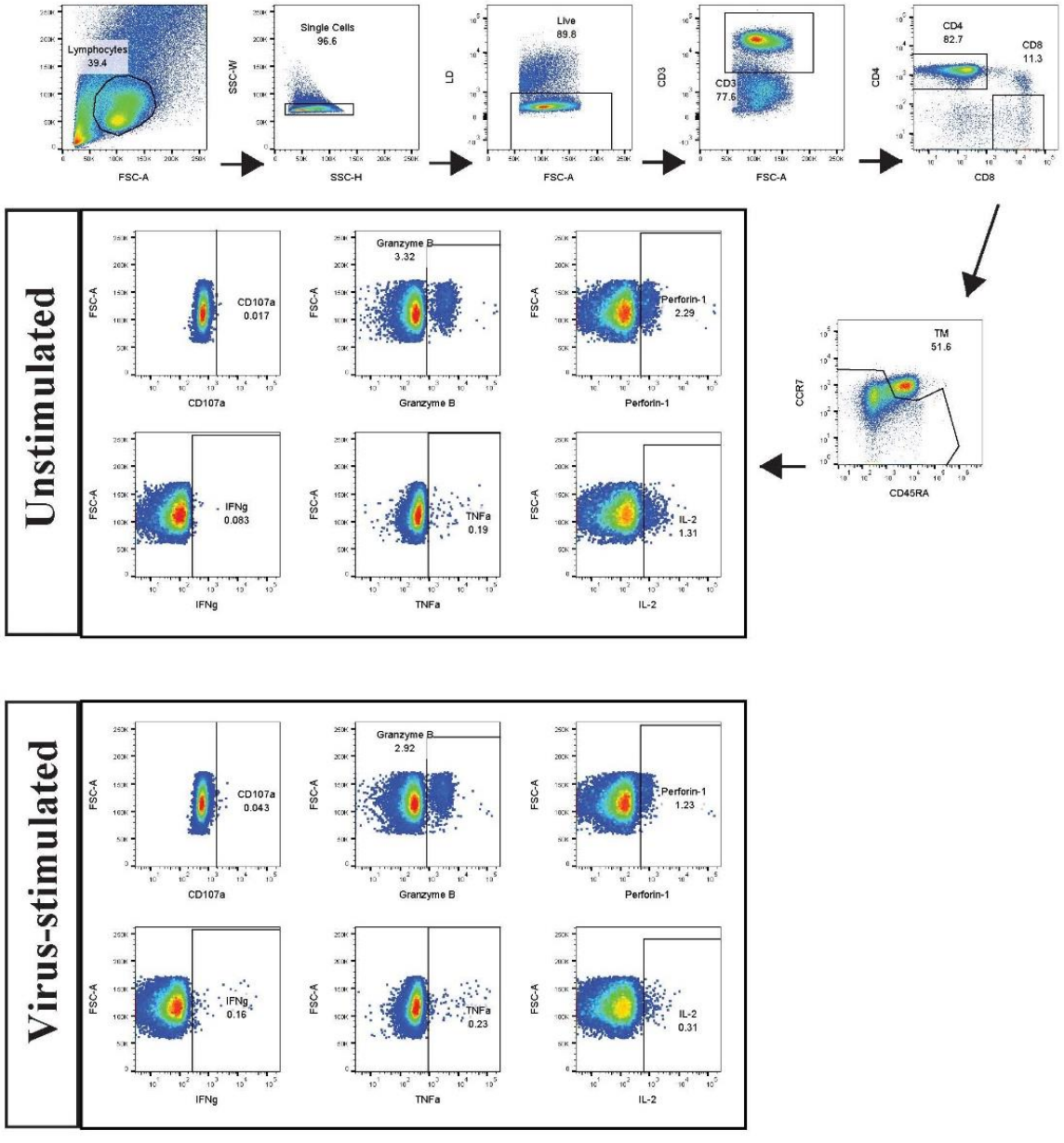
Influenza B	Vaccine	
Percentage of:	**TIV**	**aTIV**
Seroprotection BL	40	37
Seroprotection D28	62	72
Seroconversion	33	44
Seroprotection or conversion	62	74
Seroconversion p value		0.42

Supplemental Figure 4.2. Baseline humoral titer impacts fold titer change in response to both aTIV and TIV. Correlations between baseline (BL) titers and titers at D28 post vaccination were compared for standard dose (TIV, blue) and adjuvanted (aTIV, red) influenza vaccination. A) Anti-neuraminidase (anti-NA) titers were assessed using ELLA directed against strain-specific NA for both aTIV and TIV. Correlations between log-transformed titers at BL and D28 are reported below. B) Correlations of log-transformed titers against hemagglutinin (HA) at BL and D28 were assessed using hemagglutinin inhibition (HAI) assay. C) Anti-HA log-transformed titers were assessed using microneutralization (MN) assay at BL and D28. Correlation outcomes for A-C are shown in table on next page

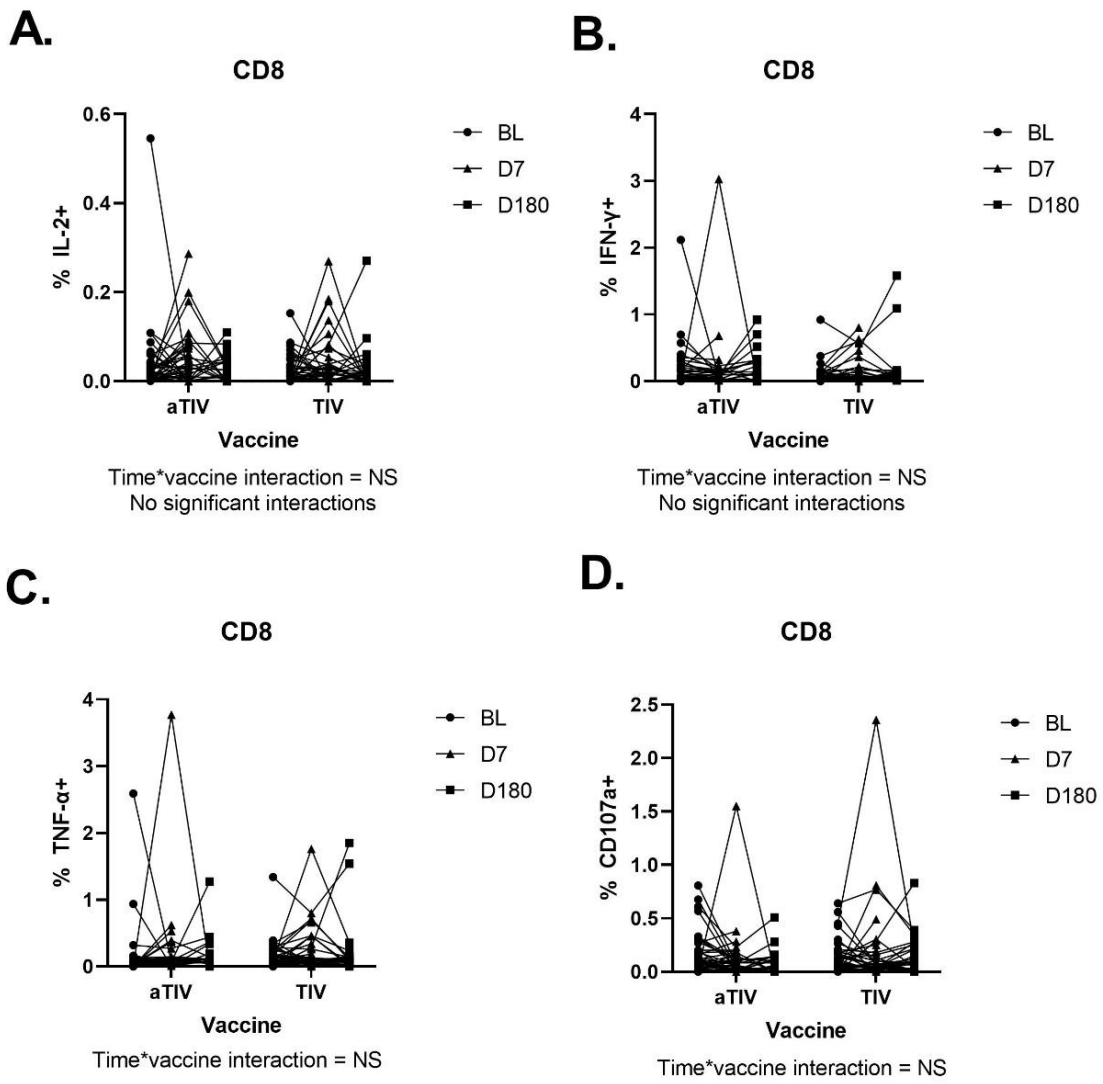


Assay	Strain	Vaccine	r value	Lower r	Upper r	p value
ELLA	H1N1	aTIV	-0.597	-0.766	-0.351	**0.00005**
ELLA	H1N1	TIV	-0.370	-0.611	-0.067	**0.019**
ELLA	H3N2	aTIV	-0.302	-0.561	0.011	0.058
ELLA	H3N2	TIV	-0.258	-0.527	0.058	0.108
HAI	H1N1	aTIV	-0.305	-0.555	-0.005	**0.047**
HAI	H1N1	TIV	-0.541	-0.726	-0.284	**0.0002**
HAI	H3N2	aTIV	-0.560	-0.736	-0.312	**0.00009**
HAI	H3N2	TIV	-0.298	-0.552	0.007	0.055
MN	H1N1	aTIV	-0.331	-0.574	-0.034	**0.030**
MN	H1N1	TIV	-0.604	-0.767	-0.367	**0.00002**
MN	H3N2	aTIV	-0.593	-0.758	-0.356	**0.00003**
MN	H3N2	TIV	-0.600	-0.764	-0.362	**0.00003**

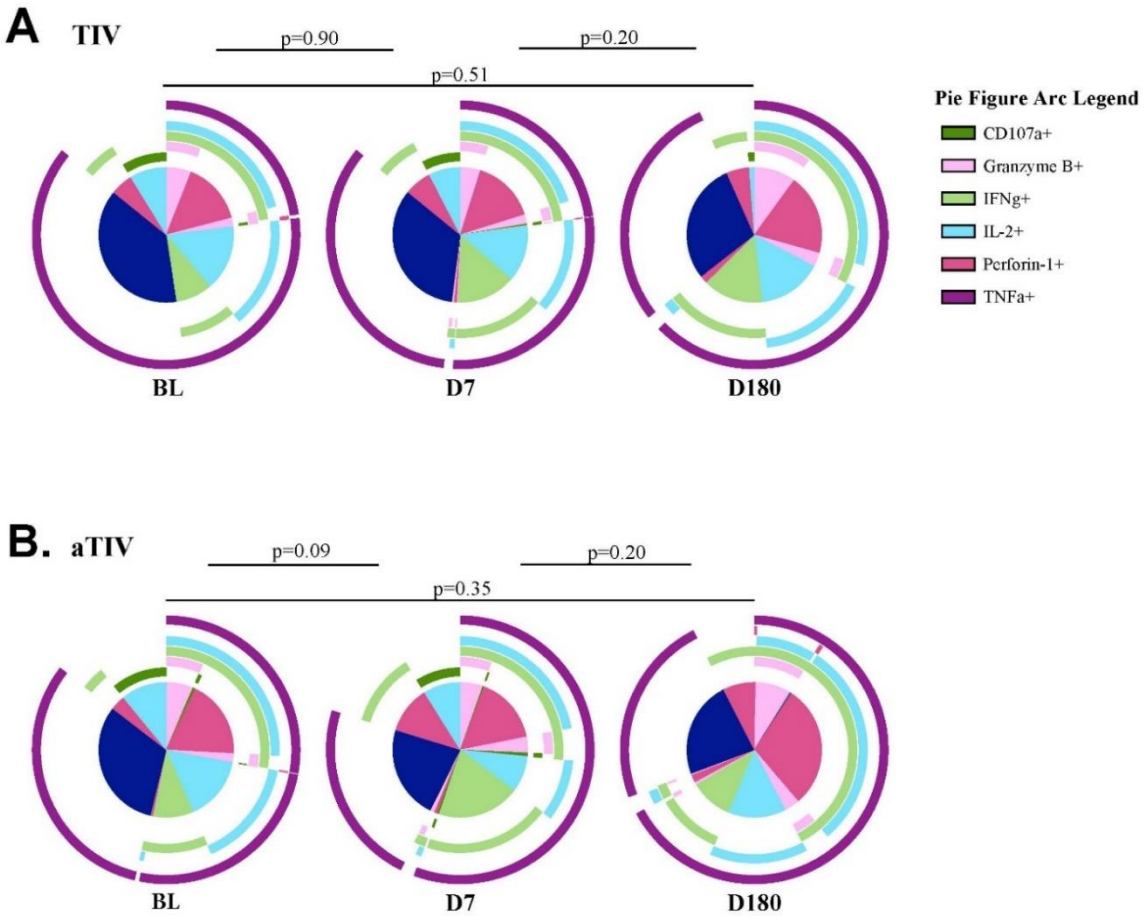
Supplemental Figure 4.3. Gating strategy for cytotoxic lymphocyte (CTL) panel. Cells were gated on live CD3+ singlet lymphocytes before gating for CD4+ or CD8+ T cells. Naive (CD45RA+CCR7+) cells were then gated out of each T cell subset and memory cells were gated for positivity of CD107a, granzyme B, IFN-γ, IL-2, perforin-1, or TNF-α. Unstimulated cells (top box) and virus-stimulated cells (bottom box) from a selected donor are shown. CD4+ and CD8+ T cells used the same gating layout for memory and effector molecule expression.



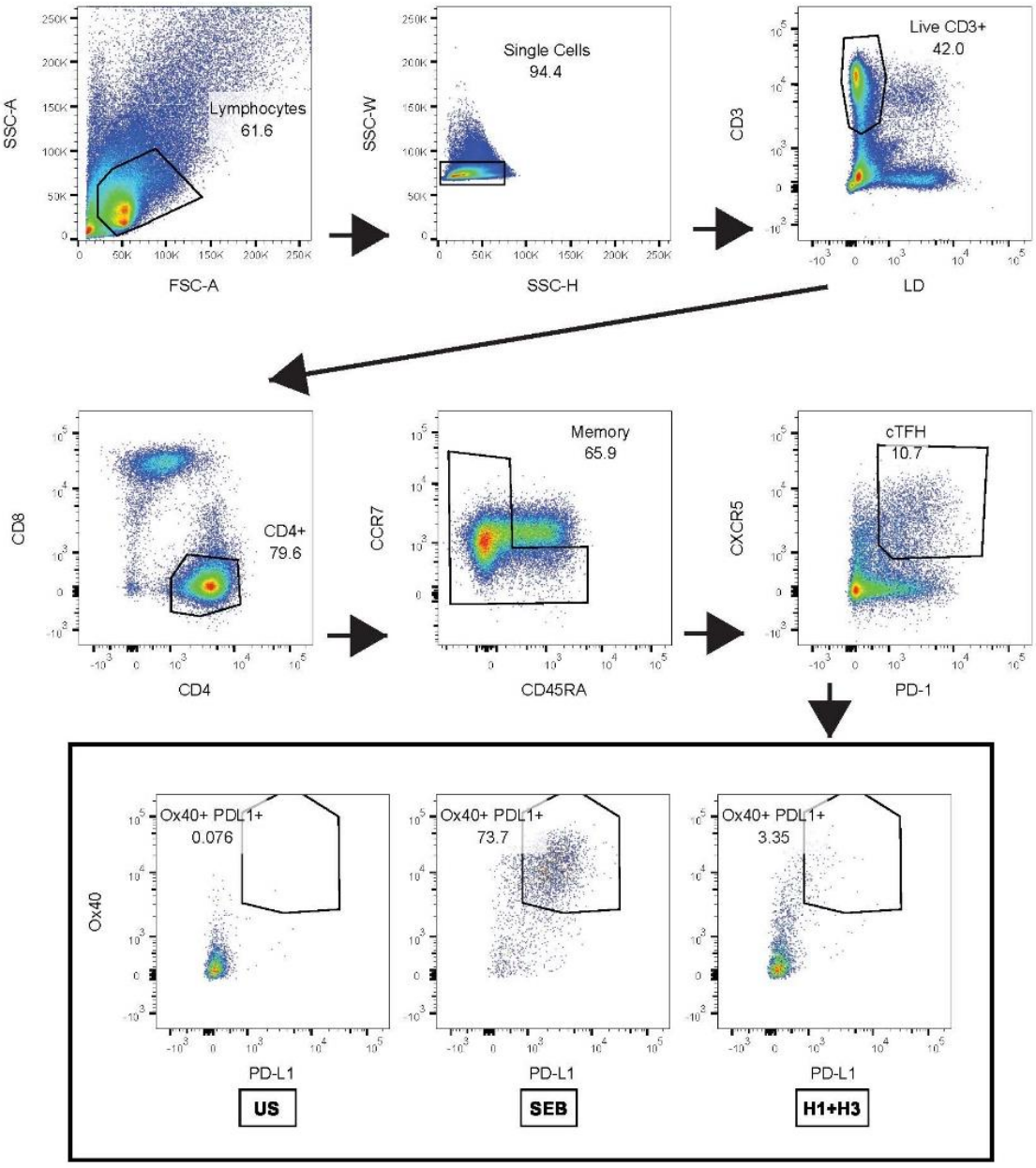
Supplemental Figure 4.4. CD8+ T cell response assessed by flow cytometry. Flow cytometry was performed on PBMC stimulated overnight with pooled A/H1N1 and A/H3N2 antigen at baseline (BL, n=81), D7 (n=75), and D180 (n=50) after vaccination. Cells were analyzed at each timepoint for expression of A) IL-2, B) IFN-γ, C) TNF-α, and D) CD107a, normalized to effector molecule expression in unstimulated cells from that timepoint. Mixed effect modeling was used to detect vaccine-specific differences between groups and differences between responses at each timepoint (α=0.05). Model results are reported below the graph.



Supplemental Figure 4.5. SPICE at baseline, D7, and D180. Polyfunctionality was examined between timepoints within a vaccine arm for either A) standard dose (TIV) or B) adjuvanted influenza vaccine (aTIV). Timepoints compared were baseline (BL), D7 post vaccination, and D180 post vaccination. Polyfunctionality pies show co-expression of effector molecules after BL subtraction when cells were stimulated by pooled influenza A antigens. P values shown were generated by Monte Carlo simulation using SPICE (α=0.05).



Supplemental Figure 4.6. Gating strategy for circulating TFH (cT_{FH}). Cells were gated on Live CD3+ lymphocytes before being gated for CD4+ T cells. Naive (CCR7+ CD45RA+) cells were then gated out. Memory cells were gated on PD-1+ CXCR5+ cells (cT_{FH}). Cells were then gated on Ox40+PDL1+ cells to detect antigen-specific cT_{FH}. Representative gating strategy shown below is from D7 T cells in unstimulated (US), SEB-stimulated (SEB), and virus-stimulated (H1+H3) conditions.



Chapter 5: Discussion and Future Directions

5.1. Discussion

While age-related immune decline is demonstrable and results in increased clinical mortality, growing evidence suggests that aged immune systems can still respond to stimuli under the correct circumstances. This thesis explores mechanisms and vaccine outcomes of two vaccines that contain adjuvants and were developed for use in older adults. While taking place in separate vaccine systems, this research draws on three major themes: the need for mechanistic studies on adjuvants in human models, lipid-based delivery mechanisms, and TLR-based adjuvants.

5.1.1. The need for mechanistic studies in primary human cells

While useful, mouse models for human immunology are limited due to key differences in murine and human immune cells. Mouse models are also merely that– models. While transcriptional regulation of inflammatory events in mice and humans are similar [304], findings in mice need to be confirmed in humans due to known differences between murine and human immune systems. One major weakness in using mouse models for vaccine studies in older adults is that the aged mouse T cell repertoire is maintained in different ways from the human. Mice do not experience thymic involution until much later in their life cycle than humans, which is reflected in the fact that peripheral maintenance is much less critical in mice than humans and is regulated differently [8]. Murine models are therefore of questionable use in vaccine studies that examine aged T cell responses, as age may not affect these compartments similarly. Another key difference between mice and humans is the expression of costimulatory

receptors on T cells. Mice express CD28 on their T cells much more extensively than humans, suggesting that B7 molecules such as CTLA-4 and CD86 have stronger impacts on murine immune responses than human [305]. This could easily lead to over-interpretation of data, particularly when vaccine candidates target specific myeloid processes like costimulation. The expression of ICOS is much more critical in humans than mice, and a key inflammasome modulator, caspase 10, is absent in mice [305,306]. These two changes in particular are of concern when evaluating potential efficacy of adjuvants like AS01, which we show in Chapter 3 to work by upregulating costimulatory marker expression (**Figure 3.6**) and involve inflammasome activation (**Figure 3.5C**).

Mice also differ from humans in the expression and activation of immune-associated genes. This includes key regulatory genes: one study identified 76 regulatory elements that were activated in mice but not human samples and 9 clear instances of divergent regulation [307]. In humans, IFN-α appears to play a role in T_H1 skewing via the activation of STAT4, while IFN-α does not induce STAT4 or play a role in T_H1 induction in mice [305]. TLR4 signaling requires IRAK1 for TLR signaling and cytokine production in human, but not mouse, macrophages. Mouse macrophages instead utilize adaptors such as IRAK2 and IRAK4 [308]. This may affect signaling induced by AS01, which contains the TLR4 ligand MPL.

Additionally, the surface markers used to define murine DC subsets differ from those used for human DCs. Several mouse DC subsets, including CD8a+ DC, do not have an obvious analogue in humans, though they share functional

similarity with CD141+ human DCs [32,309]. Murine pDCs in particular have been shown to diverge from human pDC, with a major relevant difference being that murine pDC produce IL-12 and human pDC do not [310]. In mice, *Irf8* deletion leads to a lack of pDC and CD8+ DC, but not cDC2 [311]. In contrast, humans with homozygous mutations in *Irf8* lack cDC2 [312]. This raises questions about whether human subsets considered “analogous” to mouse subsets are truly good models, particularly for work that models inflammatory signaling.

However, using human cells has its own drawbacks as a model. Human mDC are rare in blood and typically do not survive cyropreservation. To compensate for this, MDDCs are often used as models of human mDC. There is evidence that monocytes differentiate into MDDC *in vivo*, particularly in inflammatory contexts [225]. However, these cells are not perfectly analogous to primary mDC. Human MDDC have distinct ontological origins compared to mDC, and they regulate key transcription factors such as IRF4 and IRF8 differently [313]. Further, *the in vivo* conditions under which MDDC form are significantly more complex than those used to generate MDDC *in vitro* [313]. The end result is that MDDC share some traits with mDC, particularly their ability to stimulate T cells, but are able to produce pro-inflammatory cytokines more than mDC [225]. This mixed functional profile complicates direct comparisons between MDDC and mDC.

Elucidating differences between models and how cells behave *in vivo* is not just a matter of quibbling over research accuracy and the precise mechanisms of immune protection. Famously, over 80% of therapeutics

previewed in mouse models fail and are not carried forward during clinical translation to humans. These failures represent the loss of an enormous amount of time and resources for researchers seeking to develop treatments [314]. While there have been efforts to improve mouse models, with promising progress in using humanized mouse models for the immune system [315], gaps in our ability to explain the failure of vaccine candidates in human clinical trials remain. Without studies done on human samples, particularly primary cells closer to *in vivo* phenotypes, it is difficult to improve the success rate of clinical trials and quickly advance product development.

5.1.2. Lipid-based delivery mechanisms

Liposomes, such as those used in AS01 and the lipid nanoparticle COVID-19 mRNA vaccines (LNP-mRNA), are at the forefront of new vaccine designs. Liposomes are distinct from other lipid-based adjuvants such as oil-in-water emulsions like MF59 due to their three-dimensional structure. Liposomes are formed when a lipid bilayer forms around a hollow aqueous center. They have distinct particulate shapes and a hidden “payload” area that is typically where a drug or antigen is hidden [316]. In contrast, emulsions are typically solid or lamellar, and the antigen is either associated at the surface or enfolded by hydrophobic residues [317]. While both emulsions and liposomes are able to adjuvant vaccine response in animal models, they are believed to act through different mechanisms to promote antigen uptake and presentation [318–320].

The composition of the liposome is known to influence immune response, though effects vary by the characteristics of each liposome formulation. A major

physical characteristic of liposomes known to influence immune outcomes is size [321]. Smaller liposomes (<200 nm) in mouse models localize in tissues and LNs differently from larger liposomes and appear to be taken up differently by immune cells such as macrophages [321–323]. Interestingly, MF59 microvesicles formed during emulsion average 160 nm in diameter, a similar size as these smaller liposomes [324]. Fluorescently labeled MF59 is taken up by phagocytic cells such as neutrophils and monocytes after vaccination in the LN [204], but whether MF59 acts a soluble antigen that travels to the LN via the lymphatics or as a particulate antigen carried to the LN by phagocytic cells is unknown. It is also unknown if MF59 acts like a liposome in promoting phagocytosis and antigen trafficking to the LN.

Other physical characteristics of liposomes, such as lipid content and electrostatic charge, have also been shown to influence how liposomes are internalized by innate immune cells and influence downstream responses [325]. For example, QS-21, used in AS01, is not taken up by cells when not in liposomal form. The high cholesterol content of the liposomes in AS01 appears to play a role in QS-21 entry and subsequent effects on lysosome stability [220].

Another unique feature of liposomes that makes them useful as adjuvants is their ability to protect their internalized contents from degradation. In the case of lipid nanoparticle COVID-19 mRNA vaccines (LNP-mRNA), liposomal encapsulation of the mRNA is critical to ensure efficacy. The liposome helps shelter the mRNA from degradation and facilitates cellular uptake, without which

the mRNA cannot be transcribed into protein antigens [316]. Liposomes have thus been key to the development of an entire class of new vaccines.

It is currently unknown if liposomes have inherent activating activity in human primary cells, as they are not given clinically without an additional adjuvant component or antigen. Squalene adjuvants like Addavax (a commercially available substitute for MF59) weakly and non-significantly induce costimulatory molecule upregulation in human MDDC after overnight incubation, and cholesterol-based liposomes, at least, do not seem to result in immune activation (**Figure 5.1)** [220].

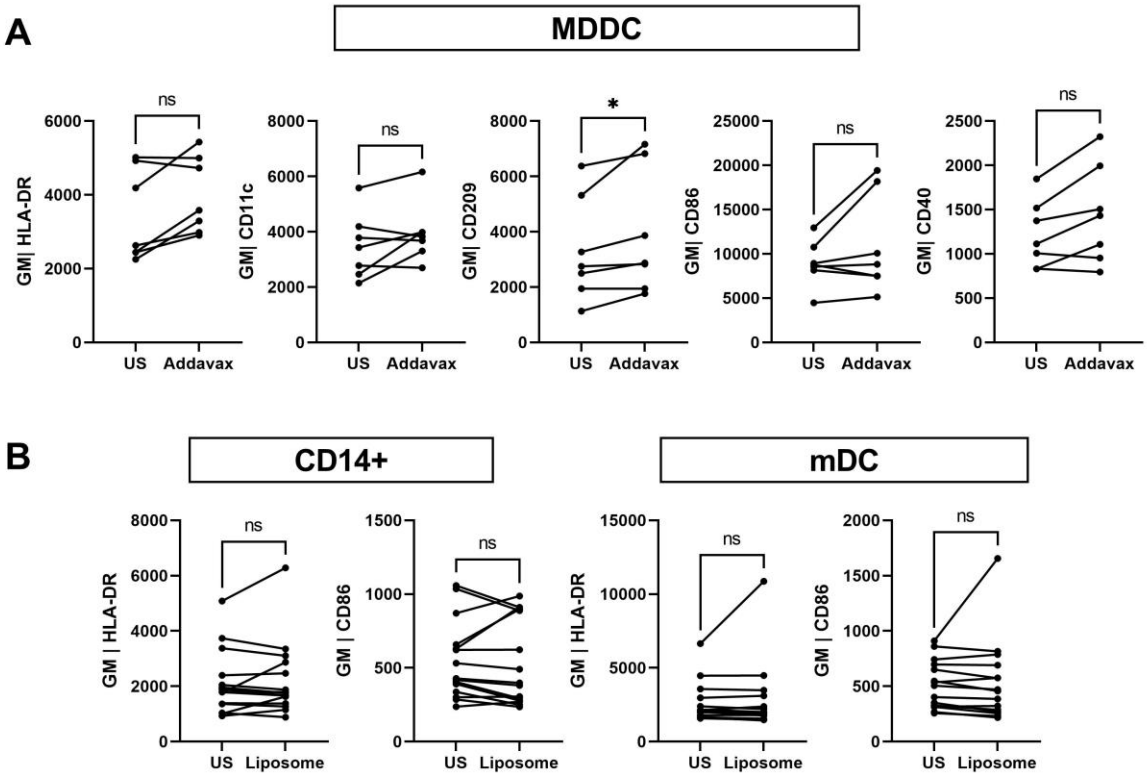


Figure 5.1. Non-significant effects of squalene adjuvant Addavax and cholesterol liposomes on human myeloid cells. A) Human MDDCs differentiated with 10% heat-treated pooled human serum, 50 ng/mL GM-CSF, and 20 ng/mL IL-4 for 6 days. MDDCs were incubated with 1:100 dilution of Addavax for 18 hours before surface marker expression was assessed with flow cytometry. B) Human PBMC were incubated with cholesterol liposomes made as

described in chapter 4, without QS-21. After 18 hours of incubation, surface molecule expression was assessed in CD14+ and mDC. Statistical tests shown are Wilcoxon t tests.

5.1.3. TLR-based adjuvants: the way forward?

Since Janeway proposed a theory for innate immune recognition in 1989, progress in developing new adjuvants has accelerated rapidly [326]. Alum was the sole adjuvant approved for human use from 1932 until 1979, when MF59 was approved. Meanwhile, since 2000, four adjuvants have been approved for use in human vaccines: AS04 (hepatitis B and human papilloma virus, 2004), AS03 (pandemic influenza vaccines, 2009), AS01 (zoster and malaria vaccines, 2017), and cytosine phosphoguanosine (CpG) 1018 (hepatitis B, 2017) [175,180]. Of these adjuvants, three utilize a TLR ligand, while the fourth, AS03, is a squalene emulsion like MF59 [175]. By pairing TLR activation with antigen recognition, TLR-adjuvanted vaccines are able to activate both the innate and adaptive arms for a protective response like a safer version of natural infection. While their role in adjuvanting vaccines in humans is incompletely understood, TLR ligands are already serving an important role in developing the next generation of adjuvants.

Other effective vaccines are likely acting through TLR-mediated activation of innate immune cells, even if they are not specifically earmarked as adjuvanted with TLR ligands. For example, mRNA vaccines such as those recently approved for the COVID-19 pandemic involve injecting relatively high concentrations of liposome-delivered mRNA molecules. These mRNA molecules are known to activate TLR signaling, particularly TLR7/8, endosomal TLRs that detect single-stranded RNA. TLR3, which detects double-stranded RNA, is also occasionally

activated, as double-stranded RNA formed as a byproduct of mRNA production is detected [327–329]. Interestingly, the adjuvanticity of mRNA is high enough that LNP-mRNA vaccines contain modified mRNA to reduce the induction of TLR signaling and lower the risk of severe side effects from the vaccine [316]. In addition to LNP-mRNA vaccines, influenza vaccines made from killed viruses can induce IFN-α production from human pDC, suggesting that they contain TLR7/9 ligands even after the virus is inactivated during production [330].

Understanding the role that TLR ligands play in adjuvanting vaccine response may result in the development of more effective vaccine adjuvants. While research has rightfully focused on TLRs that are heavily associated with bacteria and viruses, such as TLR4 and TLR7/8, TLRs and other PRRs have evolved to help coordinate responses with a vast array of pathogens. Ligands for TLRs that detect fungal (TLR2) or protozoan (TLR5) PAMPs may be useful to adjuvant vaccine responses to these pathogens, or even as an immune complement to drug treatments.

5.2. Future Directions

This thesis only examines completed projects on the intersections of age and adjuvants. Potential future directions for this research are included below. These include exploring additional effects of AS01 on human APC, particularly related to antigen presentation and autophagy; directly connecting AS01-induced innate immune activation with downstream cellular immunity; and looking for common molecular signatures across reactogenic vaccine platforms.

5.2.1. Exploring additional effects of adjuvant AS01 on human APC

In chapter 3 of this thesis, AS01's ability to induce costimulatory molecule expression in monocytes was explored. However, adjuvants can act to enhance APC function through more than upregulation of costimulatory molecules. For example, adjuvants have been reported to enhance APC migration towards the LN or injection site [192,331], increase antigen uptake by APCs [199,204,325], and improve antigen presentation to lymphocytes [182,190]. Results from RNA sequencing of AS01-treated monocytes implicate pathways known to be involved in antigen presentation. For example, autophagy is known to increase MHC Class II access to antigens, and key autophagy-related genes such as *Gsk*, *Atg4*, and *Atg16* are upregulated in AS01-treated monocytes [332]. However, AS01's impacts on antigen presentation were not assessed in that chapter.

AS01's potential effects on autophagy, and thereby antigen presentation, are a particularly promising avenue for future research. Individual genes associated with autophagy are upregulated in RNA sequencing data, but validating if autophagy is truly upregulated remains to be done. Co-localization of

autophagosome protein LC3 and lysosomal protein LAMP-1 can be examined using confocal microscopy in AS01-treated monocytes. Comparing the extent of co-localization compared to untreated controls would demonstrate autophagic flux. Fluorescent latex beads can be used to measure increase in phagocytic activity, where an increase in fluorescence indicates increased bead uptake [128]. Antigen presentation can be examined by treating APCs with AS01 followed by a pulse with a model antigen, such as CMV pp65 or SARS-CoV-2 spike peptides. T cell responses can be evaluated using a mixed lymphocyte reaction, similar to the costimulation assay used in chapter 3.

A significant limitation to the latter experiment is that the stated method does not distinguish between improved antigen processing and improved antigen-presentation/costimulation. That is, there is no way to distinguish between improved antigen uptake, antigen processing and presentation, and increased expression of costimulatory markers. While we cannot distinguish between individual methods of increasing APC stimulation of T cells, it is important to note that APCs work *in vivo* through a combination of these methods. Determining their individual contributions is less important to understanding adjuvant activity than determining if the adjuvant is capable of stimulating APCs to provide better help to T cells.

5.2.2. Directly connecting the impacts of innate immune activation to downstream effects on cellular immunity

We show in chapter 3 that costimulatory molecule upregulation on monocytes is sufficient to amplify T cell production of IFN-γ in response to SEB

(**Figure 3.7**). However, the *in vivo* context of AS01 exposure is vastly more complex. Additionally, by fixing our APCs with formaldehyde, thereby killing them, we artificially removed a key effector of APC help to T cells, cytokine production. An *in vivo* connection between innate immune activation after vaccination and improved adaptive responses has yet to be shown.

One way to demonstrate the influence of AS01 on adaptive responses *in vivo* is through longitudinal study of donors before and after vaccination with RZV. A combination of serum analysis and T cell outcomes could be used to examine this question. Briefly, donors would be recruited before and after RZV vaccination. Blood would be drawn prior to vaccination (D0) and the majority stored as frozen aliquots of PBMC. Using the remaining fresh PBMC, monocyte and mDC surface molecule response would be evaluated by ex vivo treatment with AS01, as in Chapter 3. Serum would also be collected two days after vaccination, when the innate system has had time to fully activate. Alternatively, monocytes circulating 1 day after vaccination could be examined, but that would 1) require donors to come in multiple times after vaccination, limiting enrollment and 2) rely on the assumption that activated monocytes are not egressing towards tissues such as the injected muscle or the LN.

After the full course of RZV vaccination is complete, a second blood draw would be performed. Flow cytometry would then be used to evaluate T cell responses before and after vaccination with RZV to determine high versus low responders. The degree of T cell response would then be correlated with ex vivo myeloid responses to AS01. After donors are known to be high or low

responders, Luminex could also be performed on serum markers to determine what cytokines are likely important in T cell response.

Limitations to this approach include the long time frame between initial sample collection and ability to analyze the samples. Because myeloid cell panels would need to be run on fresh PBMC to ensure mDCs could be detected, surface data would be collected months before T cell read-outs could be obtained. Additionally, this approach is highly susceptible to “batch effect” between flow cytometry runs. The length of time between clinical sample collection means that drift in cytometer laser outputs is highly likely. Addressing this will require tight control and normalization of different flow cytometry runs. There is also no way to tell if a donor will be a high or a low responder until the vaccine series is completed a minimum of 3-6 months after enrollment. This makes it difficult to predict the sample size needed to compare responses between high and low responders. While any outcomes remain mere associations, *in vivo* human data portrays a more complete picture of the mechanisms of AS01-induced vaccine efficacy.

5.2.3. Looking for molecular signatures correlated with efficacy across reactogenic vaccine platforms

While understanding the molecular mechanisms of AS01 is an interesting scientific question, AS01 is already approved for use in humans. If new adjuvants that can be used in older adults are to be identified, we need a broader understanding of which signals are associated with effective vaccine response. Studies on signals correlated with effectiveness in vaccine platforms to individual

pathogens have already been done, with a variety of results that are rarely significant for a single marker [333–335]. These studies have identified key molecules correlated with an effective response such as IL-8, soluble CD25, Type I IFNs, and IP-10 in addition to gene signatures [333,336,337]. However, studies examining common factors between vaccine platforms, especially ones already approved for clinical use, are limited. It is currently unknown if AS01 utilizes the same basic signaling mechanisms as other effective vaccines to promote T cell responses in older adults.

Another vaccine that strongly protects against mortality in older adults is the lipid nanoparticle COVID-19 mRNA vaccine (LNP-mRNA). While LNP-mRNA vaccines are protective against mortality, immunological assays performed post-vaccination show a wide range of responses in older adults (Canaday lab, unpublished data) [100,338]. Some gene and whole-blood molecular signatures associated with humoral immune response have been described [336], but it is unknown if these signatures are also expressed in aged donors. Additionally, comparisons between LNP-mRNA vaccine signatures and other vaccine platforms have not been performed to look for commonalities.

The molecular signatures induced by LNP-mRNA vaccines can be examined by two methods. First, our lab is already collecting paired PBMC samples pre- and post-vaccination. Antibody titer data and ELIspot analysis have already been collected for many of these donors, allowing us to classify them as high or low responders. As part of our lab protocol, multiple aliquots of PBMC are frozen for each donor. Sorting can be done on thawed PBMC from before and 2

weeks after vaccination to isolate monocytes. RNA can then be collected from these ex vivo samples and sent for sequencing. We can then compare gene expression data in donors who have a strong or weak T cell and humoral response. Commonalities between cytokine signatures induced by AS01 in monocytes and signatures in monocytes post LNP-mRNA vaccination can also be examined. Serum samples for these donors have been collected and can be sent for a comprehensive cytokine analysis using the Luminex platform to evaluate systemic response.

A limitation of this approach is that we are focusing on one cell type at the expense of many others involved in promoting immune responses, including cells at the site of injection [206]. While monocytes are hardy and easy to isolate, they do not represent the full breadth of the immune response. It is expected that this analysis will not be comprehensive in identifying inflammatory signals involved in effective vaccine response in older adults. Cellular senescence is also expected to complicate analysis, as older donors will have varying degrees of baseline inflammatory cytokine expression [132,141,142]. RNA sequencing is also a computationally extensive method that, while comprehensive, is not viable for rapid and high-throughput screening. Any common markers or genes that are identified as similar between vaccines will need to be validated and further refined to be truly useful as biomarkers of effective vaccine response.

References

1. Cisneros, B., García-Aguirre, I., Unzueta, J., Arrieta-Cruz, I., González-Morales, O., Domínguez-Larrieta, J. M., Tamez-González, A., Leyva-Gómez, G., & Magaña, J. J. (2022). Immune system modulation in aging: Molecular mechanisms and therapeutic targets. *Frontiers in Immunology*, *13*(December), 1–8. https://doi.org/10.3389/fimmu.2022.1059173
2. Cepeda, S., Cantu, C., Orozco, S., Xiao, Y., Brown, Z., Semwal, M. K., Venables, T., Anderson, M. S., & Griffith, A. V. (2018). Age-Associated Decline in Thymic B Cell Expression of Aire and Aire-Dependent Self-Antigens. *Cell Reports*, *22*(5), 1276–1287. https://doi.org/10.1016/j.celrep.2018.01.015
3. Turner, J. S., Benet, Z. L., & Grigorova, I. L. (2020). Signals 1, 2 and B cell fate or: Where, when and for how long? *Immunological Reviews*, *296*(1), 9–23. https://doi.org/10.1111/imr.12865
4. Michallet, M.-C., Rota, G., Maslowski, K., & Guarda, G. (2013). Innate receptors for adaptive immunity. *Current Opinion in Microbiology*, *16*(3), 296–302. https://doi.org/10.1016/j.mib.2013.04.003
5. Janeway, C. A., & Medzhitov, R. (2002). Innate immune recognition. *Annual Review of Immunology*, *20*(2), 197–216. https://doi.org/10.1146/annurev.immunol.20.083001.084359
6. Brown, B., Ojha, V., Fricke, I., Al-Sheboul, S. A., Imarogbe, C., Gravier, T., Green, M., Peterson, L., Koutsaroff, I. P., Demir, A., Andrieu, J., Leow, C. Y., & Leow, C. H. (2023). Innate and Adaptive Immunity during SARS-CoV-2

Infection: Biomolecular Cellular Markers and Mechanisms. *Vaccines*, *11*(2), 408. https://doi.org/10.3390/vaccines11020408

7. Inoue, T., Shinnakasu, R., & Kurosaki, T. (2022). Generation of High Quality Memory B Cells. *Frontiers in Immunology*, *12*(January), 1–10. https://doi.org/10.3389/fimmu.2021.825813

8. Nikolich-Žugich, J., Slifka, M. K., & Messaoudi, I. (2004). The many important facets of T-cell repertoire diversity. *Nature Reviews Immunology*, *4*(2), 123–132. https://doi.org/10.1038/nri1292

9. Olatunde, A. C., Hale, J. S., & Lamb, T. J. (2021). Cytokine-skewed Tfh cells: functional consequences for B cell help. *Trends in Immunology*, *42*(6), 536–550. https://doi.org/10.1016/j.it.2021.04.006

10. Massa, C., Wang, Y., Marr, N., & Seliger, B. (2023). Interferons and Resistance Mechanisms in Tumors and Pathogen-Driven Diseases-Focus on the Major Histocompatibility Complex (MHC) Antigen Processing Pathway. *International Journal of Molecular Sciences*, *24*(7). https://doi.org/10.3390/ijms24076736

11. Kulski, J. K., Suzuki, S., & Shiina, T. (2022). Human leukocyte antigen super-locus: nexus of genomic supergenes, SNPs, indels, transcripts, and haplotypes. *Human Genome Variation*, *9*(1). https://doi.org/10.1038/s41439-022-00226-5

12. König, R., Huang, L. Y., & Germain, R. N. (1992). MHC class II interaction with CD4 mediated by a region analogous to the MHC class I binding site for CD8. *Nature*, *356*(6372), 796–798. https://doi.org/10.1038/356796a0

13. Keeler, S. P., & Fox, J. M. (2021). Requirement of fc-fc gamma receptor interaction for antibody-based protection against emerging virus infections. *Viruses*, *13*(6), 1–23. https://doi.org/10.3390/v13061037

14. Germain, R. N., Bajénoff, M., Castellino, F., Chieppa, M., Egen, J. G., Huang, A. Y. C., Ishii, M., Koo, L. Y., & Qi, H. (2008). Making friends in out-of-the-way places: How cells of the immune system get together and how they conduct their business as revealed by intravital imaging. *Immunological Reviews*, *221*(1), 163–181. https://doi.org/10.1111/j.1600-065X.2008.00591.x

15. Dhatchinamoorthy, K., Colbert, J. D., & Rock, K. L. (2021). Cancer Immune Evasion Through Loss of MHC Class I Antigen Presentation. *Frontiers in Immunology*, *12*(March). https://doi.org/10.3389/fimmu.2021.636568

16. Hua, Z., & Hou, B. (2020). The role of B cell antigen presentation in the initiation of CD4+ T cell response. *Immunological Reviews*, *296*(1), 24–35. https://doi.org/10.1111/imr.12859

17. Tamassia, N., Bianchetto-Aguilera, F., Arruda-Silva, F., Gardiman, E., Gasperini, S., Calzetti, F., & Cassatella, M. A. (2018). Cytokine production by human neutrophils: Revisiting the “dark side of the moon.” *European Journal of Clinical Investigation*, *48*(March), 1–10. https://doi.org/10.1111/eci.12952

18. Germano, D. B., Oliveira, S. B., Bachi, A. L. L., Juliano, Y., Novo, N. F., Bussador do Amaral, J., & França, C. N. (2023). Monocyte chemokine receptors as therapeutic targets in cardiovascular diseases. *Immunology*

Letters. https://doi.org/10.1016/j.imlet.2023.03.002

19. Liu, J., Zhang, X., Cheng, Y., & Cao, X. (2021). Dendritic cell migration in inflammation and immunity. *Cellular and Molecular Immunology*, *18*(11), 2461–2471. https://doi.org/10.1038/s41423-021-00726-4
20. Betzler, A. C., Ushmorov, A., & Brunner, C. (2023). The transcriptional program during germinal center reaction - a close view at GC B cells, Tfh cells and Tfr cells. *Frontiers in Immunology*, *14*(February), 1–13. https://doi.org/10.3389/fimmu.2023.1125503
21. Batista, F. D., & Harwood, N. E. (2009). The who, how and where of antigen presentation to B cells. *Nature Reviews Immunology*, *9*(1), 15–27. https://doi.org/10.1038/nri2454
22. Victora, G. D., & Nussenzweig, M. C. (2022). Germinal Centers. *Annual Review of Immunology*, *40*, 413–442. https://doi.org/10.1146/annurev-immunol-120419-022408
23. Celli, S., Garcia, Z., Beuneu, H., & Bousso, P. (2008). Decoding the dynamics of T cell-dendritic cell interactions in vivo. *Immunological Reviews*, *221*(1), 182–187. https://doi.org/10.1111/j.1600-065X.2008.00588.x
24. Hilligan, K. L., & Ronchese, F. (2020). Antigen presentation by dendritic cells and their instruction of CD4+ T helper cell responses. *Cellular and Molecular Immunology*, *17*(6), 587–599. https://doi.org/10.1038/s41423-020-0465-0
25. Wang, X., Zhang, Y., Wang, S., Ni, H., Zhao, P., Chen, G., Xu, B., & Yuan, L. (2022). The role of CXCR3 and its ligands in cancer. *Frontiers in Oncology*, *12*(November), 1–17. https://doi.org/10.3389/fonc.2022.1022688

26. Wei, X., Narasimhan, H., Zhu, B., & Sun, J. (2023). Host Recovery from Respiratory Viral Infection. *Annual Review of Immunology*, *41*(1), 277–300. https://doi.org/10.1146/annurev-immunol-101921-040450

27. Benichou, G., Gonzalez, B., Marino, J., Ayasoufi, K., & Valujskikh, A. (2017). Role of Memory T Cells in Allograft Rejection and Tolerance. *Frontiers in Immunology*, *8*, 170. https://doi.org/10.3389/fimmu.2017.00170

28. Gupta, S. (2014). Role of dendritic cells in innate and adaptive immune response in human aging. *Experimental Gerontology*, *54*, 47–52. https://doi.org/10.1016/j.exger.2013.12.009

29. Worah, K., Mathan, T. S. M., Vu Manh, T. P., Keerthikumar, S., Schreibelt, G., Tel, J., Duiveman-de Boer, T., Sköld, A. E., van Spriel, A. B., de Vries, I. J. M., Huynen, M. A., Wessels, H. J., Gloerich, J., Dalod, M., Lasonder, E., Figdor, C. G., & Buschow, S. I. (2016). Proteomics of Human Dendritic Cell Subsets Reveals Subset-Specific Surface Markers and Differential Inflammasome Function. *Cell Reports*, *16*(11), 2953–2966. https://doi.org/10.1016/j.celrep.2016.08.023

30. Chiang, M.-C., Tullett, K. M., Lee, Y. S., Idris, A., Ding, Y., McDonald, K. J., Kassianos, A., Leal Rojas, I. M., Jeet, V., Lahoud, M. H., & Radford, K. J. (2016). Differential uptake and cross-presentation of soluble and necrotic cell antigen by human DC subsets. *European Journal of Immunology*, *46*(2), 329–339. https://doi.org/https://doi.org/10.1002/eji.201546023

31. Collin, M., Mcgovern, N., & Haniffa, M. (2013). Human dendritic cell subsets. *Immunology*, *140*(1), 22–30. https://doi.org/10.1111/imm.12117

32. Schlitzer, A., McGovern, N., Teo, P., Zelante, T., Atarashi, K., Low, D., Ho, A. W. S., See, P., Shin, A., Wasan, P. S., Hoeffel, G., Malleret, B., Heiseke, A., Chew, S., Jardine, L., Purvis, H. A., Hilkens, C. M. U., Tam, J., Poidinger, M., … Ginhoux, F. (2013). IRF4 Transcription Factor-Dependent CD11b+ Dendritic Cells in Human and Mouse Control Mucosal IL-17 Cytokine Responses. *Immunity*, *38*(5), 970–983. https://doi.org/10.1016/j.immuni.2013.04.011

33. Westman, J., & Grinstein, S. (2021). Determinants of Phagosomal pH During Host-Pathogen Interactions. *Frontiers in Cell and Developmental Biology*, *8*(January), 1–9. https://doi.org/10.3389/fcell.2020.624958

34. Hume, D. A., Irvine, K. M., & Pridans, C. (2019). The Mononuclear Phagocyte System: The Relationship between Monocytes and Macrophages. *Trends in Immunology*, *40*(2), 98–112. https://doi.org/10.1016/j.it.2018.11.007

35. Baranska, A., Shawket, A., Jouve, M., Baratin, M., Malosse, C., Voluzan, O., Vu Manh, T.-P., Fiore, F., Bajénoff, M., Benaroch, P., Dalod, M., Malissen, M., Henri, S., & Malissen, B. (2018). Unveiling skin macrophage dynamics explains both tattoo persistence and strenuous removal. *Journal of Experimental Medicine*, *215*(4), 1115–1133. https://doi.org/10.1084/jem.20171608

36. Scott, C. L., Zheng, F., De Baetselier, P., Martens, L., Saeys, Y., De Prijck, S., Lippens, S., Abels, C., Schoonooghe, S., Raes, G., Devoogdt, N., Lambrecht, B. N., Beschin, A., & Guilliams, M. (2016). Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells.

Nature Communications, *7*, 10321. https://doi.org/10.1038/ncomms10321

37. Baratin, M., Simon, L., Jorquera, A., Ghigo, C., Dembele, D., Nowak, J., Gentek, R., Wienert, S., Klauschen, F., Malissen, B., Dalod, M., & Bajénoff, M. (2017). T Cell Zone Resident Macrophages Silently Dispose of Apoptotic Cells in the Lymph Node. *Immunity*, *47*(2), 349-362.e5. https://doi.org/https://doi.org/10.1016/j.immuni.2017.07.019
38. Wolf, A. A., Yáñez, A., Barman, P. K., & Goodridge, H. S. (2019). The ontogeny of monocyte subsets. *Frontiers in Immunology*, *10*(JULY). https://doi.org/10.3389/fimmu.2019.01642
39. Verschoor, C. P., & Kohli, V. (2018). Cryopreserved whole blood for the quantification of monocyte, T-cell and NK-cell subsets, and monocyte receptor expression by multi-color flow cytometry: A methodological study based on participants from the canadian longitudinal study on aging. *Cytometry Part A*, *93*(5), 548–555. https://doi.org/10.1002/cyto.a.23372
40. Narasimhan, P. B., Marcovecchio, P., Hamers, A. A. J., & Hedrick, C. C. (2019). Nonclassical Monocytes in Health and Disease. *Annual Review of Immunology*, *37*, 439–456. https://doi.org/10.1146/annurev-immunol-042617-053119
41. Cros, J., Cagnard, N., Woollard, K., Patey, N., Zhang, S. Y., Senechal, B., Puel, A., Biswas, S. K., Moshous, D., Picard, C., Jais, J. P., D'Cruz, D., Casanova, J. L., Trouillet, C., & Geissmann, F. (2010). Human CD14dim Monocytes Patrol and Sense Nucleic Acids and Viruses via TLR7 and TLR8 Receptors. *Immunity*, *33*(3), 375–386.

https://doi.org/10.1016/j.immuni.2010.08.012

42. Chambers, E. S., & Akbar, A. N. (2020). Can blocking inflammation enhance immunity during aging? *The Journal of Allergy and Clinical Immunology*, *145*(5), 1323–1331. https://doi.org/10.1016/j.jaci.2020.03.016

43. Jakubzick, C. V., Randolph, G. J., & Henson, P. M. (2017). Monocyte differentiation and antigen-presenting functions. *Nature Reviews Immunology*, *17*(6), 349–362. https://doi.org/10.1038/nri.2017.28

44. Olingy, C. E., San Emeterio, C. L., Ogle, M. E., Krieger, J. R., Bruce, A. C., Pfau, D. D., Jordan, B. T., Peirce, S. M., & Botchwey, E. A. (2017). Non-classical monocytes are biased progenitors of wound healing macrophages during soft tissue injury. *Scientific Reports*, *7*(1), 447. https://doi.org/10.1038/s41598-017-00477-1

45. Guilliams, M., Mildner, A., & Yona, S. (2018). Developmental and Functional Heterogeneity of Monocytes. *Immunity*, *49*(4), 595–613. https://doi.org/10.1016/j.immuni.2018.10.005

46. Veninga, H., Borg, E. G. F., Vreeman, K., Taylor, P. R., Kalay, H., van Kooyk, Y., Kraal, G., Martinez-Pomares, L., & den Haan, J. M. M. (2015). Antigen targeting reveals splenic CD169 + macrophages as promoters of germinal center B-cell responses. In *European Journal of Immunology* (Vol. 45, Issue 3, pp. 747–757). https://doi.org/10.1002/eji.201444983

47. Bernhard, C. A., Ried, C., Kochanek, S., & Brocker, T. (2015). CD169+ macrophages are sufficient for priming of CTLs with specificities left out by cross-priming dendritic cells. *Proceedings of the National Academy of*

Sciences of the United States of America, *112*(17), 5461–5466. https://doi.org/10.1073/pnas.1423356112

48. Kim, C., Jadhav, R. R., Gustafson, C. E., Smithey, M. J., Alec, J., Uhrlaub, J. L., Hildebrand, W. H., Nikolich-žugich, J., Weyand, M., & Goronzy, J. J. (2020). Defects in Antiviral T Cell Responses Inflicted by Aging-Associated miR-181a Deficiency. *Cell Rep*, *29*(8), 2202–2216. https://doi.org/10.1016/j.celrep.2019.10.044.Defects
49. Detienne, S., Welsby, I., Collignon, C., Wouters, S., Coccia, M., Delhaye, S., Van Maele, L., Thomas, S., Swertvaegher, M., Detavernier, A., Elouahabi, A., Goriely, S., & Didierlaurent, A. M. (2016). Central role of CD169+ lymph node resident macrophages in the adjuvanticity of the QS-21 component of AS01. *Scientific Reports*, *6*(December), 1–14. https://doi.org/10.1038/srep39475
50. Sagoo, P., Garcia, Z., Breart, B., Lemaître, F., Michonneau, D., Albert, M. L., Levy, Y., & Bousso, P. (2016). In vivo imaging of inflammasome activation reveals a subcapsular macrophage burst response that mobilizes innate and adaptive immunity. *Nature Medicine*, *22*(1), 64–71. https://doi.org/10.1038/nm.4016
51. Amanna, I. J., Carlson, N. E., & Slifka, M. K. (2007). Duration of humoral immunity to common viral and vaccine antigens. *The New England Journal of Medicine*, *357*(19), 1903–1915. https://doi.org/10.1056/NEJMoa066092
52. Allen, H. C., & Sharma, P. (2020). Histology, Plasma Cells. In *StatPearls*. http://www.ncbi.nlm.nih.gov/pubmed/32310542

53. De Silva, N. S., & Klein, U. (2015). Dynamics of B cells in germinal centres. *Nature Reviews Immunology*, *15*(3), 137–148. https://doi.org/10.1038/nri3804

54. Herati, R. S., Reuter, M. A., Dolfi, D. V., Mansfield, K. D., Aung, H., Badwan, O. Z., Kurupati, R. K., Kannan, S., Ertl, H., Schmader, K. E., Betts, M. R., Canaday, D. H., & Wherry, E. J. (2014). Circulating CXCR5 + PD-1 + Response Predicts Influenza Vaccine Antibody Responses in Young Adults but not Elderly Adults . *The Journal of Immunology*, *193*(7), 3528–3537. https://doi.org/10.4049/jimmunol.1302503

55. Morita, R., Schmitt, N., Bentebibel, S. E., Ranganathan, R., Bourdery, L., Zurawski, G., Foucat, E., Dullaers, M., Oh, S. K., Sabzghabaei, N., Lavecchio, E. M., Punaro, M., Pascual, V., Banchereau, J., & Ueno, H. (2011). Human Blood CXCR5+CD4+ T Cells Are Counterparts of T Follicular Cells and Contain Specific Subsets that Differentially Support Antibody Secretion. *Immunity*, *34*(1), 108–121. https://doi.org/10.1016/j.immuni.2010.12.012

56. Herati, R. S., Muselman, A., Vella, L., Bengsch, B., Parkhouse, K., Alcazar, D. Del, Kotzin, J., Doyle, S. A., Tebas, P., Hensley, S. E., Su, L. F., Schmader, K. E., & Wherry, J. E. (2017). Successive annual influenza vaccination induces a recurrent oligoclonotypic memory response in circulating T follicular helper cells. *Science Immunology*, *2*(8). https://doi.org/10.1126/sciimmunol.aag2152.Successive

57. Reiss, S., Baxter, A. E., Cirelli, K. M., Dan, J. M., Morou, A., Daigneault, A.,

Brassard, N., Silvestri, G., Routy, J. P., Havenar-Daughton, C., Crotty, S., & Kaufmann, D. E. (2017). Comparative analysis of activation induced marker (AIM) assays for sensitive identification of antigen-specific CD4 T cells. *PLoS ONE*, *12*(10), 1–22. https://doi.org/10.1371/journal.pone.0186998

58. Khan, I. A., Hwang, S., & Moretto, M. (2019). Toxoplasma gondii: CD8 T cells cry for CD4 help. *Frontiers in Cellular and Infection Microbiology*, *9*(MAY), 1–8. https://doi.org/10.3389/fcimb.2019.00136
59. Zhu, J., & Paul, W. E. (2009). CD4 T cells : fates , functions , and faults ASH 50th anniversary review CD4 T cells : fates , functions , and faults. *Immunobiology*, *112*(5), 1557–1569. https://doi.org/10.1182/blood-2008-05-078154
60. Cruz, F. M., Colbert, J. D., Merino, E., Kriegsman, B. A., & Rock, K. L. (2017). The biology and underlying mechanisms of cross-presentation of exogenous antigens on MHC-I molecules. *Annual Review of Immunology*, *35*, 149–176. https://doi.org/10.1146/annurev-immunol-041015-055254
61. Lisci, M., & Griffiths, G. M. (2023). Arming a killer: mitochondrial regulation of CD8+ T cell cytotoxicity. *Trends in Cell Biology*, *33*(2), 138–147. https://doi.org/10.1016/j.tcb.2022.05.007
62. Voskoboinik, I., Smyth, M. J., & Trapani, J. A. (2006). Perforin-mediated target-cell death and immune homeostasis. *Nature Reviews Immunology*, *6*(12), 940–952. https://doi.org/10.1038/nri1983
63. Golstein, P., & Griffiths, G. M. (2018). An early history of T cell-mediated cytotoxicity. *Nature Reviews Immunology*, *18*(8), 527–535.

https://doi.org/10.1038/s41577-018-0009-3

64. Walsh, M. J., Stump, C. T., Kureshi, R., Lenehan, P., Ali, L. R., Dougan, M., Knipe, D. M., & Dougan, S. K. (2023). IFNγ is a central node of cancer immune equilibrium. In *Cell reports* (Vol. 42, Issue 3, p. 112219). https://doi.org/10.1016/j.celrep.2023.112219

65. Pizzolla, A., Nguyen, T. H. O., Sant, S., Jaffar, J., Loudovaris, T., Mannering, S. I., Thomas, P. G., Westall, G. P., Kedzierska, K., & Wakim, L. M. (2018). Influenza-specific lung-resident memory t cells are proliferative and polyfunctional and maintain diverse TCR profiles. *Journal of Clinical Investigation*, *128*(2), 721–733. https://doi.org/10.1172/JCI96957

66. Wang, Z., Wan, Y., Qiu, C., Quiñones-Parra, S., Zhu, Z., Loh, L., Tian, D., Ren, Y., Hu, Y., Zhang, X., Thomas, P. G., Inouye, M., Doherty, P. C., Kedzierska, K., & Xu, J. (2015). Recovery from severe H7N9 disease is associated with diverse response mechanisms dominated by CD8+ T cells. In *Nature Communications* (Vol. 6). https://doi.org/10.1038/ncomms7833

67. Jansen, J. M., Gerlach, T., Elbahesh, H., Rimmelzwaan, G. F., & Saletti, G. (2019). Influenza virus-specific CD4+ and CD8+ T cell-mediated immunity induced by infection and vaccination. *Journal of Clinical Virology*, *119*(June), 44–52. https://doi.org/10.1016/j.jcv.2019.08.009

68. Lefebvre, J. S., Masters, A. R., Hopkins, J. W., & Haynes, L. (2016). Age-related impairment of humoral response to influenza is associated with changes in antigen specific T follicular helper cell responses. *Scientific Reports*, *6*(December 2015), 1–14. https://doi.org/10.1038/srep25051

69. Spensieri, F., Siena, E., Borgogni, E., Zedda, L., Cantisani, R., Chiappini, N., Schiavetti, F., Rosa, D., Castellino, F., Montomoli, E., Bodinham, C. L., Lewis, D. J., Medini, D., Bertholet, S., & Del Giudice, G. (2016). Early rise of blood T follicular helper cell subsets and baseline immunity as predictors of persisting late functional antibody responses to vaccination in humans. In *PLoS ONE* (Vol. 11, Issue 6). https://doi.org/10.1371/journal.pone.0157066

70. Chen, S., Guan, F., Candotti, F., Benlagha, K., Camara, N. O. S., Herrada, A. A., James, L. K., Lei, J., Miller, H., Kubo, M., Ning, Q., & Liu, C. (2022). The role of B cells in COVID-19 infection and vaccination. *Frontiers in Immunology*, *13*(August), 1–13. https://doi.org/10.3389/fimmu.2022.988536

71. Auladell, M., Jia, X., Hensen, L., Chua, B., Fox, A., Nguyen, T. H. O., Doherty, P. C., & Kedzierska, K. (2019). Recalling the future: Immunological memory toward unpredictable influenza viruses. *Frontiers in Immunology*, *10*(JUL), 1–18. https://doi.org/10.3389/fimmu.2019.01400

72. Crooke, S. N., Ovsyannikova, I. G., Poland, G. A., & Kennedy, R. B. (2019). Immunosenescence and human vaccine immune responses. *Immunity & Ageing*, *16*(1), 1–16. https://doi.org/10.1186/s12979-019-0164-9

73. Kennedy, R. B., Ovsyannikova, I. G., Thomas, A., Larrabee, B. R., Rubin, S., & Poland, G. A. (2019). Differential durability of immune responses to measles and mumps following MMR vaccination. *Vaccine*, *37*(13), 1775–1784. https://doi.org/10.1016/j.vaccine.2019.02.030

74. Ward, B. J., Pillet, S., Charland, N., Trepanier, S., Couillard, J., & Landry, N. (2018). The establishment of surrogates and correlates of protection: Useful

tools for the licensure of effective influenza vaccines? *Human Vaccines and Immunotherapeutics*, *14*(3), 647–656. https://doi.org/10.1080/21645515.2017.1413518

75. Schwarz, T. F., & Leo, O. (2008). Immune response to human papillomavirus after prophylactic vaccination with AS04-adjuvanted HPV-16/18 vaccine: Improving upon nature. *Gynecologic Oncology*, *110*(3 SUPPL.1), S1. https://doi.org/10.1016/j.ygyno.2008.05.036
76. Pan, C., Yue, H., Zhu, L., Ma, G. hui, & Wang, H. liang. (2021). Prophylactic vaccine delivery systems against epidemic infectious diseases. *Advanced Drug Delivery Reviews*, *176*, 113867. https://doi.org/10.1016/j.addr.2021.113867
77. Ali, A., Waris, A., Khan, M. A., Asim, M., Khan, A. U., Khan, S., & Zeb, J. (2023). Recent advancement, immune responses, and mechanism of action of various vaccines against intracellular bacterial infections. *Life Sciences*, *314*(December 2022), 121332. https://doi.org/10.1016/j.lfs.2022.121332
78. Grant, E. J., Quinõnes-Parra, S. M., Clemens, E. B., & Kedzierska, K. (2016). Human influenza viruses and CD8+ T cell responses. *Current Opinion in Virology*, *16*(Icv), 132–142. https://doi.org/10.1016/j.coviro.2016.01.016
79. Zens, K. D., Chen, J. K., & Farber, D. L. (2019). Vaccine-generated lung tissue–resident memory T cells provide heterosubtypic protection to influenza infection. *JCI Insight*, *1*(10), 1–12. https://doi.org/10.1172/jci.insight.85832
80. Phan, J. M., Layton, E. D., Yu, K. K. Q., Aguilar, M. S., Golez, I., Franko, N.

M., Logue, J. K., Rodda, L. B., Howard, C. A., Pepper, M., Gale, M., Chu, H. Y., & Seshadri, C. (2023). Cytotoxic T Cells Targeting Spike Glycoprotein Are Associated with Hybrid Immunity to SARS-CoV-2. *Journal of Immunology (Baltimore, Md. : 1950)*, *210*(9), 1236–1246. https://doi.org/10.4049/jimmunol.2200815

81. Levin, M. J., Kroehl, M. E., Johnson, M. J., Hammes, A., Reinhold, D., Lang, N., & Weinberg, A. (2018). Th1 memory differentiates recombinant from live herpes zoster vaccines. *The Journal of Clinical Investigation*, *128*(10), 4429–4440. https://doi.org/10.1172/JCI121484
82. Harbour, J. C., Abdelbary, M., Schell, J. B., Fancher, S. P., McLean, J. J., Nappi, T. J., Liu, S., Nice, T. J., Xia, Z., Früh, K., & Nolz, J. C. (2023). T helper 1 effector memory CD4+ T cells protect the skin from poxvirus infection. *Cell Reports*, *42*(5), 112407. https://doi.org/https://doi.org/10.1016/j.celrep.2023.112407
83. Mcelhaney, J. E., Ewen, C., Zhou, X., Kane, K. P., Xie, D., David, W., Beth, M., Kleppinger, A., Wang, Y., & Bleackley, R. C. (2010). Response To Influenza Vaccination in Older Adults. *Vaccine*, *27*(18), 2418–2425. https://doi.org/10.1016/j.vaccine.2009.01.136.Granzyme
84. McElhaney, J. E., Xie, D., Hager, W. D., Barry, M. B., Wang, Y., Kleppinger, A., Ewen, C., Kane, K. P., & Bleackley, R. C. (2006). T Cell Responses Are Better Correlates of Vaccine Protection in the Elderly. *The Journal of Immunology*, *176*(10), 6333–6339. https://doi.org/10.4049/jimmunol.176.10.6333

85. Koutsakos, M., Reynaldi, A., Lee, W. S., Nguyen, J., Amarasena, T., Taiaroa, G., Kinsella, P., Liew, K. C., Tran, T., Kent, H. E., Tan, H.-X., Rowntree, L. C., Nguyen, T. H. O., Thomas, P. G., Kedzierska, K., Petersen, J., Rossjohn, J., Williamson, D. A., Khoury, D., … Juno, J. A. (2023). SARS-CoV-2 breakthrough infection induces rapid memory and de novo T cell responses. *Immunity*, *56*(4), 879-892.e4. https://doi.org/10.1016/j.immuni.2023.02.017

86. Alcendor, D. J., Matthews-Juarez, P., Smoot, D., Hildreth, J. E. K., Lamar, K., Tabatabai, M., Wilus, D., & Juarez, P. D. (2022). Breakthrough COVID-19 Infections in the US: Implications for Prolonging the Pandemic. *Vaccines*, *10*(5). https://doi.org/10.3390/vaccines10050755

87. Bi, X., & Tong, S. (2018). Impact of immune escape mutations and N-linked glycosylation on the secretion of hepatitis B virus virions and subviral particles: Role of the small envelope protein. *Virology*, *518*, 358–368. https://doi.org/10.1016/j.virol.2018.03.011

88. Horvath, S., Pirazzini, C., Bacalini, M. G., Gentilini, D., Di Blasio, A. M., Delledonne, M., Mari, D., Arosio, B., Monti, D., Passarino, G., De Rango, F., D'Aquila, P., Giuliani, C., Marasco, E., Collino, S., Descombes, P., Garagnani, P., & Franceschi, C. (2015). Decreased epigenetic age of PBMCs from Italian semi-supercentenarians and their offspring. *Aging*, *7*(12), 1159–1170. https://doi.org/10.18632/aging.100861

89. Fulop, T., Witkowski, J. M., Le Page, A., Fortin, C., Pawelec, G., & Larbi, A. (2017). Intracellular signalling pathways: targets to reverse immunosenescence. *Clinical and Experimental Immunology*, *187*(1), 35–43.

https://doi.org/10.1111/cei.12836

90. Yi, G., de Kraker, M. E. A., Buetti, N., Zhong, X., Li, J., Yuan, Z., Zhu, W., Zhou, J., & Zhou, H. (2023). Risk factors for in-hospital mortality and secondary bacterial pneumonia among hospitalized adult patients with community-acquired influenza: a large retrospective cohort study. *Antimicrobial Resistance and Infection Control*, *12*(1), 25. https://doi.org/10.1186/s13756-023-01234-y

91. Oxman, M. N., Levin, M. J., Johnson, G. R., Schmader, K. E., Straus, S. E., Gelb, L. D., Arbeit, R. D., Simberkoff, M. S., Gershon, A. A., Davis, L. E., Weinberg, A., Boardman, K. D., Williams, H. M., Zhang, J. H., Peduzzi, P. N., Beisel, C. E., Morrison, V. A., Guatelli, J. C., Brooks, P. A., … Silber, J. L. (2005). A Vaccine to Prevent Herpes Zoster and Postherpetic Neuralgia in Older Adults. *New England Journal of Medicine*, *352*(22), 2271–2284. https://doi.org/10.1056/NEJMoa051016

92. Grohskopf, L. A., Blanton, L. H., Ferdinands, J. M., Chung, J. R., Broder, K. R., Talbot, H. K., Morgan, R. L., & Fry, A. M. (2022). Prevention and Control of Seasonal Influenza with Vaccines: Recommendations of the Advisory Committee on Immunization Practices - United States, 2022-23 Influenza Season. *MMWR. Recommendations and Reports : Morbidity and Mortality Weekly Report. Recommendations and Reports*, *71*(1), 1–28. https://doi.org/10.15585/mmwr.rr7101a1

93. Mullooly, J. P., Bridges, C. B., Thompson, W. W., Chen, J., Weintraub, E., Jackson, L. A., Black, S., & Shay, D. K. (2007). Influenza- and RSV-

associated hospitalizations among adults. *Vaccine*, *25*(5), 846–855. https://doi.org/10.1016/j.vaccine.2006.09.041

94. Grasselli, G., Zangrillo, A., Zanella, A., Antonelli, M., Cabrini, L., Castelli, A., Cereda, D., Coluccello, A., Foti, G., Fumagalli, R., Iotti, G., Latronico, N., Lorini, L., Merler, S., Natalini, G., Piatti, A., Ranieri, M. V., Scandroglio, A. M., Storti, E., … Pesenti, A. (2020). Baseline Characteristics and Outcomes of 1591 Patients Infected With SARS-CoV-2 Admitted to ICUs of the Lombardy Region, Italy. *JAMA*, *323*(16), 1574–1581. https://doi.org/10.1001/jama.2020.5394

95. Docherty, A. B., Harrison, E. M., Green, C. A., Hardwick, H. E., Pius, R., Norman, L., Holden, K. A., Read, J. M., Dondelinger, F., Carson, G., Merson, L., Lee, J., Plotkin, D., Sigfrid, L., Halpin, S., Jackson, C., Gamble, C., Horby, P. W., Nguyen-Van-Tam, J. S., … Semple, M. G. (2020). Features of 20 133 UK patients in hospital with covid-19 using the ISARIC WHO Clinical Characterisation Protocol: prospective observational cohort study. *BMJ (Clinical Research Ed.)*, *369*, m1985. https://doi.org/10.1136/bmj.m1985

96. Bureau, U. S. C. (n.d.). *Table 1. Population by Age and Sex: 2020.* U.S. Department of Commerce. Retrieved April 5, 2023, from https://www.census.gov/data/tables/2020/demo/age-and-sex/2020-age-sex-composition.html

97. United Nations, Department of Economic and Social Affairs, P. D. (2022). *Probabilistic Population Projections based on the World Population*

Prospects 2022. World Population Prospects 2022, Online Edition. https://population.un.org/wpp/Download/

98. Stiasny, K., Aberle, J. H., Keller, M., Grubeck-Loebenstein, B., & Heinz, F. X. (2012). Age affects quantity but not quality of antibody responses after vaccination with an inactivated flavivirus vaccine against tick-borne encephalitis. *PLoS ONE*, *7*(3), 1–7. https://doi.org/10.1371/journal.pone.0034145

99. Sasaki, S., Sullivan, M., Narvaez, C. F., Holmes, T. H., Furman, D., Zheng, N. Y., Nishtala, M., Wrammert, J., Smith, K., James, J. A., Dekker, C. L., Davis, M. M., Wilson, P. C., Greenberg, H. B., & He, X. S. (2011). Limited efficacy of inactivated influenza vaccine in elderly individuals is associated with decreased production of vaccine-specific antibodies. *Journal of Clinical Investigation*, *121*(8), 3109–3119. https://doi.org/10.1172/JCI57834

100. Canaday, D. H., Carias, L., Oyebanji, O. A., Keresztesy, D., Wilk, D., Payne, M., Aung, H., St. Denis, K., Lam, E. C., Rowley, C. F., Berry, S. D., Cameron, C. M., Cameron, M. J., Wilson, B., Balazs, A. B., Gravenstein, S., & King, C. L. (2021). Reduced BNT162b2 Messenger RNA Vaccine Response in Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)-Naive Nursing Home Residents. *Clinical Infectious Diseases*, *73*(11), 2112–2115. https://doi.org/10.1093/cid/ciab447

101. Lee, J. Le, & Linterman, M. A. (2022). Mechanisms underpinning poor antibody responses to vaccines in ageing. *Immunology Letters*, *241*(October 2021), 1–14. https://doi.org/10.1016/j.imlet.2021.11.001

102. Zhang, H., Weyand, C. M., & Goronzy, J. J. (2021). Hallmarks of the aging T-cell system. *FEBS Journal*, *288*(24), 7123–7142. https://doi.org/10.1111/febs.15770

103. Cho, H., Kang, H., Lee, H. H., & Kim, C. W. (2017). Programmed cell death 1 (PD-1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) in viral hepatitis. *International Journal of Molecular Sciences*, *18*(7), 1–20. https://doi.org/10.3390/ijms18071517

104. Morou, A., Palmer, B. E., & Kaufmann, D. E. (2014). Distinctive features of CD4+ T cell dysfunction in chronic viral infections. *Current Opinion in HIV and AIDS*, *9*(5), 446–451. https://doi.org/10.1097/COH.0000000000000094

105. Haynes, L., & Eaton, S. M. (2005). The effect of age on the cognate function of CD4+ T cells. *Immunological Reviews*, *205*, 220–228. https://doi.org/10.1111/j.0105-2896.2005.00255.x

106. Lorenzo, E. C., Bartley, J. M., & Haynes, L. (2018). The impact of aging on CD4+ T cell responses to influenza infection. *Biogerontology*, *19*(6), 437–466. https://doi.org/10.1007/s10522-018-9754-8

107. Perkey, E., Miller, R. A., & Garcia, G. G. (2012). Ex Vivo Enzymatic Treatment of Aged CD4 T Cells Restores Cognate T Cell Helper Function and Enhances Antibody Production in Mice. *The Journal of Immunology*, *189*(12), 5582–5589. https://doi.org/10.4049/jimmunol.1200487

108. Tamir, A., Eisenbraun, M. D., Garcia, G. G., & Miller, R. A. (2000). Age-Dependent Alterations in the Assembly of Signal Transduction Complexes at the Site of T Cell/APC Interaction. *The Journal of Immunology*, *165*(3),

1243–1251. https://doi.org/10.4049/jimmunol.165.3.1243

109. Yu, M., Li, G., Lee, W. W., Yuan, M., Cui, D., Weyand, C. M., & Goronzy, J. J. (2012). Signal inhibition by the dual-specific phosphatase 4 impairs T cell-dependent B-cell responses with age. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(15). https://doi.org/10.1073/pnas.1109797109

110. Richner, J. M., Gmyrek, G. B., Govero, J., Tu, Y., van der Windt, G. J. W., Metcalf, T. U., Haddad, E. K., Textor, J., Miller, M. J., & Diamond, M. S. (2015). Age-Dependent Cell Trafficking Defects in Draining Lymph Nodes Impair Adaptive Immunity and Control of West Nile Virus Infection. *PLoS Pathogens*, *11*(7), 1–29. https://doi.org/10.1371/journal.ppat.1005027

111. Hill, D. L., Whyte, C. E., Innocentin, S., Le Lee, J., Dooley, J., Wang, J., James, E. A., Lee, J. C., Kwok, W. W., Zand, M. S., Liston, A., Carr, E. J., & Linterman, M. A. (2021). Impaired HA-specific T follicular helper cell and antibody responses to influenza vaccination are linked to inflammation in humans. *ELife*, *10*, 1–30. https://doi.org/10.7554/eLife.70554

112. Zöphel, D., Kaschek, L., Steiner, R., Janku, S., Chang, H. F., & Lis, A. (2023). Heterozygous OT-I mice reveal that antigen-specific CD8+ T cells shift from apoptotic to necrotic killers in the elderly. *Aging Cell*, *January*, 1–12. https://doi.org/10.1111/acel.13824

113. Jergović, M., Smithey, M. J., & Nikolich-Žugich, J. (2018). Intrinsic and extrinsic contributors to defective CD8+ T cell responses with aging. *Experimental Gerontology*, *105*(January), 140–145.

https://doi.org/10.1016/j.exger.2018.01.011

114. Wang, D., Du, J., Song, Y., Wang, B., Song, R., Hao, Y., Zeng, Y., Xiao, J., Zheng, H., Zeng, H., Zhao, H., & Kong, Y. (2020). CD70 contributes to age-associated T cell defects and overwhelming inflammatory responses. *Aging*, *12*(12), 12032–12050. https://doi.org/10.18632/aging.103368

115. Briceño, O., Lissina, AnBriceño, O., Lissina, A., Wanke, K., Afonso, G., von Braun, A., Ragon, K., Miquel, T., Gostick, E., Papagno, L., Stiasny, K., Price, D. A., Mallone, R., Sauce, D., Karrer, U., & Appay, V. (2016). Reduced naïve CD8+ T-cell priming efficacy in el, pp. 14–21). https://doi. org/10. 1111/acel. 12384n., Wanke, K., Afonso, G., von Braun, A., Ragon, K., Miquel, T., Gostick, E., Papagno, L., Stiasny, K., Price, D. A., Mallone, R., Sauce, D., Karrer, U., & Appay, V. (2016). Reduced naïve CD8+ T-cell priming efficacy in elderly adults. In *Aging Cell* (Vol. 15, Issue 1, pp. 14–21). https://doi.org/10.1111/acel.12384

116. Gallerani, E., Proietto, D., Dallan, B., Campagnaro, M., Pacifico, S., Albanese, V., Marzola, E., Marconi, P., Caputo, A., Appay, V., Gavioli, R., & Nicoli, F. (2021). Impaired Priming of SARS-CoV-2-Specific Naive CD8+ T Cells in Older Subjects. *Frontiers in Immunology*, *12*(July), 1–10. https://doi.org/10.3389/fimmu.2021.693054

117. Quinn, K. M., Fox, A., Harland, K. L., Russ, B. E., Li, J., Nguyen, T. H. O., Loh, L., Olshanksy, M., Naeem, H., Tsyganov, K., Wiede, F., Webster, R., Blyth, C., Sng, X. Y. X., Tiganis, T., Powell, D., Doherty, P. C., Turner, S. J., Kedzierska, K., & La Gruta, N. L. (2018). Age-Related Decline in Primary

CD8+ T Cell Responses Is Associated with the Development of Senescence in Virtual Memory CD8+ T Cells. *Cell Reports*, *23*(12), 3512–3524. https://doi.org/10.1016/j.celrep.2018.05.057

118. Jiang, J., Bennett, A. J., Fisher, E., Williams-Bey, Y., Shen, H., & Murasko, D. M. (2009). Limited expansion of virus-specific CD8 T cells in the aged environment. *Mechanisms of Ageing and Development*, *130*(11), 713–721. https://doi.org/https://doi.org/10.1016/j.mad.2009.08.007
119. Li, G., Smithey, M. J., Rudd, B. D., & Nikolich-Žugich, J. (2012). Age-associated alterations in CD8α+ dendritic cells impair CD8 T-cell expansion in response to an intracellular bacterium. *Aging Cell*, *11*(6), 968–977. https://doi.org/https://doi.org/10.1111/j.1474-9726.2012.00867.x
120. Hester, A. K., Semwal, M. K., Cepeda, S., Xiao, Y., Wimberly, K., Venables, T., Dileepan, T., & Griffith, A. V. (2022). Redox regulation of age-associated defects in generation and maintenance of T cell self-tolerance and immunity to foreign antigens Allison. *Cell Rep*, *38*(7). https://doi.org/10.1016/j.celrep.2022.110363.Redox
121. Agrawal, A., Agrawal, S., Cao, J.-N., Su, H., Osann, K., & Gupta, S. (2007). Altered Innate Immune Functioning of Dendritic Cells in Elderly Humans: A Role of Phosphoinositide 3-Kinase-Signaling Pathway. *The Journal of Immunology*, *178*(11), 6912–6922. https://doi.org/10.4049/jimmunol.178.11.6912
122. Qian, F., Wan, X., Zhang, L., Lin, A., Zhao, H., Fikrig, E., & Montgomery, R. R. (2011). Impaired interferon signaling in dendritic cells from older donors

infected in vitro with west nile virus. *Journal of Infectious Diseases*, *203*(10), 1415–1424. https://doi.org/10.1093/infdis/jir048

123. Oh, S. J., Lee, J. K., & Shin, O. S. (2019). Aging and the immune system: The impact of immunosenescence on viral infection, immunity and vaccine immunogenicity. *Immune Network*, *19*(6), 1–18. https://doi.org/10.4110/in.2019.19.e37

124. Metcalf, T. U., Wilkinson, P. A., Cameron, M. J., Ghneim, K., Chiang, C., Wertheimer, A. M., Hiscott, J. B., Nikolich-Zugich, J., & Haddad, E. K. (2017). Human Monocyte Subsets Are Transcriptionally and Functionally Altered in Aging in Response to Pattern Recognition Receptor Agonists. *Journal of Immunology (Baltimore, Md. : 1950)*, *199*(4), 1405–1417. https://doi.org/10.4049/jimmunol.1700148

125. Nyugen, J., Agrawal, S., Gollapudi, S., & Gupta, S. (2010). Impaired functions of peripheral blood monocyte subpopulations in aged humans. *Journal of Clinical Immunology*, *30*(6), 806–813. https://doi.org/10.1007/s10875-010-9448-8

126. Molony, R. D., Nguyen, J. T., Kong, Y., Montgomery, R. R., Shaw, A. C., & Iwasaki, A. (2017). Aging impairs both primary and secondary RIG-I signaling for interferon induction in human monocytes. *Science Signaling*, *10*(509). https://doi.org/10.1126/scisignal.aan2392

127. Pence, B. D., & Yarbro, J. R. (2019). Classical monocytes maintain ex vivo glycolytic metabolism and early but not later inflammatory responses in older adults. *Immunity and Ageing*, *16*(1), 1–8. https://doi.org/10.1186/s12979-

019-0143-1

128. Yarbro, J. R., & Pence, B. D. (2019). Classical monocytes from older adults maintain capacity for metabolic compensation during glucose deprivation and lipopolysaccharide stimulation. *Mechanisms of Ageing and Development*, *183*(August), 111146. https://doi.org/10.1016/j.mad.2019.111146

129. Costantini, A., Viola, N., Berretta, A., Galeazzi, R., Matacchione, G., Sabbatinelli, J., Storci, G., De Matteis, S., Butini, L., Rippo, M. R., Procopio, A. D., Caraceni, D., Antonicelli, R., Olivieri, F., & Bonafè, M. (2018). Age-related M1/M2 phenotype changes in circulating monocytes from healthy/unhealthy individuals. *Aging*, *10*(6), 1268–1280. https://doi.org/10.18632/aging.101465

130. Vink, P., Delgado Mingorance, I., Maximiano Alonso, C., Rubio-Viqueira, B., Jung, K. H., Rodriguez Moreno, J. F., Grande, E., Marrupe Gonzalez, D., Lowndes, S., Puente, J., Kristeleit, H., Farrugia, D., McNeil, S. A., Campora, L., Di Paolo, E., El Idrissi, M., Godeaux, O., López-Fauqued, M., Salaun, B., … Oostvogels, L. (2019). Immunogenicity and safety of the adjuvanted recombinant zoster vaccine in patients with solid tumors, vaccinated before or during chemotherapy: A randomized trial. *Cancer*, *125*(8), 1301–1312. https://doi.org/10.1002/cncr.31909

131. Schietinger, A., & Greenberg, P. D. (2014). Tolerance and exhaustion: defining mechanisms of T cell dysfunction. *Trends in Immunology*, *35*(2), 51–60. https://doi.org/10.1016/j.it.2013.10.001

132. Coppé, J.-P., Patil, C. K., Rodier, F., Sun, Y., Muñoz, D. P., Goldstein, J., Nelson, P. S., Desprez, P.-Y., & Campisi, J. (2008). Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biology*, *6*(12), 2853–2868. https://doi.org/10.1371/journal.pbio.0060301

133. Akbar, A. N., & Fletcher, J. M. (2005). Memory T cell homeostasis and senescence during aging. *Current Opinion in Immunology*, *17*(5), 480–485. https://doi.org/10.1016/j.coi.2005.07.019

134. Xu, W., & Larbi, A. (2017). Markers of T cell senescence in humans. In *International Journal of Molecular Sciences* (Vol. 18, Issue 8). https://doi.org/10.3390/ijms18081742

135. Haq, K., Fulop, T., Tedder, G., Gentleman, B., Garneau, H., Meneilly, G. S., Kleppinger, A., Pawelec, G., & McElhaney, J. E. (2017). Cytomegalovirus Seropositivity Predicts a Decline in the T Cell But Not the Antibody Response to Influenza in Vaccinated Older Adults Independent of Type 2 Diabetes Status. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, *72*(9), 1163–1170. https://doi.org/10.1093/gerona/glw216

136. Yang, X., Zheng, Y., Han, Z., & Zhang, X. (2021). Functions and clinical significance of KLRG1 in the development of lung adenocarcinoma and immunotherapy. *BMC Cancer*, *21*(1), 752. https://doi.org/10.1186/s12885-021-08510-3

137. Tessmer, M. S., Fugere, C., Stevenaert, F., Naidenko, O. V, Chong, H. J.,

Leclercq, G., & Brossay, L. (2007). KLRG1 binds cadherins and preferentially associates with SHIP-1. *International Immunology*, *19*(4), 391–400. https://doi.org/10.1093/intimm/dxm004

138. Kared, H., Martelli, S., Ng, T. P., Pender, S. L. F., & Larbi, A. (2016). CD57 in human natural killer cells and T-lymphocytes. *Cancer Immunology, Immunotherapy : CII*, *65*(4), 441–452. https://doi.org/10.1007/s00262-016-1803-z

139. Le Priol, Y., Puthier, D., Lécureuil, C., Combadière, C., Debré, P., Nguyen, C., & Combadière, B. (2006). High cytotoxic and specific migratory potencies of senescent CD8+ CD57+ cells in HIV-infected and uninfected individuals. *Journal of Immunology (Baltimore, Md. : 1950)*, *177*(8), 5145–5154. https://doi.org/10.4049/jimmunol.177.8.5145

140. Chattopadhyay, P. K., Betts, M. R., Price, D. A., Gostick, E., Horton, H., Roederer, M., & De Rosa, S. C. (2009). The cytolytic enzymes granyzme A, granzyme B, and perforin: expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression. *Journal of Leukocyte Biology*, *85*(1), 88–97. https://doi.org/10.1189/jlb.0208107

141. Sanada, F., Taniyama, Y., Muratsu, J., Otsu, R., Shimizu, H., Rakugi, H., & Morishita, R. (2018). Source of Chronic Inflammation in Aging. *Frontiers in Cardiovascular Medicine*, *5*, 12. https://doi.org/10.3389/fcvm.2018.00012

142. Shaw, A. C., Goldstein, D. R., & Montgomery, R. R. (2013). Age-dependent dysregulation of innate immunity. *Nature Reviews. Immunology*, *13*(12), 875–887. https://doi.org/10.1038/nri3547

143. Pereira, B., Xu, X.-N., & Akbar, A. N. (2020). Targeting Inflammation and Immunosenescence to Improve Vaccine Responses in the Elderly. *Frontiers in Immunology*, *11*, 583019. https://doi.org/10.3389/fimmu.2020.583019

144. Csiszar, A., Sosnowska, D., Wang, M., Lakatta, E. G., Sonntag, W. E., & Ungvari, Z. (2012). Age-associated proinflammatory secretory phenotype in vascular smooth muscle cells from the non-human primate Macaca mulatta: reversal by resveratrol treatment. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, *67*(8), 811–820. https://doi.org/10.1093/gerona/glr228

145. Franceschi, C., Garagnani, P., Vitale, G., Capri, M., & Salvioli, S. (2017). Inflammaging and 'Garb-aging.' *Trends in Endocrinology & Metabolism*, *28*(3), 199–212. https://doi.org/https://doi.org/10.1016/j.tem.2016.09.005

146. Tang, X., Deng, B., Zang, A., He, X., Zhou, Y., Wang, D., Li, D., Dai, X., Chen, J., Zhang, X., Liu, Y., Xu, Y., Chen, J., Zheng, W., Zhang, L., Gao, C., Yang, H., Li, B., & Wang, X. (2022). Characterization of age-related immune features after autologous NK cell infusion: Protocol for an open-label and randomized controlled trial. *Frontiers in Immunology*, *13*, 940577. https://doi.org/10.3389/fimmu.2022.940577

147. Li, J., Huang, H.-H., Tu, B., Zhou, M.-J., Hu, W., Fu, Y.-L., Li, X.-Y., Yang, T., Song, J.-W., Fan, X., Jiao, Y.-M., Xu, R.-N., Zhang, J.-Y., Zhou, C.-B., Yuan, J.-H., Zhen, C., Shi, M., Wang, F.-S., & Zhang, C. (2021). Reversal of the CD8(+) T-Cell Exhaustion Induced by Chronic HIV-1 Infection Through Combined Blockade of the Adenosine and PD-1 Pathways. *Frontiers in*

Immunology, *12*, 687296. https://doi.org/10.3389/fimmu.2021.687296

148. Vick, L. V, Collins, C. P., Khuat, L. T., Wang, Z., Dunai, C., Aguilar, E. G., Stoffel, K., Yendamuri, S., Smith, R. J., Mukherjee, S., Barbi, J., Canter, R. J., Monjazeb, A. M., & Murphy, W. J. (2022). Aging augments obesity-induced thymic involution and peripheral T cell exhaustion altering the "obesity paradox". *Frontiers in Immunology*, *13*, 1012016. https://doi.org/10.3389/fimmu.2022.1012016

149. Porsche, C. E., Delproposto, J. B., Geletka, L., O'Rourke, R., & Lumeng, C. N. (2021). Obesity results in adipose tissue T cell exhaustion. *JCI Insight*, *6*(8). https://doi.org/10.1172/jci.insight.139793

150. Ahn, E., Araki, K., Hashimoto, M., Li, W., Riley, J. L., Cheung, J., Sharpe, A. H., Freeman, G. J., Irving, B. A., & Ahmed, R. (2018). Role of PD-1 during effector CD8 T cell differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(18), 4749–4754. https://doi.org/10.1073/pnas.1718217115

151. Wang, Y., Chung, Y. R., Eitzinger, S., Palacio, N., Gregory, S., Bhattacharyya, M., & Penaloza-MacMaster, P. (2019). TLR4 signaling improves PD-1 blockade therapy during chronic viral infection. *PLoS Pathogens*, *15*(2), 1–23. https://doi.org/10.1371/journal.ppat.1007583

152. Bally, Alexander P.R, Austin, James W., and Boss, J. M. (2016). Genetic and epigenetic regulation of PD-1 expression HHS Public Access. *J. Immunol.*, *196*(6), 2431–2437. https://doi.org/10.4049/jimmunol.1502643

153. Herati, R. S., Knorr, D. A., Vella, L. A., Silva, L. V., Chilukuri, L., Apostolidis,

S. A., Huang, A. C., Muselman, A., Manne, S., Kuthuru, O., Staupe, R. P., Adamski, S. A., Kannan, S., Kurupati, R. K., Ertl, H. C. J., Wong, J. L., Bournazos, S., McGettigan, S., Schuchter, L. M., … Wherry, E. J. (2022). PD-1 directed immunotherapy alters Tfh and humoral immune responses to seasonal influenza vaccine. *Nature Immunology*, *23*(8), 1183–1192. https://doi.org/10.1038/s41590-022-01274-3

154. Han, S., Asoyan, A., Rabenstein, H., Nakano, N., & Obst, R. (2010). Role of antigen persistence and dose for CD4+ T-cell exhaustion and recovery. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(47), 20453–20458. https://doi.org/10.1073/pnas.1008437107

155. Abdolmaleki, M., Mojtabavi, N., Zavvar, M., Vaezi, M., Noorbakhsh, F., & Nicknam, M. H. (2018). Scrutinizing the expression and blockade of inhibitory molecules expressed on T cells from acute myeloid leukemia patients. *Iranian Journal of Allergy, Asthma and Immunology*, *17*(3), 265–273.

156. Zou, W., Wolchok, J. D., Chen, L., Weiping, Z., Jedd D., W., & Lieping, C. (2016). PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy. *Science Translational Medicine*, *8*(328), 1–34. https://doi.org/10.1126/scitranslmed.aad7118.PD-L1

157. Pauken, K. E., & Wherry, E. J. (2015). Overcoming T cell exhaustion in infection and cancer. *Trends in Immunology*, *36*(4), 265–276. https://doi.org/10.1016/j.it.2015.02.008

158. Motta, F., Sica, A., & Selmi, C. (2020). Frailty in Rheumatic Diseases.

Frontiers in Immunology, *11*(October), 1–15. https://doi.org/10.3389/fimmu.2020.576134

159. Hoogendijk, E. O., Afilalo, J., Ensrud, K. E., Kowal, P., Onder, G., & Fried, L. P. (2019). Frailty: implications for clinical practice and public health. *The Lancet*, *394*(10206), 1365–1375. https://doi.org/10.1016/S0140-6736(19)31786-6

160. Fried, L. P., Tangen, C. M., Walston, J., Newman, A. B., Hirsch, C., Gottdiener, J., Seeman, T., Tracy, R., Kop, W. J., Burke, G., & McBurnie, M. A. (2001). Frailty in older adults: Evidence for a phenotype. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, *56*(3), 146–157. https://doi.org/10.1093/gerona/56.3.m146

161. Ciarambino, T., Crispino, P., Minervini, G., & Giordano, M. (2023). COVID-19 and Frailty. *Vaccines*, *11*(3). https://doi.org/10.3390/vaccines11030606

162. Bleve, A., Motta, F., Durante, B., Pandolfo, C., Selmi, C., & Sica, A. (2022). Immunosenescence, Inflammaging, and Frailty: Role of Myeloid Cells in Age-Related Diseases. *Clinical Reviews in Allergy and Immunology*, *0123456789*. https://doi.org/10.1007/s12016-021-08909-7

163. Verschoor, C. P., & Kuchel, G. A. (2023). Parsing chronological and biological age effects on vaccine responses. *Aging*, *15*(5), 1235–1236. https://doi.org/10.18632/aging.204572

164. Weinberger, B. (2021). Vaccination of older adults: Influenza, pneumococcal disease, herpes zoster, COVID-19 and beyond. *Immunity and Ageing*, *18*(1), 1–18. https://doi.org/10.1186/s12979-021-00249-6

165. Kojima, G. (2015). Prevalence of Frailty in Nursing Homes: A Systematic Review and Meta-Analysis. *Journal of the American Medical Directors Association*, *16*(11), 940–945. https://doi.org/10.1016/j.jamda.2015.06.025

166. Menec, V. H., MacWilliam, L., & Aoki, F. Y. (2002). Hospitalizations and deaths due to respiratory illnesses during influenza seasons: A comparison of community residents, senior housing residents, and nursing home residents. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, *57*(10), 629–635. https://doi.org/10.1093/gerona/57.10.M629

167. Mylotte, J. M. (2002). Nursing home - Acquired pneumonia. *Clinical Infectious Diseases*, *35*(10), 1205–1211. https://doi.org/10.1086/344281

168. Poscia, A., Collamati, A., Carfí, A., Topinkova, E., Richter, T., Denkinger, M., Pastorino, R., Landi, F., Ricciardi, W., Bernabei, R., & Onder, G. (2017). Influenza and pneumococcal vaccination in older adults living in nursing home: A survival analysis on the shelter study. *European Journal of Public Health*, *27*(6), 1016–1020. https://doi.org/10.1093/eurpub/ckx150

169. Fulop, T., Larbi, A., Dupuis, G., Page, A. Le, Frost, E. H., Cohen, A. A., Witkowski, J. M., & Franceschi, C. (2018). Immunosenescence and inflamm-aging as two sides of the same coin: Friends or Foes? *Frontiers in Immunology*, *8*(JAN). https://doi.org/10.3389/fimmu.2017.01960

170. Vukmanovic-Stejic, M., Chambers, E. S., Suárez-Fariñas, M., Sandhu, D., Fuentes-Duculan, J., Patel, N., Agius, E., Lacy, K. E., Turner, C. T., Larbi, A., Birault, V., Noursadeghi, M., Mabbott, N. A., Rustin, M. H. A., Krueger, J.

G., & Akbar, A. N. (2018). Enhancement of cutaneous immunity during aging by blocking p38 mitogen-activated protein (MAP) kinase–induced inflammation. *Journal of Allergy and Clinical Immunology*, *142*(3), 844–856. https://doi.org/10.1016/j.jaci.2017.10.032

171. Picard, E., Armstrong, S., Andrew, M. K., Haynes, L., Loeb, M., Pawelec, G., Kuchel, G. A., McElhaney, J. E., & Verschoor, C. P. (2022). Markers of systemic inflammation are positively associated with influenza vaccine antibody responses with a possible role for ILT2(+)CD57(+) NK-cells. *Immunity & Ageing : I & A*, *19*(1), 26. https://doi.org/10.1186/s12979-022-00284-x
172. Fan, J., Jin, S., Gilmartin, L., Toth, I., Hussein, W. M., & Stephenson, R. J. (2022). Advances in Infectious Disease Vaccine Adjuvants. *Vaccines*, *10*(7). https://doi.org/10.3390/vaccines10071120
173. Nordén, R., Nilsson, J., Samuelsson, E., Risinger, C., Sihlbom, C., Blixt, O., Larson, G., Olofsson, S., & Bergström, T. (2019). Recombinant glycoprotein E of varicella zoster virus contains glycan-peptide motifs that modulate B cell epitopes into discrete immunological signatures. *International Journal of Molecular Sciences*, *20*(4). https://doi.org/10.3390/ijms20040954
174. Pollard, A. J., & Bijker, E. M. (2021). A guide to vaccinology: from basic principles to new developments. *Nature Reviews Immunology*, *21*(2), 83–100. https://doi.org/10.1038/s41577-020-00479-7
175. Pulendran, B., S. Arunachalam, P., & O'Hagan, D. T. (2021). Emerging concepts in the science of vaccine adjuvants. *Nature Reviews Drug*

Discovery, *20*(6), 454–475. https://doi.org/10.1038/s41573-021-00163-y

176. Zhang, C., Maruggi, G., Shan, H., & Li, J. (2019). Advances in mRNA Vaccines for Infectious Diseases. *Frontiers in Immunology*, *10*, 594. https://doi.org/10.3389/fimmu.2019.00594

177. Baden, L. R., El Sahly, H. M., Essink, B., Kotloff, K., Frey, S., Novak, R., Diemert, D., Spector, S. A., Rouphael, N., Creech, C. B., McGettigan, J., Khetan, S., Segall, N., Solis, J., Brosz, A., Fierro, C., Schwartz, H., Neuzil, K., Corey, L., … Zaks, T. (2021). Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *The New England Journal of Medicine*, *384*(5), 403–416. https://doi.org/10.1056/NEJMoa2035389

178. Polack, F. P., Thomas, S. J., Kitchin, N., Absalon, J., Gurtman, A., Lockhart, S., Perez, J. L., Pérez Marc, G., Moreira, E. D., Zerbini, C., Bailey, R., Swanson, K. A., Roychoudhury, S., Koury, K., Li, P., Kalina, W. V., Cooper, D., Frenck, R. W., Hammitt, L. L., … Gruber, W. C. (2020). Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *New England Journal of Medicine*, *383*(27), 2603–2615. https://doi.org/10.1056/nejmoa2034577

179. Canaday, D. H., Oyebanji, O. A., Keresztesy, D., Payne, M., Wilk, D., Carias, L., Aung, H., St. Denis, K., Lam, E. C., Rowley, C. F., Berry, S. D., Cameron, C. M., Cameron, M. J., Wilson, B. M., Balazs, A. B., King, C. L., & Gravenstein, S. (2022). Significant Reduction in Vaccine-Induced Antibody Levels and Neutralization Activity Among Healthcare Workers and Nursing Home Residents 6 Months Following Coronavirus Disease 2019 BNT162b2 mRNA Vaccination. In *Clinical Infectious Diseases* (Vol. 75, Issue 1, pp.

e884–e887). https://doi.org/10.1093/cid/ciab963

180. Firdaus, F. Z., Skwarczynski, M., & Toth, I. (2022). *Developments in Vaccine AdjuvantsAdjuvants BT - Vaccine Design: Methods and Protocols, Volume 3. Resources for Vaccine Development* (S. Thomas (ed.); pp. 145–178). Springer US. https://doi.org/10.1007/978-1-0716-1892-9_8

181. Herbert, W. J. (1966). Antigenicity of soluble protein in the presence of high levels of antibody: a possible mode of action of the antigen adjuvants. *Nature*, *210*(5037), 747–748. https://doi.org/10.1038/210747a0

182. Kooijman, S., Brummelman, J., van Els, C. A. C. M., Marino, F., Heck, A. J. R., Mommen, G. P. M., Metz, B., Kersten, G. F. A., Pennings, J. L. A., & Meiring, H. D. (2018). Novel identified aluminum hydroxide-induced pathways prove monocyte activation and pro-inflammatory preparedness. *Journal of Proteomics*, *175*(January), 144–155. https://doi.org/10.1016/j.jprot.2017.12.021

183. Ulanova, M., Tarkowski, A., Hahn-Zoric, M., & Hanson, L. A. (2001). The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. *Infection and Immunity*, *69*(2), 1151–1159. https://doi.org/10.1128/IAI.69.2.1151-1159.2001

184. Oleszycka, E., McCluskey, S., Sharp, F. A., Muñoz-Wolf, N., Hams, E., Gorman, A. L., Fallon, P. G., & Lavelle, E. C. (2018). The vaccine adjuvant alum promotes IL-10 production that suppresses Th1 responses. *European Journal of Immunology*, *48*(4), 705–715.

https://doi.org/10.1002/eji.201747150

185. Groft, S. G., Nagy, N., Boom, W. H., & Harding, C. V. (2020). Toll-Like Receptor 2-Tpl2-Dependent ERK Signaling Drives Inverse Interleukin 12 Regulation in Dendritic Cells and Macrophages. *Infection and Immunity*, *89*(1). https://doi.org/10.1128/IAI.00323-20

186. Richardson, E. T., Shukla, S., Sweet, D. R., Wearsch, P. A., Tsichlis, P. N., Boom, W. H., & Harding, C. V. (2015). Toll-like receptor 2-dependent extracellular signal-regulated kinase signaling in Mycobacterium tuberculosis-infected macrophages drives anti-inflammatory responses and inhibits Th1 polarization of responding T cells. *Infection and Immunity*, *83*(6), 2242–2254. https://doi.org/10.1128/IAI.00135-15

187. Rose, W. A. 2nd, Okragly, A. J., Patel, C. N., & Benschop, R. J. (2015). IL-33 released by alum is responsible for early cytokine production and has adjuvant properties. *Scientific Reports*, *5*, 13146. https://doi.org/10.1038/srep13146

188. Sasaki, E., Asanuma, H., Momose, H., Furuhata, K., Mizukami, T., & Hamaguchi, I. (2021). Nasal alum-adjuvanted vaccine promotes IL-33 release from alveolar epithelial cells that elicits IgA production via type 2 immune responses. *PLoS Pathogens*, *17*(8 August), 1–25. https://doi.org/10.1371/journal.ppat.1009890

189. Stephen, J., Scales, H. E., Benson, R. A., Erben, D., Garside, P., & Brewer, J. M. (2017). Neutrophil swarming and extracellular trap formation play a significant role in Alum adjuvant activity. *Npj Vaccines*, *2*(1), 0–1.

https://doi.org/10.1038/s41541-016-0001-5

190. McKee, A. S., Burchill, M. A., Munks, M. W., Jin, L., Kappler, J. W., Friedman, R. S., Jacobelli, J., & Marrack, P. (2013). Host DNA released in response to aluminum adjuvant enhances MHC class II-mediated antigen presentation and prolongs CD4 T-cell interactions with dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(12). https://doi.org/10.1073/pnas.1300392110

191. Karacs, J., Reithofer, M., Kitzmüller, C., Kraller, M., Schmalz, S., Bleichert, S., Huppa, J. B., Stockinger, H., Bohle, B., & Jahn-Schmid, B. (2021). Adjuvants and vaccines used in allergen-specific immunotherapy induce neutrophil extracellular traps. *Vaccines*, *9*(4). https://doi.org/10.3390/vaccines9040321

192. Reithofer, M., Karacs, J., Strobl, J., Kitzmüller, C., Polak, D., Seif, K., Kamalov, M., Becker, C. F. W., Greiner, G., Schmetterer, K., Stary, G., Bohle, B., & Jahn-Schmid, B. (2020). Alum triggers infiltration of human neutrophils ex vivo and causes lysosomal destabilization and mitochondrial membrane potential-dependent NET-formation. In *FASEB Journal* (Vol. 34, Issue 10, pp. 14024–14041). https://doi.org/10.1096/fj.202001413R

193. Desai, J., Foresto-Neto, O., Honarpisheh, M., Steiger, S., Nakazawa, D., Popper, B., Buhl, E. M., Boor, P., Mulay, S. R., & Anders, H. J. (2017). Particles of different sizes and shapes induce neutrophil necroptosis followed by the release of neutrophil extracellular trap-like chromatin. *Scientific Reports*, *7*(1), 1–10. https://doi.org/10.1038/s41598-017-15106-0

194. Sharp, F. A., Ruane, D., Claass, B., Creagh, E., Harris, J., Malyala, P., Singh, M., O'Hagan, D. T., Pétrilli, V., Tschopp, J., O'Neill, L. A. J., & Lavelle, E. C. (2009). Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. In *Proceedings of the National Academy of Sciences of the United States of America* (Vol. 106, Issue 3, pp. 870–875). https://doi.org/10.1073/pnas.0804897106

195. Kool, M., Petrilli, V., Smedt, T. De, Rolaz, A., Hammad, H., van Nimwegen, M., Bergen, I. M., Castillo, R., Lambrecht, B. N., & Tschopp, J. (2008). Alum Adjuvant Stimulates Inflammatory Dendritic Cells through Activation of the NALP3 Inflammasome. *Journal of Immunology*, *181*(6), 3755–3759. https://doi.org/https://doi.org/10.4049/jimmunol.181.6.3755

196. Franchi, L., & Núñez, G. (2008). The Nlrp3 inflammasome is critical for aluminum hydroxide-mediated IL-1β secretion but dispensable for adjuvant activity. In *European Journal of Immunology* (Vol. 38, Issue 8, pp. 2085–2089). https://doi.org/10.1002/eji.200838549

197. Petrovsky, N., & Aguilar, J. C. (2004). Vaccine adjuvants: current state and future trends. *Immunology and Cell Biology*, *82*(5), 488–496. https://doi.org/10.1111/j.0818-9641.2004.01272.x

198. Ko, E.-J., Lee, Y.-T., Kim, K.-H., Jung, Y.-J., Lee, Y., Denning, T. L., & Kang, S.-M. (2016). Effects of MF59 Adjuvant on Induction of Isotype-Switched IgG Antibodies and Protection after Immunization with T-Dependent Influenza Virus Vaccine in the Absence of CD4 + T Cells . *Journal of Virology*, *90*(15), 6976–6988. https://doi.org/10.1128/jvi.00339-16

199. Kim, E. H., Woodruff, M. C., Grigoryan, L., Maier, B., Lee, S. H., Mandal, P., Cortese, M., Natrajan, M. S., Ravindran, R., Ma, H., Merad, M., Gitlin, A. D., Mocarski, E. S., Jacob, J., & Pulendran, B. (2020). Squalene emulsion-based vaccine adjuvants stimulate CD8 T cell, but not antibody responses, through a RIPK3-dependent pathway. *ELife*, *9*, 1–27. https://doi.org/10.7554/eLife.52687

200. Mendes, A., Azevedo-Silva, J., & Fernandes, J. C. (2022). From Sharks to Yeasts: Squalene in the Development of Vaccine Adjuvants. *Pharmaceuticals*, *15*(3). https://doi.org/10.3390/ph15030265

201. Seubert, A., Calabro, S., Santini, L., Galli, B., Genovese, A., Valentini, S., Aprea, S., Colaprico, A., D'Oro, U., Giuliani, M. M., Pallaoro, M., Pizza, M., O'Hagan, D. T., Wack, A., Rappuoli, R., & De Gregorio, E. (2011). Adjuvanticity of the oil-in-water emulsion MF59 is independent of Nlrp3 inflammasome but requires the adaptor protein MyD88. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(27), 11169–11174. https://doi.org/10.1073/pnas.1107941108

202. Calabro, S., Tortoli, M., Baudner, B. C., Pacitto, A., Cortese, M., O'Hagan, D. T., De Gregorio, E., Seubert, A., & Wack, A. (2011). Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. *Vaccine*, *29*(9), 1812–1823. https://doi.org/10.1016/j.vaccine.2010.12.090

203. Liang, F., Lindgren, G., Sandgren, K. J., Thompson, E. A., Francica, J. R., Seubert, A., Gregorio, E. De, Barnett, S., O'hagan, D. T., Sullivan, N. J.,

Koup, R. A., Seder, R. A., & Loré, K. (2017). Vaccine priming is restricted to draining lymph nodes & controlled by adjuvant-mediated antigen uptake. *Science Translational Medicine*, *9*(393). https://doi.org/10.1126/scitranslmed.aal2094

204. Mastelic Gavillet, B., Eberhardt, C. S., Auderset, F., Castellino, F., Seubert, A., Tregoning, J. S., Lambert, P.-H., de Gregorio, E., Del Giudice, G., & Siegrist, C.-A. (2015). MF59 Mediates Its B Cell Adjuvanticity by Promoting T Follicular Helper Cells and Thus Germinal Center Responses in Adult and Early Life. *The Journal of Immunology*, *194*(10), 4836–4845. https://doi.org/10.4049/jimmunol.1402071

205. Spensieri, F., Borgogni, E., Zedda, L., Bardelli, M., Buricchi, F., Volpini, G., Fragapane, E., Tavarini, S., Finco, O., Rappuoli, R., Giudice, G. Del, Galli, G., & Castellino, F. (2013). Human circulating influenza-CD4+ ICOS1+IL-21 + T cells expand after vaccination, exert helper function, and predict antibody responses. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(35), 14330–14335. https://doi.org/10.1073/pnas.1311998110

206. Vono, M., Taccone, M., Caccin, P., Gallotta, M., Donvito, G., Falzoni, S., Palmieri, E., Pallaoro, M., Rappuoli, R., Di Virgilio, F., De Gregorio, E., Montecucco, C., & Seubert, A. (2013). The adjuvant MF59 induces ATP release from muscle that potentiates response to vaccination. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(52), 21095–21100. https://doi.org/10.1073/pnas.1319784110

207. Cantisani, R., Pezzicoli, A., Cioncada, R., Malzone, C., De Gregorio, E., D'Oro, U., & Piccioli, D. (2015). Vaccine Adjuvant MF59 Promotes Retention of Unprocessed Antigen in Lymph Node Macrophage Compartments and Follicular Dendritic Cells. *The Journal of Immunology*, *194*(4), 1717–1725. https://doi.org/10.4049/jimmunol.1400623

208. Lofano, G., Mancini, F., Salvatore, G., Cantisani, R., Monaci, E., Carrisi, C., Tavarini, S., Sammicheli, C., Rossi Paccani, S., Soldaini, E., Laera, D., Finco, O., Nuti, S., Rappuoli, R., De Gregorio, E., Bagnoli, F., & Bertholet, S. (2015). Oil-in-Water Emulsion MF59 Increases Germinal Center B Cell Differentiation and Persistence in Response to Vaccination. *The Journal of Immunology*, *195*(4), 1617–1627. https://doi.org/10.4049/jimmunol.1402604

209. Khurana, S., Verma, N., Yewdell, J. W., Hilbert, A. K., Castellino, F., Lattanzi, M., Del Giudice, G., Rappuoli, R., & Golding, H. (2011). MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines. *Science Translational Medicine*, *3*(85), 1–10. https://doi.org/10.1126/scitranslmed.3002336

210. Vesikari, T., Kirstein, J., Devota Go, G., Leav, B., Ruzycky, M. E., Isakov, L., de Bruijn, M., Oberye, J., & Heijnen, E. (2018). Efficacy, immunogenicity, and safety evaluation of an MF59-adjuvanted quadrivalent influenza virus vaccine compared with non-adjuvanted influenza vaccine in children: a multicentre, randomised controlled, observer-blinded, phase 3 trial. *The Lancet Respiratory Medicine*, *6*(5), 345–356. https://doi.org/10.1016/S2213-2600(18)30108-5

211. Khurana, S., Coyle, E. M., Dimitrova, M., Castellino, F., Nicholson, K., Del Giudice, G., & Golding, H. (2014). Heterologous prime-boost vaccination with MF59-adjuvanted H5 vaccines promotes antibody affinity maturation towards the hemagglutinin HA1 domain and broad H5N1 cross-clade neutralization. *PLoS ONE*, *9*(4). https://doi.org/10.1371/journal.pone.0095496

212. Dendouga, N., Fochesato, M., Lockman, L., Mossman, S., & Giannini, S. L. (2012). Cell-mediated immune responses to a varicella-zoster virus glycoprotein E vaccine using both a TLR agonist and QS21 in mice. *Vaccine*, *30*(20), 3126–3135. https://doi.org/10.1016/j.vaccine.2012.01.088

213. Moncunill, G., Mpina, M., Nhabomba, A. J., Aguilar, R., Ayestaran, A., Sanz, H., Campo, J. J., Jairoce, C., Barrios, D., Dong, Y., Díez-Padrisa, N., Fernandes, J. F., Abdulla, S., Sacarlal, J., Williams, N. A., Harezlak, J., Mordmüller, B., Agnandji, S. T., Aponte, J. J., … Dobaño, C. (2017). Distinct helper t cell type 1 and 2 responses associated with malaria protection and risk in rts,s/as01e vaccinees. *Clinical Infectious Diseases*, *65*(5), 746–755. https://doi.org/10.1093/cid/cix429

214. Coccia, M., Collignon, C., Hervé, C., Chalon, A., Welsby, I., Detienne, S., Van Helden, M. J., Dutta, S., Genito, C. J., Waters, N. C., Van Deun, K., Smilde, A. K., Van Den Berg, R. A., Franco, D., Bourguignon, P., Morel, S., Garçon, N., Lambrecht, B. N., Goriely, S., … Didierlaurent, A. M. (2017). Cellular and molecular synergy in AS01-adjuvanted vaccines results in an early IFNγ response promoting vaccine immunogenicity. *Npj Vaccines*, *2*(1).

https://doi.org/10.1038/s41541-017-0027-3

215. Swanson, K. V., Deng, M., & Ting, J. P. Y. (2019). The NLRP3 inflammasome: molecular activation and regulation to therapeutics. *Nature Reviews Immunology*, *19*(8), 477–489. https://doi.org/10.1038/s41577-019-0165-0

216. Tannahill, G., Curtis, A., Adamik, J., Palsson-McDermott, E., McGettrick, A., Goel, G., Frezza, C., Bernard, N., Kelly, B., Foley, N., Zheng, L., Gardet, A., Tong, Z., Jany, S., Corr, S., Haneklaus, M., Caffery, B., Pierce, K., Walmsley, S., … O'Neill, L. (2013). Succinate is a danger signal that induces IL-1β via HIF-1α. *Nature*, *496*(7444), 238–242. https://doi.org/10.1038/nature11986.Succinate

217. Xing, Y., Yao, X., Li, H., Xue, G., Guo, Q., Yang, G., An, L., Zhang, Y., & Meng, G. (2017). Cutting Edge: TRAF6 Mediates TLR/IL-1R Signaling–Induced Nontranscriptional Priming of the NLRP3 Inflammasome. *The Journal of Immunology*, *199*(5), 1561–1566. https://doi.org/10.4049/jimmunol.1700175

218. Savva, A., & Roger, T. (2013). Targeting Toll-like receptors: Promising therapeutic strategies for the management of sepsis-associated pathology and infectious diseases. *Frontiers in Immunology*, *4*(NOV), 1–16. https://doi.org/10.3389/fimmu.2013.00387

219. Marty-Roix, R., Vladimer, G. I., Pouliot, K., Weng, D., Buglione-Corbett, R., West, K., MacMicking, J. D., Chee, J. D., Wang, S., Lu, S., & Lien, E. (2016). Identification of QS-21 as an inflammasome-activating molecular component

of saponin adjuvants. *Journal of Biological Chemistry*, *291*(3), 1123–1136. https://doi.org/10.1074/jbc.M115.683011

220. Welsby, I., Detienne, S., N'Kuli, F., Thomas, S., Wouters, S., Bechtold, V., De Wit, D., Gineste, R., Reinheckel, T., Elouahabi, A., Courtoy, P. J., Didierlaurent, A. M., & Goriely, S. (2017). Lysosome-dependent activation of human dendritic cells by the vaccine adjuvant QS-21. *Frontiers in Immunology*, *7*(JAN), 1–16. https://doi.org/10.3389/fimmu.2016.00663

221. Didierlaurent, A. M., Collignon, C., Bourguignon, P., Wouters, S., Fierens, K., Fochesato, M., Dendouga, N., Langlet, C., Malissen, B., Lambrecht, B. N., Garçon, N., Van Mechelen, M., & Morel, S. (2014). Enhancement of Adaptive Immunity by the Human Vaccine Adjuvant AS01 Depends on Activated Dendritic Cells. *The Journal of Immunology*, *193*(4), 1920–1930. https://doi.org/10.4049/jimmunol.1400948

222. Benlahrech, A., Gotch, F., Kelleher, P., & Patterson, S. (2011). Loss of NK stimulatory capacity by plasmacytoid and monocyte-derived DC but not myeloid DC in HIV-1 infected patients. *PloS One*, *6*(3), e17525. https://doi.org/10.1371/journal.pone.0017525

223. Satpathy, A. T., KC, W., Albring, J. C., Edelson, B. T., Kretzer, N. M., Bhattacharya, D., Murphy, T. L., & Murphy, K. M. (2012). Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. *The Journal of Experimental Medicine*, *209*(6), 1135–1152. https://doi.org/10.1084/jem.20120030

224. Guermonprez, P., Gerber-Ferder, Y., Vaivode, K., Bourdely, P., & Helft, J.

(2019). Chapter One - Origin and development of classical dendritic cells. In C. Lhuillier & L. B. T.-I. R. of C. and M. B. Galluzzi (Eds.), *Immunobiology of Dendritic Cells Part B* (Vol. 349, pp. 1–54). Academic Press. https://doi.org/https://doi.org/10.1016/bs.ircmb.2019.08.002

225. Coillard, A., & Segura, E. (2021). Antigen presentation by mouse monocyte-derived cells: Re-evaluating the concept of monocyte-derived dendritic cells. *Molecular Immunology*, *135*, 165–169. https://doi.org/https://doi.org/10.1016/j.molimm.2021.04.012
226. Cunningham, A. L., Lal, H., Kovac, M., Chlibek, R., Hwang, S. J., Díez-Domingo, J., Godeaux, O., Levin, M. J., McElhaney, J. E., Puig-Barberà, J., Vanden Abeele, C., Vesikari, T., Watanabe, D., Zahaf, T., Ahonen, A., Athan, E., Barba-Gomez, J. F., Campora, L., De Looze, F., … Heineman, T. C. (2016). Efficacy of the herpes zoster subunit vaccine in adults 70 years of age or older. *New England Journal of Medicine*, *375*(11), 1019–1032. https://doi.org/10.1056/NEJMoa1603800
227. Izurieta, H. S., Wu, X., Forshee, R., Lu, Y., Sung, H. M., Agger, P. E., Chillarige, Y., Link-Gelles, R., Lufkin, B., Wernecke, M., MacUrdy, T. E., Kelman, J., & Dooling, K. (2021). Recombinant Zoster Vaccine (Shingrix): Real-World Effectiveness in the First 2 Years Post-Licensure. *Clinical Infectious Diseases*, *73*(6), 941–948. https://doi.org/10.1093/cid/ciab125
228. Johnson, M. J., Liu, C., Ghosh, D., Lang, N., Levin, M. J., & Weinberg, A. (2022). Cell-Mediated Immune Responses After Administration of the Live or the Recombinant Zoster Vaccine: 5-Year Persistence. *Journal of Infectious*

Diseases, *225*(8), 1477–1481. https://doi.org/10.1093/infdis/jiab580

229. Oxman, MN, Levin, MJ, Johnson, GR, Schmader, KE, Straus, SE, Gelb, LD, Arbeit, RD, Simberkoff, MS, Gershon, AA, Davis, LE, Weinberg, A, Boardman, KD, Williams, HM, Hongyuan Zhang, J, Peduzzi, PN, Beisel, CE, Morrison, VA, Guatelli, JC, Brooks, C.A. Kauffm, and J. L. S. (2006). A vaccine to prevent herpes zoster and postherpetic neuralgia in older adults. In *Consultant Pharmacist* (Vol. 21, Issue 2, pp. 149–150).

230. Weinberg, A., Pang, L., Johnson, M. J., Caldas, Y., Cho, A., Tovar-Salazar, A., Canniff, J., Schmader, K. E., Popmihajlov, Z., & Levin, M. J. (2019). The Effect of Age on the Immunogenicity of the Live Attenuated Zoster Vaccine Is Predicted by Baseline Regulatory T Cells and Varicella-Zoster Virus-Specific T Cell Immunity. *Journal of Virology*, *93*(15), 1–17. https://doi.org/10.1128/jvi.00305-19

231. Buchan, S. A., Daneman, N., Wang, J., Wilson, S. E., Garber, G., Wormsbecker, A. E., Antoniou, T., & Deeks, S. L. (2021). Herpes zoster in older adults in Ontario, 2002-2016: Investigating incidence and exploring equity. *PloS One*, *16*(2), e0246086. https://doi.org/10.1371/journal.pone.0246086

232. Grupping, K., Campora, L., Douha, M., Heineman, T. C., Klein, N. P., Lal, H., Peterson, J., Vastiau, I., & Oostvogels, L. (2017). Immunogenicity and safety of the Hz/su adjuvanted herpes zoster subunit vaccine in adults previously vaccinated with a live attenuated herpes zoster vaccine. *Journal of Infectious Diseases*, *216*(11), 1343–1351.

https://doi.org/10.1093/infdis/jix482

233. Burny, W., Marchant, A., Hervé, C., Callegaro, A., Caubet, M., Fissette, L., Gheyle, L., Legrand, C., Ndour, C., Tavares Da Silva, F., van der Most, R., Willems, F., Didierlaurent, A. M., & Yarzabal, J. (2019). Inflammatory parameters associated with systemic reactogenicity following vaccination with adjuvanted hepatitis B vaccines in humans. *Vaccine*, *37*(14), 2004–2015. https://doi.org/10.1016/j.vaccine.2019.02.015

234. Didierlaurent, A. M., Laupèze, B., Di Pasquale, A., Hergli, N., Collignon, C., & Garçon, N. (2017). Adjuvant system AS01: helping to overcome the challenges of modern vaccines. *Expert Review of Vaccines*, *16*(1), 55–63. https://doi.org/10.1080/14760584.2016.1213632

235. Partnership, R. . C. T. (2015). Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: Final results of a phase 3, individually randomised, controlled trial. *The Lancet*, *386*(9988), 31–45. https://doi.org/10.1016/S0140-6736(15)60721-8

236. Kazmin, D., Nakaya, H. I., Lee, E. K., Johnson, M. J., Van Der Most, R., Van Den Berg, R. A., Ballou, W. R., Jongert, E., Wille-Reece, U., Ockenhouse, C., Aderem, A., Zak, D. E., Sadoff, J., Hendriks, J., Wrammert, J., Ahmed, R., & Pulendran, B. (2017). Systems analysis of protective immune responses to RTS,S malaria vaccination in humans. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(9), 2425–2430. https://doi.org/10.1073/pnas.1621489114

237. Brunner, L., Barnier-Quer, C., & Collin, N. (2017). QS-21 Adjuvant:

Laboratory-Scale Purification Method and Formulation Into Liposomes. *Methods in Molecular Biology (Clifton, N.J.)*, *1494*, 73–86. https://doi.org/10.1007/978-1-4939-6445-1_5

238. Bowman, E. R., Cameron, C. M., Richardson, B., Kulkarni, M., Gabriel, J., Cichon, M. J., Riedl, K. M., Mustafa, Y., Cartwright, M., Snyder, B., Raman, S. V., Zidar, D. A., Koletar, S. L., Playford, M. P., Mehta, N. N., Sieg, S. F., Freeman, M. L., Lederman, M. M., Cameron, M. J., & Funderburg, N. T. (2020). Macrophage maturation from blood monocytes is altered in people with HIV, and is linked to serum lipid profiles and activation indices: A model for studying atherogenic mechanisms. *PLoS Pathogens*, *16*(10), 1–24. https://doi.org/10.1371/journal.ppat.1008869

239. Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research*, *43*(7), e47. https://doi.org/10.1093/nar/gkv007

240. Hänzelmann, S., Castelo, R., & Guinney, J. (2013). GSVA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinformatics*, *14*, 7. https://doi.org/10.1186/1471-2105-14-7

241. Komai-Koma, M., Ji, Y., Cao, H., Liu, Z., McSharry, C., & Xu, D. (2021). Monophosphoryl lipid A directly regulates Th1 cytokine production in human CD4+ T-cells through Toll-like receptor 2 and 4. *Immunobiology*, *226*(5), 152132. https://doi.org/10.1016/j.imbio.2021.152132

242. Sun, J., Wu, G., Pastor, F., Rahman, N., Wang, W.-H., Zhang, Z., Merle, P.,

Hui, L., Salvetti, A., Durantel, D., Yang, D., & Andrisani, O. (2022). RNA helicase DDX5 enables STAT1 mRNA translation and interferon signalling in hepatitis B virus replicating hepatocytes. *Gut*, *71*(5), 991–1005. https://doi.org/10.1136/gutjnl-2020-323126

243. Giza, H. M., & Bozzacco, L. (2021). Unboxing dendritic cells: Tales of multi-faceted biology and function. In *Immunology* (Vol. 164, Issue 3, pp. 433–449). https://doi.org/10.1111/imm.13394

244. Gill, P. S., Ozment, T. R., Lewis, N. H., Sherwood, E. R., & Williams, D. L. (2022). Trained Immunity Enhances Human Monocyte Function in Aging and Sepsis. *Frontiers in Immunology*, *13*, 872652. https://doi.org/10.3389/fimmu.2022.872652

245. McGettrick, A. F., & O'Neill, L. A. J. (2020). The Role of HIF in Immunity and Inflammation. *Cell Metabolism*, *32*(4), 524–536. https://doi.org/10.1016/j.cmet.2020.08.002

246. Wang, T., Liu, H., Lian, G., Zhang, S.-Y., Wang, X., & Jiang, C. (2017). HIF1*α*-Induced Glycolysis Metabolism Is Essential to the Activation of Inflammatory Macrophages. *Mediators of Inflammation*, *2017*, 9029327. https://doi.org/10.1155/2017/9029327

247. Sánchez-Ramón, S., Conejero, L., Netea, M. G., Sancho, D., Palomares, Ó., & Subiza, J. L. (2018). Trained Immunity-Based Vaccines: A New Paradigm for the Development of Broad-Spectrum Anti-infectious Formulations. *Frontiers in Immunology*, *9*, 2936. https://doi.org/10.3389/fimmu.2018.02936

248. Netea, M. G., Domínguez-Andrés, J., Barreiro, L. B., Chavakis, T., Divangahi, M., Fuchs, E., Joosten, L. A. B., van der Meer, J. W. M., Mhlanga, M. M., Mulder, W. J. M., Riksen, N. P., Schlitzer, A., Schultze, J. L., Stabell Benn, C., Sun, J. C., Xavier, R. J., & Latz, E. (2020). Defining trained immunity and its role in health and disease. *Nature Reviews. Immunology*, *20*(6), 375–388. https://doi.org/10.1038/s41577-020-0285-6

249. Sun, J., Gan, L., & Sun, J. (2023). Identification and Validation of Three m6A Regulators: FTO, HNRNPC, and HNRNPA2B1 as Potential Biomarkers for Endometriosis. *Genes*, *14*(1). https://doi.org/10.3390/genes14010086

250. Zhao, W., Wang, Z., Sun, Z., He, Y., Jian, D., Hu, X., Zhang, W., & Zheng, L. (2018). RNA helicase DDX5 participates in oxLDL-induced macrophage scavenger receptor 1 expression by suppressing mRNA degradation. *Experimental Cell Research*, *366*(2), 114–120. https://doi.org/10.1016/j.yexcr.2018.03.003

251. Subbannayya, Y., Pinto, S. M., Bösl, K., Keshava Prasad, T. S., & Kandasamy, R. K. (2019). Dynamics of dual specificity phosphatases and their interplay with protein kinases in immune signaling. *International Journal of Molecular Sciences*, *20*(9). https://doi.org/10.3390/ijms20092086

252. Ma, Q., Yu, J., Zhang, X., Wu, X., & Deng, G. (2023). Wnt/β-catenin signaling pathway-a versatile player in apoptosis and autophagy. *Biochimie*, *211*, 57–67. https://doi.org/10.1016/j.biochi.2023.03.001

253. Choi, S. H., Gonen, A., Diehl, C. J., Kim, J., Almazan, F., Witztum, J. L., & Miller, Y. I. (2015). SYK regulates macrophage MHC-II expression via

activation of autophagy in response to oxidized LDL. *Autophagy*, *11*(5), 785–795. https://doi.org/10.1080/15548627.2015.1037061

254. You, R. I., & Chu, C. L. (2017). SHP-1 (PTPN6) keeps the inflammation at bay: Limiting IL-1α-mediated neutrophilic dermatoses by preventing Syk kinase activation. *Cellular and Molecular Immunology*, *14*(11), 881–883. https://doi.org/10.1038/cmi.2017.59

255. Boesteanu, A. C., & Katsikis, P. D. (2009). Memory T cells need CD28 costimulation to remember. *Seminars in Immunology*, *21*(2), 69–77. https://doi.org/10.1016/j.smim.2009.02.005

256. Richter, L., Landsverk, O. J. B., Atlasy, N., Bujko, A., Yaqub, S., Horneland, R., Øyen, O., Aandahl, E. M., Lundin, K. E. A., Stunnenberg, H. G., Bækkevold, E. S., & Jahnsen, F. L. (2018). Transcriptional profiling reveals monocyte-related macrophages phenotypically resembling DC in human intestine. *Mucosal Immunology*, *11*(5), 1512–1523. https://doi.org/10.1038/s41385-018-0060-1

257. Guillaume, J., Leufgen, A., Hager, F. T., Pabst, O., & Cerovic, V. (2023). MHCII expression on gut macrophages supports T cell homeostasis and is regulated by microbiota and ontogeny. *Scientific Reports*, *13*(1), 1–13. https://doi.org/10.1038/s41598-023-28554-8

258. Nakano, H., Lin, K. L., Yanagita, M., Charbonneau, C., Cook, D. N., Kakiuchi, T., & Gunn, M. D. (2009). Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses. *Nature Immunology*, *10*(4), 394–402. https://doi.org/10.1038/ni.1707

259. Burny, W., Callegaro, A., Bechtold, V., Clement, F., Delhaye, S., Fissette, L., Janssens, M., Leroux-Roels, G., Marchant, A., van den Berg, R. A., Garçon, N., van der Most, R., Didierlaurent, A. M., Carletti, I., Esen, M., Gabor, J., Haelterman, E., Hervé, C., Horsmans, Y., … Zuchner, D. (2017). Different adjuvants induce common innate pathways that are associated with enhanced adaptive responses against a model antigen in humans. *Frontiers in Immunology*, *8*(AUG). https://doi.org/10.3389/fimmu.2017.00943

260. Jing, Y., Shaheen, E., Drake, R. R., Chen, N., Gravenstein, S., & Deng, Y. (2009). Aging is associated with a numerical and functional decline in plasmacytoid dendritic cells, whereas myeloid dendritic cells are relatively unaltered in human peripheral blood. *Human Immunology*, *70*(10), 777–784. https://doi.org/10.1016/j.humimm.2009.07.005

261. Prakash, S., Agrawal, S., Cao, J. N., Gupta, S., & Agrawal, A. (2013). Impaired secretion of interferons by dendritic cells from aged subjects to influenza. *Age*, *35*(5), 1785–1797. https://doi.org/10.1007/s11357-012-9477-8

262. Cao, Y., Fan, Y., Li, F., Hao, Y., Kong, Y., Chen, C., Hao, X., Han, D., Li, G., Wang, Z., Song, C., Han, J., & Zeng, H. (2022). Phenotypic and functional alterations of monocyte subsets with aging. *Immunity and Ageing*, *19*(1), 1–11. https://doi.org/10.1186/s12979-022-00321-9

263. Seidler, S., Zimmermann, H. W., Bartneck, M., Trautwein, C., & Tacke, F. (2010). Age-dependent alterations of monocyte subsets and monocyte-related chemokine pathways in healthy adults. *BMC Immunology*, *11*.

https://doi.org/10.1186/1471-2172-11-30

264. Cheng, A., Hsieh, M.-J., Chang, S.-Y., Ieong, S.-M., Cheng, C.-Y., Sheng, W.-H., & Chang, S.-C. (2022). Correlation of adverse effects and antibody responses following homologous and heterologous COVID19 prime-boost vaccinations. *Journal of the Formosan Medical Association = Taiwan Yi Zhi*. https://doi.org/10.1016/j.jfma.2022.12.002

265. Oyebanji, O. A., Wilson, B., Keresztesy, D., Carias, L., Wilk, D., Payne, M., Aung, H., Denis, K. S., Lam, E. C., Rowley, C. F., Berry, S. D., Cameron, C. M., Cameron, M. J., Schmader, K. E., Balazs, A. B., King, C. L., Canaday, D. H., & Gravenstein, S. (2021). Does a lack of vaccine side effects correlate with reduced BNT162b2 mRNA vaccine response among healthcare workers and nursing home residents? *Aging Clinical and Experimental Research*, *33*(11), 3151–3160. https://doi.org/10.1007/s40520-021-01987-9

266. Callegaro, A., Burny, W., Hervé, C., Hyung Kim, J., Levin, M. J., Zahaf, T., Cunningham, A. L., & Didierlaurent, A. M. (2021). Association Between Immunogenicity and Reactogenicity: A Post Hoc Analysis of 2 Phase 3 Studies With the Adjuvanted Recombinant Zoster Vaccine. *The Journal of Infectious Diseases*, *226*, 1943–1948. https://doi.org/10.1093/infdis/jiab536

267. Hagen, S. R., Thompson, J. D., Snyder, D. S., & Myers, K. R. (1997). Analysis of a monophosphoryl lipid A immunostimulant preparation from Salmonella minnesota R595 by high-performance liquid chromatography. *Journal of Chromatography A*, *767*(1–2), 53–61. https://doi.org/10.1016/S0021-9673(97)00041-1

268. Wang, Y. Q., Bazin-Lee, H., Evans, J. T., Casella, C. R., & Mitchell, T. C. (2020). MPL Adjuvant Contains Competitive Antagonists of Human TLR4. *Frontiers in Immunology*, *11*(October), 1–12. https://doi.org/10.3389/fimmu.2020.577823

269. Heo, J. Y., Song, J. Y., Noh, J. Y., Choi, M. J., Yoon, J. G., Lee, S. N., Cheong, H. J., & Kim, W. J. (2018). Effects of influenza vaccination on pneumonia in the elderly. *Human Vaccines and Immunotherapeutics*, *14*(3), 744–749. https://doi.org/https://doi.org/10.1080/21645515.2017.1405200

270. Reed, C., Chaves, S. S., Kirley, P. D., Emerson, R., Aragon, D., Hancock, E. B., Butler, L., Baumbach, J., Hollick, G., Bennett, N. M., Laidler, M. R., Thomas, A., Meltzer, M. I., & Finelli, L. (2015). Estimating influenza disease burden from population-based surveillance data in the United States. *PLoS ONE*, *10*(3). https://doi.org/10.1371/journal.pone.0118369

271. Monto, A. S., Rotthoff, J., Teich, E., Herlocher, M. L., Truscon, R., Yen, H. L., Elias, S., & Ohmit, S. E. (2004). Detection and control of influenza outbreaks in well-vaccinated nursing home populations. *Clinical Infectious Diseases*, *39*(4), 459–464. https://doi.org/10.1086/422646

272. McConeghy, K. W., Davidson, H. E., Canaday, D. H., Han, L., Saade, E., Mor, V., & Gravenstein, S. (2021). Cluster-randomized Trial of Adjuvanted Versus Nonadjuvanted Trivalent Influenza Vaccine in 823 US Nursing Homes. *Clinical Infectious Diseases*, *73*(11), E4237–E4243. https://doi.org/10.1093/cid/ciaa1233

273. Frey, S. E., Reyes, M. R. A. D. L., Reynales, H., Bermal, N. N., Nicolay, U.,

Narasimhan, V., Forleo-Neto, E., & Arora, A. K. (2014). Comparison of the safety and immunogenicity of an MF59®-adjuvanted with a non-adjuvanted seasonal influenza vaccine in elderly subjects. *Vaccine*, *32*(39), 5027–5034. https://doi.org/10.1016/j.vaccine.2014.07.013

274. Nicolay, U., Heijnen, E., Nacci, P., Patriarca, P. A., & Leav, B. (2019). Immunogenicity of aIIV3, MF59-adjuvanted seasonal trivalent influenza vaccine, in older adults ≥65 years of age: Meta-analysis of cumulative clinical experience. *International Journal of Infectious Diseases*, *85*(2019), S1–S9. https://doi.org/10.1016/j.ijid.2019.03.026

275. Dolfi, D. V., Mansfield, K. D., Kurupati, R. K., Kannan, S., Doyle, S. A., Ertl, H. C. J., Schmader, K. E., & Wherry, E. J. (2013). Vaccine-Induced Boosting of Influenza Virus-Specific CD4 T Cells in Younger and Aged Humans. *PLoS ONE*, *8*(10), 2–12. https://doi.org/10.1371/journal.pone.0077164

276. Klimov, Alexander Balish, Amanda Veguilla, Vic Sun, Hong Schiffer, Jarad Lu, Xiuhua Katz, Jacqueline M Hancock, K. (2012). Influenza virus titration, antigenic characterization, and serological methods for antibody detection. In *Methods in Molecular Biology* (Vol. 865, pp. 25–51). https://doi.org/10.1007/978-1-61779-621-0

277. Couzens, L., Gao, J., Westgeest, K., Sandbulte, M., Lugovtsev, V., Fouchier, R., & Eichelberger, M. (2014). An optimized enzyme-linked lectin assay to measure influenza A virus neuraminidase inhibition antibody titers in human sera. *Journal of Virological Methods*, *210*, 7–14. https://doi.org/10.1016/j.jviromet.2014.09.003

278. Zhou, X., & McElhaney, J. E. (2011). Age-related changes in memory and effector T cells responding to influenza A/H3N2 and pandemic A/H1N1 strains in humans. *Vaccine*, *29*(11), 2169–2177. https://doi.org/10.1016/j.vaccine.2010.12.029

279. Roederer, M., Nozzi, J. L., & Nason, M. C. (2011). SPICE: Exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry Part A*, *79 A*(2), 167–174. https://doi.org/10.1002/cyto.a.21015

280. CDC. (2022). *Advisory Committee on Immunization Practice: Recommendations*. https://www.cdc.gov/vaccines/acip/recommendations.html

281. Van Buynder, P. G., Konrad, S., Van Buynder, J. L., Brodkin, E., Krajden, M., Ramler, G., & Bigham, M. (2013). The comparative effectiveness of adjuvanted and unadjuvanted trivalent inactivated influenza vaccine (TIV) in the elderly. *Vaccine*, *31*(51), 6122–6128. https://doi.org/10.1016/j.vaccine.2013.07.059

282. Gravenstein, S., McConeghy, K. W., Saade, E., Davidson, H. E., Canaday, D. H., Han, L., Rudolph, J., Joyce, N., Dahabreh, I. J., & Mor, V. (2021). Adjuvanted Influenza Vaccine and Influenza Outbreaks in US Nursing Homes: Results From a Pragmatic Cluster-Randomized Clinical Trial. *Clinical Infectious Diseases*, *73*(11), E4229–E4236. https://doi.org/10.1093/cid/ciaa1916

283. Memoli, M. J., Shaw, P. A., Han, A., Czajkowski, L., Reed, S., Athota, R., Bristol, T., Fargis, S., Risos, K., Powers, J. H., Davey, R. T., &

Taubenberger, J. K. (2016). Evaluation of antihemagglutinin and antineuraminidase antibodies as correlates of protection in an influenza A/H1N1 virus healthy human challenge model. In *mBio* (Vol. 7, Issue 2). https://doi.org/10.1128/mBio.00417-16

284. Dunning, A. J., DiazGranados, C. A., Voloshen, T., Hu, B., Landolfi, V. A., & Talbot, H. K. (2016). Correlates of Protection against Influenza in the Elderly: Results from an Influenza Vaccine Efficacy Trial. *Clinical and Vaccine Immunology*, *23*(3), 228–235. https://doi.org/10.1128/CVI.00604-15

285. Skarlupka, A. L., Bebin-Blackwell, A.-G., Sumner, S. F., & Ross, T. M. (2021). Universal Influenza Virus Neuraminidase Vaccine Elicits Protective Immune Responses against Human Seasonal and Pre-Pandemic Strains. *Journal of Virology*, *95*(17), 1–16.

286. Kelvin, D. J., & Farooqui, A. (2013). Extremely low vaccine effectiveness against influenza H3N2 in the elderly during the 2012/2013 flu season. In *Journal of Infection in Developing Countries* (Vol. 7, Issue 3, pp. 299–301). https://doi.org/10.3855/jidc.3544

287. Rondy, M., Gherasim, A., Casado, I., Launay, O., Rizzo, C., Pitigoi, D., Mickiene, A., Sierk, D., Descartes, U. P., Cité, S. P., Cochin-pasteur, C. I. C., National, C., Recherche, D., Cnrs, S., Nationale, E., Ens, S., Claude, U., & Lyon, B. (2017). Low 2016/17 season vaccine effectiveness against hospitalised influenza A (H3N2) among elderly: awareness warranted for 2017/18 season. *Euro Surveill*, *22*(41), 1–6. https://doi.org/https://doi.org/10.2807/1560-7917.ES.2017.22.41.17-00645

288. Shapiro, J. R., Li, H., Morgan, R., Chen, Y., Kuo, H., Ning, X., Shea, P., Wu, C., Merport, K., Saldanha, R., Liu, S., Abrams, E., Chen, Y., Kelly, D. C., Sheridan-Malone, E., Wang, L., Zeger, S. L., Klein, S. L., & Leng, S. X. (2021). Sex-specific effects of aging on humoral immune responses to repeated influenza vaccination in older adults. *Npj Vaccines*, *6*(1), 1–11. https://doi.org/10.1038/s41541-021-00412-6

289. Sung, M. H., Shen, Y., Handel, A., Bahl, J., & Ross, T. M. (2021). Longitudinal Assessment of Immune Responses to Repeated Annual Influenza Vaccination in a Human Cohort of Adults and Teenagers. *Frontiers in Immunology*, *12*(March), 1–10. https://doi.org/10.3389/fimmu.2021.642791

290. CDC. (2022). *Vaccination Coverage among Nursing Home Residents*. FluVaxView. https://www.cdc.gov/flu/fluvaxview/interactive-nursing-home.htm?CDC_AA_refVal=https%3A%2F%2Fwww.cdc.gov%2Fflu%2Ffluvaxview%2Fnursinghome%2Ftrends%2Findex.html

291. Khurana, S., Hahn, M., Coyle, E. M., King, L. R., Lin, T. L., Treanor, J., Sant, A., & Golding, H. (2019). Repeat vaccination reduces antibody affinity maturation across different influenza vaccine platforms in humans. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-019-11296-5

292. McLean, H. Q., Thompson, M. G., Sundaram, M. E., Meece, J. K., McClure, D. L., Friedrich, T. C., & Belongia, E. A. (2014). Impact of repeated vaccination on vaccine effectiveness against influenza A(H3N2) and B during 8 seasons. *Clinical Infectious Diseases*, *59*(10), 1375–1385.

https://doi.org/10.1093/cid/ciu680

293. Ohmit, S. E., Thompson, M. G., Petrie, J. G., Thaker, S. N., Jackson, M. L., Belongia, E. A., Zimmerman, R. K., Gaglani, M., Lamerato, L., Spencer, S. M., Jackson, L., Meece, J. K., Nowalk, M. P., Song, J., Zervos, M., Cheng, P. Y., Rinaldo, C. R., Clipper, L., Shay, D. K., … Monto, A. S. (2014). Influenza vaccine effectiveness in the 2011-2012 season: Protection against each circulating virus and the effect of prior vaccination on estimates. *Clinical Infectious Diseases*, *58*(3), 319–327. https://doi.org/10.1093/cid/cit736

294. Kumar, A., McElhaney, J. E., Walrond, L., Cyr, T. D., Merani, S., Kollmann, T. R., Halperin, S. A., & Scheifele, D. W. (2017). Cellular immune responses of older adults to four influenza vaccines: Results of a randomized, controlled comparison. *Human Vaccines and Immunotherapeutics*, *13*(9), 2048–2057. https://doi.org/10.1080/21645515.2017.1337615

295. Sridhar, S. (2016). Heterosubtypic T-cell immunity to influenza in humans: Challenges for universal T-cell influenza vaccines. *Frontiers in Immunology*, *7*(MAY), 1–12. https://doi.org/10.3389/fimmu.2016.00195

296. Shahid, Z., Kleppinger, A., Gentleman, B., Falsey, A. R., & McElhaney, J. E. (2010). Clinical and immunologic predictors of influenza illness among vaccinated older adults. *Vaccine*, *28*(38), 6145–6151. https://doi.org/10.1016/j.vaccine.2010.07.036

297. Wilkinson, T. M., Li, C. K. F., Chui, C. S. C., Huang, A. K. Y., Perkins, M., Liebner, J. C., Lambkin-Williams, R., Gilbert, A., Oxford, J., Nicholas, B.,

Staples, K. J., Dong, T., Douek, D. C., McMichael, A. J., & Xu, X. N. (2012). Preexisting influenza-specific CD4 + T cells correlate with disease protection against influenza challenge in humans. *Nature Medicine*, *18*(2), 274–280. https://doi.org/10.1038/nm.2612

298. Boyd, A., Almeida, J. R., Darrah, P. A., Sauce, D., Seder, R. A., Appay, V., Gorochov, G., & Larsen, M. (2015). Pathogen-specific T cell polyfunctionality is a correlate of T cell efficacy and immune protection. *PLoS ONE*, *10*(6), 1–18. https://doi.org/10.1371/journal.pone.0128714

299. Darrah, P. A., Patel, D. T., De Luca, P. M., Lindsay, R. W. B., Davey, D. F., Flynn, B. J., Hoff, S. T., Andersen, P., Reed, S. G., Morris, S. L., Roederer, M., & Seder, R. A. (2007). Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nature Medicine*, *13*(7), 843–850. https://doi.org/10.1038/nm1592

300. Betts, M. R., Nason, M. C., West, S. M., De Rosa, S. C., Migueles, S. A., Abraham, J., Lederman, M. M., Benito, J. M., Goepfert, P. A., Connors, M., Roederer, M., & Koup, R. A. (2006). HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood*, *107*(12), 4781–4789. https://doi.org/10.1182/blood-2005-12-4818

301. Dan, J. M., Lindestam Arlehamn, C. S., Weiskopf, D., da Silva Antunes, R., Havenar-Daughton, C., Reiss, S. M., Brigger, M., Bothwell, M., Sette, A., & Crotty, S. (2016). A Cytokine-Independent Approach To Identify Antigen-Specific Human Germinal Center T Follicular Helper Cells and Rare Antigen-Specific CD4 + T Cells in Blood . *The Journal of Immunology*, *197*(3), 983–

993. https://doi.org/10.4049/jimmunol.1600318

302. Koutsakos, M., Wheatley, A. K., Loh, L., Clemens, E. B., Sant, S., Nüssing, S., Fox, A., Chung, A. W., Laurie, K. L., Hurt, A. C., Rockman, S., Lappas, M., Loudovaris, T., Mannering, S. I., Westall, G. P., Elliot, M., Tangye, S. G., Wakim, L. M., Kent, S. J., … Kedzierska, K. (2018). Circulating TFH cells, serological memory, and tissue compartmentalization shape human influenza-specific B cell immunity. *Science Translational Medicine*, *10*(428), 1–16. https://doi.org/10.1126/scitranslmed.aan8405

303. Cohen, J. (1992). *Quantitative Methods in Psychology: A Power Primer*. *112*(1), 155–159. https://doi.org/10.1037/0033-2909.112.1.155

304. Takao, K., & Miyakawa, T. (2015). Genomic responses in mouse models greatly mimic human inflammatory diseases. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(4), 1167–1172. https://doi.org/10.1073/pnas.1401965111

305. Mestas, J., & Hughes, C. C. W. (2004). Of Mice and Not Men: Differences between Mouse and Human Immunology. *The Journal of Immunology*, *172*(5), 2731–2738. https://doi.org/10.4049/jimmunol.172.5.2731

306. Horn, S., Hughes, M. A., Schilling, R., Sticht, C., Tenev, T., Ploesser, M., Meier, P., Sprick, M. R., MacFarlane, M., & Leverkus, M. (2017). Caspase-10 Negatively Regulates Caspase-8-Mediated Cell Death, Switching the Response to CD95L in Favor of NF-κB Activation and Cell Survival. *Cell Reports*, *19*(4), 785–797. https://doi.org/10.1016/j.celrep.2017.04.010

307. Shay, T., Jojic, V., Zuk, O., Rothamel, K., Puyraimond-Zemmour, D., Feng,

T., Wakamatsu, E., Benoist, C., Koller, D., & Regev, A. (2013). Conservation and divergence in the transcriptional programs of the human and mouse immune systems. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(8), 2946–2951. https://doi.org/10.1073/pnas.1222738110

308. Sun, J., Li, N., Oh, K.-S., Dutta, B., Vayttaden, S. J., Lin, B., Ebert, T. S., De Nardo, D., Davis, J., Bagirzadeh, R., Lounsbury, N. W., Pasare, C., Latz, E., Hornung, V., & Fraser, I. D. C. (2016). Comprehensive RNAi-based screening of human and mouse TLR pathways identifies species-specific preferences in signaling protein use. *Science Signaling*, *9*(409), ra3. https://doi.org/10.1126/scisignal.aab2191
309. Shortman, K., & Liu, Y.-J. (2002). Mouse and human dendritic cell subtypes. *Nature Reviews. Immunology*, *2*(3), 151–161. https://doi.org/10.1038/nri746
310. Fitzgerald-Bocarsly, P., Dai, J., & Singh, S. (2008). Plasmacytoid dendritic cells and type I IFN: 50 years of convergent history. *Cytokine & Growth Factor Reviews*, *19*(1), 3–19. https://doi.org/10.1016/j.cytogfr.2007.10.006
311. Heger, L., Hofer, T. P., Bigley, V., de Vries, I. J. M., Dalod, M., Dudziak, D., & Ziegler-Heitbrock, L. (2020). Subsets of CD1c(+) DCs: Dendritic Cell Versus Monocyte Lineage. *Frontiers in Immunology*, *11*, 559166. https://doi.org/10.3389/fimmu.2020.559166
312. Hambleton, S., Salem, S., Bustamante, J., Bigley, V., Boisson-Dupuis, S., Azevedo, J., Fortin, A., Haniffa, M., Ceron-Gutierrez, L., Bacon, C. M., Menon, G., Trouillet, C., McDonald, D., Carey, P., Ginhoux, F., Alsina, L.,

Zumwalt, T. J., Kong, X.-F., Kumararatne, D., … Gros, P. (2011). IRF8 mutations and human dendritic-cell immunodeficiency. *The New England Journal of Medicine*, *365*(2), 127–138. https://doi.org/10.1056/NEJMoa1100066

313. Collin, M., & Bigley, V. (2018). Human dendritic cell subsets: an update. *Immunology*, *154*(1), 3–20. https://doi.org/10.1111/imm.12888

314. Perrin, S. (2014). Preclinical research: Make mouse studies work. *Nature*, *507*(7493), 423–425. https://doi.org/10.1038/507423a

315. Brunetti, J. E., Kitsera, M., Muñoz-Fontela, C., & Rodríguez, E. (2023). Use of Hu-PBL Mice to Study Pathogenesis of Human-Restricted Viruses. *Viruses*, *15*(1). https://doi.org/10.3390/v15010228

316. Schoenmaker, L., Witzigmann, D., Kulkarni, J. A., Verbeke, R., Kersten, G., Jiskoot, W., & Crommelin, D. J. A. (2021). mRNA-lipid nanoparticle COVID-19 vaccines: Structure and stability. *International Journal of Pharmaceutics*, *601*, 120586. https://doi.org/https://doi.org/10.1016/j.ijpharm.2021.120586

317. Corthésy, B., & Bioley, G. (2018). Lipid-Based Particles: Versatile Delivery Systems for Mucosal Vaccination against Infection. *Frontiers in Immunology*, *9*, 431. https://doi.org/10.3389/fimmu.2018.00431

318. Techawiwattanaboon, T., Courant, T., Brunner, L., Sathean-Anan-Kun, S., Krangvichian, P., Iadsee, N., Nakornpakdee, Y., Sangjun, N., Komanee, P., Collin, N., Ruxrungtham, K., & Patarakul, K. (2022). Designing Adjuvant Formulations to Promote Immunogenicity and Protective Efficacy of Leptospira Immunoglobulin-Like Protein A Subunit Vaccine. *Frontiers in*

Cellular and Infection Microbiology, *12*, 918629. https://doi.org/10.3389/fcimb.2022.918629

319. Sliepen, K., Schermer, E., Bontjer, I., Burger, J. A., Lévai, R. F., Mundsperger, P., Brouwer, P. J. M., Tolazzi, M., Farsang, A., Katinger, D., Moore, J. P., Scarlatti, G., Shattock, R. J., Sattentau, Q. J., & Sanders, R. W. (2021). Interplay of diverse adjuvants and nanoparticle presentation of native-like HIV-1 envelope trimers. *NPJ Vaccines*, *6*(1), 103. https://doi.org/10.1038/s41541-021-00364-x

320. Janitzek, C. M., Carlsen, P. H. R., Thrane, S., Khanna, V. M., Jakob, V., Barnier-Quer, C., Collin, N., Theander, T. G., Salanti, A., Nielsen, M. A., & Sander, A. F. (2021). The Immunogenicity of Capsid-Like Particle Vaccines in Combination with Different Adjuvants Using Different Routes of Administration. *Vaccines*, *9*(2). https://doi.org/10.3390/vaccines9020131

321. Brewer, J. M., Pollock, K. G. J., Tetley, L., & Russell, D. G. (2004). Vesicle size influences the trafficking, processing, and presentation of antigens in lipid vesicles. *Journal of Immunology (Baltimore, Md. : 1950)*, *173*(10), 6143–6150. https://doi.org/10.4049/jimmunol.173.10.6143

322. Manolova, V., Flace, A., Bauer, M., Schwarz, K., Saudan, P., & Bachmann, M. F. (2008). Nanoparticles target distinct dendritic cell populations according to their size. *European Journal of Immunology*, *38*(5), 1404–1413. https://doi.org/https://doi.org/10.1002/eji.200737984

323. Carstens, M. G., Camps, M. G. M., Henriksen-Lacey, M., Franken, K., Ottenhoff, T. H. M., Perrie, Y., Bouwstra, J. A., Ossendorp, F., & Jiskoot, W.

(2011). Effect of vesicle size on tissue localization and immunogenicity of liposomal DNA vaccines. *Vaccine*, *29*(29), 4761–4770. https://doi.org/https://doi.org/10.1016/j.vaccine.2011.04.081

324. Schultze, V., D'Agosto, V., Wack, A., Novicki, D., Zorn, J., & Hennig, R. (2008). Safety of MF59^{TM} adjuvant. *Vaccine*, *26*(26), 3209–3222. https://doi.org/https://doi.org/10.1016/j.vaccine.2008.03.093

325. Perrie, Y., Crofts, F., Devitt, A., Griffiths, H. R., Kastner, E., & Nadella, V. (2016). Designing liposomal adjuvants for the next generation of vaccines. *Advanced Drug Delivery Reviews*, *99*(Pt A), 85–96. https://doi.org/10.1016/j.addr.2015.11.005

326. Medzhitov, R. (2003). Charles A. Janeway, Jr. 1943-2003. *Nature Immunology*, *4*(6), 502. https://doi.org/10.1038/ni0603-502

327. Vlatkovic, I. (2021). Non-Immunotherapy Application of LNP-mRNA: Maximizing Efficacy and Safety. *Biomedicines*, *9*(5). https://doi.org/10.3390/biomedicines9050530

328. Oladipo, E. K., Adeniyi, M. O., Ogunlowo, M. T., Irewolede, B. A., Adekanola, V. O., Oluseyi, G. S., Omilola, J. A., Udoh, A. F., Olufemi, S. E., Adediran, D. A., Olonade, A., Idowu, U. A., Kolawole, O. M., Oloke, J. K., & Onyeaka, H. (2022). Bioinformatics Designing and Molecular Modelling of a Universal mRNA Vaccine for SARS-CoV-2 Infection. *Vaccines*, *10*(12). https://doi.org/10.3390/vaccines10122107

329. Shahsavandi, S., & Hariri, A. A. (2023). SARS-CoV-2 Variant-Specific mRNA Vaccine: Pros and Cons. *Viral Immunology*.

https://doi.org/10.1089/vim.2022.0121

330. Damjanovska, S., Smith, C., Sayin, I., Burant, C. J., Gravenstein, S., & Canaday, D. H. (2021). Adjuvant effect of type I interferon induced by many but not all commercial influenza vaccines. *Vaccine*, *39*(5), 786–789. https://doi.org/10.1016/j.vaccine.2020.12.051

331. Cioncada, R., Maddaluno, M., Vo, H. T. M., Woodruff, M., Tavarini, S., Sammicheli, C., Tortoli, M., Pezzicoli, A., De Gregorio, E., Carroll, M. C., D'Oro, U., & Piccioli, D. (2017). Vaccine adjuvant MF59 promotes the intranodal differentiation of antigen-loaded and activated monocyte-derived dendritic cells. *PLoS ONE*, *12*(10), 1–19. https://doi.org/10.1371/journal.pone.0185843

332. Crotzer, V. L., & Blum, J. S. (2009). Autophagy and its role in MHC-mediated antigen presentation. *Journal of Immunology (Baltimore, Md. : 1950)*, *182*(6), 3335–3341. https://doi.org/10.4049/jimmunol.0803458

333. Tang, W., Xie, H., Ye, Z., Eick-Cost, A. A., Scheckelhoff, M., Gustin, C. E., Bream, J. H., & Plant, E. P. (2023). Post-vaccination serum cytokines levels correlate with breakthrough influenza infections. *Scientific Reports*, *13*(1), 1174. https://doi.org/10.1038/s41598-023-28295-8

334. Wright, P. F., Hoen, A. G., Ilyushina, N. A., Brown, E. P., Ackerman, M. E., Wieland-Alter, W., Connor, R. I., Jegaskanda, S., Rosenberg-Hasson, Y., Haynes, B. C., Luke, C. J., Subbarao, K., & Treanor, J. J. (2016). Correlates of Immunity to Influenza as Determined by Challenge of Children with Live, Attenuated Influenza Vaccine. *Open Forum Infectious Diseases*, *3*(2),

ofw108. https://doi.org/10.1093/ofid/ofw108

335. Olotu, A., Moris, P., Mwacharo, J., Vekemans, J., Kimani, D., Janssens, M., Kai, O., Jongert, E., Lievens, M., Leach, A., Villafana, T., Savarese, B., Marsh, K., Cohen, J., & Bejon, P. (2011). Circumsporozoite-specific T cell responses in children vaccinated with RTS,S/AS01E and protection against P falciparum clinical malaria. *PloS One*, *6*(10), e25786. https://doi.org/10.1371/journal.pone.0025786

336. Severa, M., Rizzo, F., Sinigaglia, A., Ricci, D., Etna, M. P., Cola, G., Landi, D., Buscarinu, M. C., Valdarchi, C., Ristori, G., Riccetti, S., Piubelli, C., Palmerini, P., Rosato, A., Gobbi, F., Balducci, S., Marfia, G. A., Salvetti, M., Barzon, L., & Coccia, E. M. (2023). A specific anti-COVID-19 BNT162b2 vaccine-induced early innate immune signature positively correlates with the humoral protective response in healthy and multiple sclerosis vaccine recipients. *Clinical & Translational Immunology*, *12*(3), e1434. https://doi.org/10.1002/cti2.1434

337. Howard, L. M., Hoek, K. L., Goll, J. B., Samir, P., Galassie, A., Allos, T. M., Niu, X., Gordy, L. E., Creech, C. B., Prasad, N., Jensen, T. L., Hill, H., Levy, S. E., Joyce, S., Link, A. J., & Edwards, K. M. (2017). Cell-Based Systems Biology Analysis of Human AS03-Adjuvanted H5N1 Avian Influenza Vaccine Responses: A Phase I Randomized Controlled Trial. *PloS One*, *12*(1), e0167488. https://doi.org/10.1371/journal.pone.0167488

338. Maringer, Y., Nelde, A., Schroeder, S. M., Schuhmacher, J., Hörber, S., Peter, A., Karbach, J., Jäger, E., & Walz, J. S. (2022). Durable spike-specific

T cell responses after different COVID-19 vaccination regimens are not further enhanced by booster vaccination. *Science Immunology*, *7*(78), 1–13. https://doi.org/10.1126/SCIIMMUNOL.ADD3899