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Immunity to Influenza Infection in Humans

David J. Topham,¹ Marta L. DeDiego,² Aitor Nogales,³ Mark Y. Sangster,¹ and Andrea Sant¹

¹David H. Smith Center for Vaccine Biology and Immunology, Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, New York 14642, USA

²Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, 28049 Madrid, Spain

³Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, 28040 Madrid, Spain

Correspondence: david_topham@urmc.rochester.edu

This review discusses the human immune responses to influenza infection with some insights from studies using animal models, such as experimental infection of mice. Recent technological advances in the study of human immune responses have greatly added to our knowledge of the infection and immune responses, and therefore much of the focus is on recent studies that have moved the field forward. We consider the complexity of the adaptive response generated by many sequential encounters through infection and vaccination.

Despite the widespread and readily available seasonal influenza vaccination in the United States and other countries, influenza continues to cause annual epidemics. In the United States alone, influenza viruses are estimated to infect between 9.2 million and 35.6 million people, resulting in 140,000–710,000 hospitalizations and 12,000–56,000 deaths (see cdc.gov/flu/about/burden/index.html). Currently, there are four strains of the seasonal virus that widely circulate, two are of the influenza A (IAV) lineage, and two are of the influenza B lineage (see cdc.gov/flu/about/burden/index.html). Human seasonal IAVs are comprised of two antigenically distinct variants as characterized by their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), with H1N1 and H3N2 IAVs in circulation. Influenza B viruses fall into two lineages, influenza B/Yamagata and B/Victoria. H3N2 viruses are the most antigenically variable and frequent drift variants emerge

(Both et al. 1983; DeDiego et al. 2016a; Allen and Ross 2018). H1N1 is relatively more stable antigenically, although drift still occurs (Carrat and Flahault 2007; Clark et al. 2017a; Suptawiwat et al. 2018). Influenza B viruses have been the most antigenically stable, although the reasons for these differences in antigenic stability among influenza A and B viruses are not known. There is an influenza C virus strain that can infect humans, but it is rare and infection does not result in any significant disease (see cdc.gov/flu/about/burden/index.html).

Currently, licensed vaccines against influenza carry either three (H1N1, H3N2, and B) or four (H1N1, H3N2, and two Bs) strains of the virus (see cdc.gov/flu/about/burden/index.html). Even in areas with widespread vaccination, infections continue to occur as a result of poor antigenic match to the circulating strains (particularly for H3N2), as well as poor responses to vaccination (DeDiego et al. 2016a).

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People most at risk for severe disease and complications from influenza infection tend to be the very young (<6 yr of age [yoa]), older adults (>65 yoa), and those with underlying health conditions such as diabetes, cardiovascular disease, and pregnancy (Abelleira et al. 2019; Kytömaa et al. 2019; Mertz et al. 2019).

Immunity to influenza is comprised of both the innate and adaptive components. Humans typically acquire adaptive immunity to influenza in the first years of life (birth to 10 yoa) consisting of antiviral antibodies, memory B cells (MBCs), and memory CD4⁺ and CD8⁺ T cells. Innate immune responses are the first line of defense during infection and consist of types I and III interferon (IFN) production by infected cells and immune cells such as macrophages and specialized dendritic cells (DCs) (Fonteneau et al. 2003; Lui et al. 2009). The interferons induce a paracrine antiviral response, making cells more resistant to infection (Ramos et al. 2019), as well as working in concert with inflammatory chemokines that recruit innate immune cells such as neutrophils to the site of infection (Sprenger et al. 1996; Ichikawa et al. 2013; Lim et al. 2015). These innate immune responses can restrain virus replication until effector T cells and antibodies reach the tissue. Infections can last as long as 10–21 d in young naive subjects, and typically 5–7 d in those with preexisting immunity (Little et al. 1994; Ivan et al. 2013). Human influenza viruses have strong countermeasures to the antiviral effects of the innate immune response, which vary in effectiveness and affect viral pathogenesis and transmission (DeDiego et al. 2016b; Nogales et al. 2017a, 2018b).

HOST INNATE IMMUNE RESPONSES AFTER IAV INFECTION

Host Innate Immune Responses and IAV Pathogenesis

Innate immune responses lead to the expression of interferon-stimulated genes (ISGs) and inflammatory cytokines, which restrict virus replication. However, an excessive production of these proteins, mainly inflammatory cytokines, is associated with severe pathogenesis or mortality.

An exacerbated proinflammatory response can lead to acute respiratory distress syndrome (ARDS), which is the main cause of death in IAV-infected patients (Mauad et al. 2010; To et al. 2010). Most of the patients infected with the 2009 H1N1 pandemic virus experienced a mild disease, which usually resolved in few weeks. However, severe disease occurred in some patients. The levels of interleukin (IL)-1 receptor antagonist protein (IL1RA), IL-6, tumor necrosis factor (TNF), IL-8, monocyte chemoattractant protein 1 (MCP-1)/CCL2, macrophage inflammatory protein 1B (MIP-1β)/CCL4, and IFN-γ-inducible protein 10 (IP-10)/CXCL10 and apoptosis were up-regulated in the lungs from these fatal cases (Gao et al. 2013). Similarly, significantly higher levels of granulocyte colony-stimulating factor (G-CSF), IL-1, IL-6, IL-10, IL-15, MCP-1, and TNF were measured in serum of fatal cases (To et al. 2010). IFN-γ, IL-8, IL-9, IL-17, IL-6, TNF-α, IL-15, and IL-12p70 responses were found in hospitalized patients versus those with mild disease (Bermejo-Martin et al. 2009). Severe disease in subjects infected with the H5N1 virus was also correlated with the levels of IL-8, IP-10, and monokine induced by IFN-γ (MIG)/CXCL9 and MCP-1 (chemoattractants of monocytes and macrophages [de Jong et al. 2006]). H7N9 IAV infection is associated with early high levels of IL-6, IL-8, and MIP-1β in serum (Wang et al. 2014) and is correlated with pharyngeal virus load (Shen et al. 2014). Similarly, the high mortality rate observed in young adults during the 1918 pandemic has been attributed to the induction of a dysregulated pro-inflammatory response based upon experimental studies in various naive animal models using the reconstructed 1918 influenza virus (Kash et al. 2006; Kobasa et al. 2007; de Wit et al. 2018).

IAV NS1 Protein

The NS1 protein is translated from the mRNA synthesized from IAV segment 8 (Lamb and Lai 1980). This protein is most often a 230-amino acid protein (Marc 2014). However, mutations that either suppress the stop codon at position 231 or create a premature stop codon result

in length variations (Marc 2014). For instance, from the late 1940s until the middle of the 1980s, NS1 of human H1N1 IAV encoded at the carboxyl terminus, a 7-amino acid extension, being this protein 237 amino acids in length (Lohrmann et al. 2013; Marc 2014). In contrast, the NS1 protein of the 2009 pH1N1, like that of most swine H1N1 IAV, has only 219 amino acids (Lohrmann et al. 2013; Marc 2014; Chauché et al. 2018).

The major function of NS1 is to antagonize host innate immune responses during infection, and this occurs at multiple stages of IFN production and signaling cascades. IAVs lacking or expressing truncated forms of NS1 (García-Sastre et al. 1998; Talon et al. 2000b), expressing reduced levels of NS1 (Nogales et al. 2014), or encoding amino acid mutations affecting NS1 functions (Nogales et al. 2014, 2017b; DeDiego et al. 2016a) are severely impaired in cells competent in the production of type I IFN, whereas they show similar levels of replication compared with viruses with wild-type NS1 in type I IFN-deficient cells (e.g., Vero cells) (García-Sastre et al. 1998).

NS1 inhibits RIG-I-mediated signaling and subsequent induction of type I IFNs. Specifically, the NS1 protein inhibits retinoic acid-inducible gene I (RIG-I) activation by sequestration of this RNA helicase and its activating ligand (Mibayashi et al. 2007; Opitz et al. 2007). Furthermore, the NS1 protein inhibits RIG-I ubiquitination mediated by TRIM25 and riplet, which is crucial for maximal type I and III IFNs' expression during viral infection (Gack et al. 2009; Rajsbaum et al. 2012; Koliopoulos et al. 2018). NS1 also interferes with functions of important intracellular antiviral ISGs, including protein kinase R (PKR) and oligoadenylate synthase (OAS). PKR is activated by double-strand RNA (dsRNA) or by the cellular protein activator of the interferon-induced PKR (PACT), resulting in autophosphorylation and phosphorylation of cellular proteins, including the α subunit of the eukaryotic initiation factor 2 (eIF2 α) (Patel and Sen 1998). This phosphorylation leads to inhibition of protein synthesis, including viral proteins, in infected cells. The RNA-binding domain of NS1 can

bind to viral RNA and avoid detection by PKR (Lu et al. 1995). It also binds to PKR itself via residues 123–127 and 35 and 46 and inhibits PKR-mediated viral mRNA suppression and apoptosis (Bergmann et al. 2000; Li et al. 2006; Min et al. 2007; Schierhorn et al. 2017). Activation of OAS by dsRNA produces poly(A) chains with 2'-5' phosphodiester bonds (Clemens and Williams 1978), which bind to and activate constitutively expressed ribonuclease L (RNaseL), leading to the cleavage of viral and cellular single-stranded RNA (ssRNA), resulting in inhibition of virus replication (Silverman and Weiss 2014). OAS can also be blocked by influenza NS1. The RNA-binding domain of NS1 can out-compete the RNA binding capacity of OAS, thereby inhibiting the antiviral response (Min and Krug 2006).

NS1 blocks the inhibitor of kappa β kinase (IKK) subunit β (IKK- β), inhibiting the activation of the nuclear factor (NF)- κ B pathway, and preventing the expression of antiviral genes (Wang et al. 2000; Gao et al. 2012). In addition, NS1 inhibits the IRF-3 transcription factor (Talon et al. 2000a) and Jun amino-terminal kinase (JNK), a kinase that phosphorylates transcription factors of the AP-1 family, increasing their activity (Ludwig et al. 2002). In addition, NS1 activates the phosphatidylinositol-3-kinase (PI3K) pathway by direct interaction with the p85 β subunit (Hale et al. 2006), causing the phosphorylation of a downstream mediator of PI3K signal transduction, Akt. The NS1-PI3K interaction increases the rate of viral internalization, inhibits apoptosis (Ehrhardt et al. 2007) and enhances type I IFN and proinflammatory production by enhancing the activity of IRF3 (Hrincius et al. 2011; Lu et al. 2011).

IAV NS1 interacts with NLRP3, inhibiting the NLRP3 inflammasome activation, and leading to impaired IL-1 β and IL-18 responses (Cheong et al. 2015; Moriyama et al. 2016; Park et al. 2018). In addition, NS1 inhibits host gene expression, including innate immune response genes, through different mechanisms. NS1 inhibits host pre-mRNA endonucleolytic cleavage and polyadenylation by direct interaction and inhibition of cleavage and polyadenylation specificity factor-30 subunit (CPSF30)

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and the recruitment of the poly(A) polymerase to add the poly(A) tail (Nemeroff et al. 1998; Chen et al. 1999; Noah et al. 2003). Additionally, the NS1 protein can bind cellular double-stranded DNA (dsDNA), preventing loading of the transcriptional machinery to the host DNA, and thus inhibiting the expression of antiviral genes (Anastasina et al. 2016). Additionally, the NS1 of IAV binds many components of the mRNA export machinery: nuclear RNA export factor 1 (NXF1), p15, ribonucleic acid export 1 (RAE1), and adenovirus early region 1B-associated protein 5 (E1B-AP5), which interacts with both mRNAs and nucleoporins to direct mRNAs through the nuclear pore complex, blocking their function (Satterly et al. 2007). In addition to modulating innate immunity, the NS1 protein attenuates human DCs maturation and the capacity of DCs to induce T-cell responses (Fernandez-Sesma et al. 2006).

IAV PA-X Protein

IAV segment 3 encodes the PA and the PA-X proteins (Jagger et al. 2012). PA-X is translated as a +1 frameshift open reading frame (ORF), from the PA viral segment (Jagger et al. 2012), resulting in a protein with the amino-terminal 191 amino acid originating from PA and a carboxy-terminal region of 61 or 41 codons encoded by an overlapping ORF (X-ORF), which is accessed by one ribosomal frame shift in the PA gene (Shi et al. 2012).

PA-X induces the shutoff of host protein expression in infected cells, contributing to the blocking of cellular antiviral responses (Jagger et al. 2012; Hayashi et al. 2015; Hu et al. 2015, 2018; Khaperskyy and McCormick 2015; Khaperskyy et al. 2016; Levene and Gaglia 2018). PA-X selectively degrades host RNA polymerase II (Pol II)-transcribed mRNAs and noncoding RNAs in the nucleus of infected cells, thereby ensuring successful viral replication and counteracting the antiviral response in the host (Khaperskyy et al. 2016; Levene and Gaglia 2018). Complete degradation of host mRNAs following PA-X-mediated endonucleolytic cleavage is dependent on the host 5'→3'-exonuclease Xrn1 (Khaperskyy et al. 2016).

The PA-X-mediated shutoff activity involves the amino-terminal endonucleolytic domain (region shared with the PA protein) (Yuan et al. 2009), resulting in the degradation of host mRNAs. Accordingly, mutations in the endonuclease active site decreases the protein's ability to induce host cellular shutoff (Hara et al. 2006; Oishi et al. 2018). Two independent studies showed that the initial 15 amino acids (positions 192–206) in the PA-X carboxy-terminal region are sufficient for the full shutoff activity of PA-X (Oishi et al. 2015; Hayashi et al. 2016). In addition, additional studies reported on the contribution of the last 20 carboxy-terminal residues in PA-X-mediated shutoff activity. Amino acids at positions 233–252 of the PA-X carboxyl terminus also strongly suppress gene expression and enhance viral replication and virulence in human pandemic H1N1 2009, avian H5N1, and avian H9N2 (Gao et al. 2015a). Simultaneously, Bavagnoli et al. (2015) also provided direct evidence that the last 20 amino acids in the PA-X carboxy-terminal region are important for endonuclease activity, and this was assumed to contribute to host shutoff by the virus. The loss of PA-X expression can increase or diminish viral replication and virulence, depending on the viral strain (Jagger et al. 2012; Gao et al. 2015b, 2015c; Hayashi et al. 2015; Hu et al. 2016; Lee et al. 2017).

IAV NS1 and PA-X Protein Evolution

These preceding studies suggest that the effect of PA-X on virus replication and pathogenesis may be host- and strain-specific, likely reflecting adaptation to a given host. IAV NS1 and PA-X proteins have the synergistic ability to inhibit host gene expression, although through different mechanisms. The NS1 protein of the highly pathogenic H5N1 influenza virus isolated from humans in 1997 did not bind CPSF30 in vitro and caused high β interferon mRNA production and reduced virus replication. In contrast, the NS1 proteins of H5N1 viruses isolated from humans after 1998 bind CPSF30 in vitro and do not attenuate virus replication (Twu et al. 2007).

Similarly, the NS1 protein from pH1N1 2009 isolated from humans in 2009, early after

the pandemic began, did not bind CPSF30 and did not inhibit general gene expression (Hale et al. 2010; Clark et al. 2017b). Interestingly, the NS1 protein of currently circulating pH1N1 IAV have gained the ability to inhibit host gene expression (Clark et al. 2017b), suggesting again that inhibiting host gene expression is beneficial for the virus. Recombinant influenza pH1N1 viruses encoding NS1 and PA-X proteins that alternate in the ability inhibit host gene expression are impaired in viral growth in culture and are attenuated in vivo, compared with viruses in which only one of the viral proteins (NS1 or PA-X) inhibited host gene expression (Nogales et al. 2017c). Currently circulating viruses show increased ability to inhibit host gene expression mediated by the NS1 protein (Nogales et al. 2018a) and multiple amino acid changes in the PA-X protein that decreased PA-X-mediated inhibition of host gene expression (Nogales et al. 2018a). Balance in NS1 and PA-X activities is important for viral fitness, not only in cultured cells but also in vivo (Nogales et al. 2017c, 2018a).

IAV-PB1-F2

PB1-F2 is an accessory protein that is translated from an alternative +1 ORF of PB1 (Chen et al. 2001). PB1-F2 promotes cellular apoptosis through a mitochondrial pathway (Zamarin et al. 2005). By binding to the inner and outer mitochondrial membrane transport protein adenine nucleotide translocator 3 (ANT3) and voltage-dependent anion channel 1 (VDAC1), PB1-F2 disrupts mitochondrial integrity, releasing cytochrome *c*, and leading to apoptosis (Zamarin et al. 2005). Furthermore, PB1-F2 completely translocates into the mitochondrial inner membrane space via Tom40 channels, and its accumulation accelerates mitochondrial fragmentation caused by reduced membrane potential ($\Delta\psi_m$) (Yoshizumi et al. 2014). PB1-F2 variants lacking a carboxy-terminal polypeptide, often found in low pathogenic subtypes, do not affect mitochondrial function. PB1-F2-mediated attenuation of $\Delta\psi_m$ suppresses the RIG-I signaling pathway and activation of NLRP3 inflammasomes (Yoshizumi et al. 2014). PB1-F2

has also been shown to inhibit the RIG-I-TRIM25-mediated antiviral signaling pathway by direct interaction with the mitochondrial antiviral-signaling protein (MAVS) (Varga et al. 2011, 2012). PB1-F2 promotes viral pathogenicity especially with respect to highly pathogenic viruses like the 1918 H1N1 and H5N1 strains (Conenello et al. 2007; Le Goffic et al. 2011) and replication (Mazur et al. 2008).

Different mechanisms may account for the effect of PB1-F2 on promoting virus pathogenesis. Recently, it has been shown that the PB1-F2 protein of IAV 1918 pandemic virus also acquired a novel IFN antagonist function by targeting the DEAD-box helicase DDX3, a key downstream mediator in antiviral interferon signaling, toward proteasome-dependent degradation (Park et al. 2019), providing an explanation for the role of PB1-F2 in increasing virus pathogenesis. Additionally, PB1-F2 contributes to increased susceptibility to bacterial superinfection (McAuley et al. 2007). Also, PB1-F2-mediated IAV pathogenicity could result from its interaction with NLRP3, leading to inflammasome activation and increased production of cytokine IL-1 β (Zamarin et al. 2005; McAuley et al. 2013). PB1-F2 has also been shown to increase mitochondrial reactive oxygen species (ROS) and calcium (Ca^{2+}) efflux, which contributed to the activation of NLRP3 inflammasome (Pinar et al. 2017).

IAV PB1-F2 Evolution

A specific set of amino acid residues present in the “inflammatory motif” present within the PB1-F2 carboxyl terminus of pandemic 1968 (H3N2) IAV increases inflammation and promotes secondary bacterial pneumonia in mice (Alymova et al. 2011). Furthermore, studies indicate that enhancement of inflammation by this motif might involve activation of the NLRP3 inflammasome (McAuley et al. 2013; Pinar et al. 2017). Other carboxy-terminal PB1-F2 residues present in the “cytotoxic motif” promote development of secondary bacterial pneumonia (Alymova et al. 2014). These virulent “inflammatory and cytotoxic” motifs have been present in natural isolates from human sea-

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sonal IAV of all subtypes, but there has been a trend toward a gradual reduction in the number of virulent residues over time (Alymova et al. 2018), suggesting an evolution in human hosts.

IAV PB2

The polymerase basic protein 2 (PB2), a subunit of the viral RNA polymerase complex, interacts with MAVS, and inhibits MAVS-mediated IFN- β expression (Graef et al. 2010; Iwai et al. 2010), likely because of the mitochondrial localization of certain PB2 proteins (Graef et al. 2010). The PB2 proteins of seasonal human influenza viruses localize to the mitochondria, in contrast to the PB2 proteins of avian influenza viruses. Using two recombinant human influenza viruses encoding PB2 proteins that do or do not localize to mitochondria, it was found that the virus encoding the nonmitochondrial PB2 protein induces higher levels of IFN- β and, in an animal model, is attenuated compared with the virus encoding a mitochondrial PB2 (Graef et al. 2010). A mutation in the PB2 (T588I), found in swine pH1N1 viruses, but rarely detected in human pH1N1 isolates, exacerbated PB2 inhibition of MAVS-mediated IFN- β expression, and increased the binding of PB2 to MAVS. The T588I variant induced lower levels of host IFN- β expression than the WT strain during infection (Zhao et al. 2014).

THE ROLE OF CD4 T CELLS IN PROTECTIVE IMMUNITY TO INFLUENZA

CD4 T cells contribute importantly and in diverse ways to protective immunity to influenza. The most well-known and documented function of CD4 T cells is provision of “help” for neutralizing antibody production by B cells. CD4 T cells also play key functions that are distinct from delivery of help to influenza-specific B cells. First, during influenza infection, CD4 T cells are critical for the protective immunity conveyed by cytotoxic CD8 T cells; the main effector T cells that eliminate infected host cells in the respiratory tract (Schmidt and Varga 2018; Topham and Reilly 2018). Influenza-specific CD4 T cells that are elicited by infection are

known to enhance the initial priming, expansion and establishment of long-lived memory CD8 T cells (Bevan 2004; Bedoui et al. 2016). A recent study (Ahrends et al. 2017) also provided strong evidence that CD4 T-cell help is critical for development of effector CD8 T cells that have cytotoxic potential, and for expression of cell surface molecules important for homing and extravasation, all features required for protective immunity delivered by influenza-specific CD8 T cells. At the site of infection, CD4 T cells also promote positioning of memory CD8 T cells within the infected respiratory tract (Laidlaw et al. 2014). In addition to enhancing adaptive immunity, a potentially critical function of influenza-specific CD4 T-cell memory is the ability to accelerate recruitment of innate effectors to the lung. The role of the early innate response to infection is well-documented (for review, see Iwasaki and Pillai 2014; Chen et al. 2018), and recent studies have shown influenza-specific CD4 T memory promotes this early response and blunts virus infection (Strutt et al. 2010; Teijaro et al. 2010).

CD4 T cells also provide direct effector functions in the respiratory tract. Local production of IFN- γ from CD4 T cells can provide direct antiviral activity in mice (Ito et al. 2011; McKinstry et al. 2012). Although influenza-specific human CD4 T cells are enriched in IFN- γ production (Richards et al. 2018) and thus can be considered “Th1-like,” in general, cytokine production in humans is highly complex (Roti et al. 2008; Purwar et al. 2011; Mosmann et al. 2014; Liechti and Roederer 2019). This complexity in humans may be caused by the multitude and diverse types of exposure of humans to influenza antigens via both infection and vaccination. Therefore, the contribution of individual CD4 T cell cytokines to antiviral effects in the respiratory tract in humans is not yet clear. Beyond cytokine production, an additional function of CD4 T cells that has been increasingly validated is cytotoxicity, which has the potential to directly eliminate influenza-infected cells (for review, see Brown et al. 2016; Juno et al. 2017; Takeuchi and Saito 2017), discussed in detail below. A final activity of influenza-specific CD4 T cells is regulatory and repair function.

These activities are contributed by diverse functional subsets of CD4 T cells, including conventional Treg, as well as IL-10- or amphiregulin-producing cells, which can diminish the damage associated with the profound inflammatory responses (Brincks et al. 2013; Arpaia et al. 2015; Zaiss et al. 2015; Egarnes and Gosselin 2018) that occurs on infection with highly pathogenic strains of influenza (Pulendran and Madhur 2015; Betakova et al. 2017; Ong et al. 2017; Wong et al. 2018).

Follicular Helper Cells

Because of the importance of neutralizing antibodies, as well as other antibody-mediated effector mechanisms (de Jong et al. 2003; Treanor and Wright 2003; Rimmelzwaan and McElhane 2008; Neu et al. 2016; Boudreau and Alter 2019), there has been increasing emphasis on the subset of CD4 T cells termed T follicular helper cells (Tfh). This subset of CD4 T cells promotes the generation and maintenance of the germinal center (GC) reaction and the production of high affinity, class-switched antibody and B-cell memory. Tfh are characterized by the expression of the chemokine receptor CXCR5, which directs localization of the primed CD4 T cells to the B-cell zone of secondary lymphoid organs. Here, Tfh also express high levels of ICOS and PD-1 (Breitfeld et al. 2000; Schaerli et al. 2000; for reviews, see Crotty 2015; Hale and Ahmed 2015; Vinuesa et al. 2016). The transcriptional repressor Bcl-6 directs lineage commitment (Nurieva et al. 2009; Yu et al. 2009). Cytokines, including IL-6 and IL-21 (Nurieva et al. 2008), and T-cell receptor (TcR) signal strength (Fazilleau et al. 2009; Tubo et al. 2013; Keck et al. 2014; Krishnamoorthy et al. 2017) have all been shown to influence Tfh differentiation. Interestingly, beyond TcR ligand interactions and cytokine milieu, particular DC subsets may also be critical in priming of Tfh. Lung CD11b⁺ migratory dendritic cells (cDC2) have been implicated in priming following intranasal immunization (Krishnaswamy et al. 2017), and late appearing antigen presenting cells (APCs) have been identified in the Tfh response following influenza virus infection (Yoo et al. 2012). In

human circulation, CXCR5⁺ cells have lower expression of Bcl-6 as well ICOS and PD-1 (Chevalier et al. 2011; Locci et al. 2013; Heit et al. 2017). However, despite these phenotypic differences, circulating CXCR5⁺ CD4 T cells show a superior capacity to help B cells on activation (Chevalier et al. 2011; Morita et al. 2011; Locci et al. 2013). Circulating CXCR5⁺ cells can be further distinguished by expression of CCR6, CXCR3, ICOS, and PD-1, with helper activity concentrated within the CXCR3⁻ cells that are PD1⁺ and/or ICOS⁺ (Morita et al. 2011; Benteibibel et al. 2013; He et al. 2013; Locci et al. 2013; Herati et al. 2014).

There has been much recent progress in understanding the human Tfh response following influenza vaccination. Increases in Tfh-expressing activation markers (ICOS⁺ or ICOS⁺ PD1⁺) correlate with the magnitude (Benteibibel et al. 2013; He et al. 2013; Herati et al. 2014; Spensieri et al. 2016) and avidity (Benteibibel et al. 2016) of influenza-specific antibody. Circulating human Tfh that are evident after vaccination appear to be clonally related to those within the GC and transition to a more quiescent phenotype at memory (Heit et al. 2017; Herati et al. 2017). Recent data has shown a close correlation between the elicited antibody response to vaccination and the emergence of cells in peripheral blood with markers reminiscent of follicular helper CD4 T cells (for review, see Hale and Ahmed 2015; Linterman and Hill 2016; Ueno 2016), arguing that CD4 T-cell help for vaccine responses can be tracked soon after vaccination (Herati et al. 2014). It is much more difficult to track CD4 Tfh responses in humans after infection because, first, the kinetics of responses are typically heterogeneous after diagnosis of infection and, second, many of the responding CD4 T cells are recruited to the lung after infection, making sampling and quantification extremely difficult.

The antigen specificity in CD4 T-cell help is a critical parameter for antibody responses to both infection and vaccination. Recent studies in both animal models of infection (Nayak et al. 2013a; Alam et al. 2014, 2017) and human vaccination studies (Nayak et al. 2013b, 2015) suggest that the most effective help for antibody

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responses occurs when the antigen specificity of the CD4 T cells matches that of the B cell. This obligate linkage likely reflects that nature of the antigen taken up by the immunoglobulin receptor, and the resulting human leukocyte antigen (HLA) class II:peptide complexes that are displayed at the cell surface of the antigen-specific B cells that recruit CD4 T-cell help in secondary lymphoid tissue. This constraint likely limits the CD4 T-cell help available in humans for novel avian strains of influenza, although recent novel vaccine constructs have described strategies to overcome this limitation (Moise et al. 2018; DiPiazza et al. 2019).

Cytolytic CD4 T Cells

There has been increasing appreciation of a distinct subset of CD4 T cells with cytolytic function that may also contribute to protective immunity to influenza. In many respects, cytotoxic CD4 T cells resemble CD8 T cells in terms of key transcriptional regulators (Takeuchi et al. 2016) and efficiency of killing (Hildemann et al. 2013) and in primary cytotoxic mechanisms involving perforin and granzymes (Brown et al. 2016; Takeuchi and Saito 2017). Both IL-2 and inflammatory signals, abundant in responses to viral infections, are thought to be central elements that promote the cytolytic potential of CD4 T cells (Hua et al. 2013; Workman et al. 2014). Recent studies in human peripheral blood CD4 T cells have identified the transcription factor HOBIT (homolog of Blimp-1 in T cells) as a unique identifier of cells expressing granzymes, perforin and other markers linked to cytolytic function (Oja et al. 2017). Until recently, CD4 T cells with cytotoxic function have been quantified by expression of stored cytolytic mediators, such as granzyme, requiring that cells be permeabilized before analyses. However, recently, cell surface markers, such as CRTAM (class I-restricted T cell-associated molecule) (Takeuchi et al. 2016) and the natural killer cell marker NKG2C/E (Marshall et al. 2017) have been defined that have the potential to quantify and permit isolation of intact cells with cytotoxic potential. The role of cytolytic CD4 T cells in influenza immunity has been

supported by the prominence of this phenotype in the infected lung (Workman et al. 2014; Brown et al. 2016; Marshall et al. 2017) and has been found as correlate of protection in human challenge studies (Wilkinson et al. 2012).

One intriguing unresolved question is whether cytolytic CD4 T cells perform a unique function that is distinct from cytotoxic CD8 T cells. Because of their similarity in function and development, why does the host need the apparent redundant mechanism for cell-mediated cytotoxicity? Two possible non-mutually exclusive possibilities can be envisioned. Cytotoxic CD4 T cells may serve as a complementary mechanism, eliminating infected cells at selected sites within the lung, perhaps controlled by a unique array of lung positioning molecules (Richter et al. 2007; Takamura 2017). Recent studies have revealed the diversity of infected and antigen-bearing cells detectable in the lung early after infection (DiPiazza et al. 2017) that may be located at distinct sites in the respiratory tract. In addition, studies of lung tissue by microscopy have indicated that CD4 and CD8 T cells localize to different subregions in the lung (for review, see Szabo et al. 2019). Alternatively or additionally, CD4 T cells may serve as a fail-safe mechanism when CD8 T cell epitopes are lost from circulating influenza strains (Voeten et al. 2000), which may be less likely to occur in influenza-specific CD4 T-cell repertoire because of their broader epitope specificity (for review, see Sant et al. 2018a).

Abundance and Specificity of Human Influenza-Specific CD4 T Cells

Recent efforts have sought to quantify and characterize the repertoire of circulating influenza-specific CD4 T cells from human subjects (Assarsson et al. 2008; Babon et al. 2009; Richards et al. 2010, 2015; Wilkinson et al. 2012; Chen et al. 2014; Savic et al. 2016; Uchtenhagen et al. 2016). In general, these studies have found that in most healthy adults, there are detectable influenza-specific CD4 T cells, but that the abundance is highly variable (Assarsson et al. 2008; Wilkinson et al. 2012; Hayward et al. 2015; Richards et al. 2015; Savic et al. 2016).

With the increased interest in the design of universal influenza vaccines (Krammer and Palese 2015; Paules et al. 2017), there has been particular interest in candidate epitopes that would elicit broadly cross-reactive CD4 T cells that detect genetically conserved peptide epitopes reactive shared among diverse viral strains (Eliasson et al. 2018; Lee et al. 2018; Tutykhina et al. 2018). In healthy adults, there is prominent CD4 T-cell reactivity toward epitopes from the internal virion M1, NP (Wilkinson et al. 2012; Chen et al. 2014; Hayward et al. 2015; Savic et al. 2016), and polymerase (Assarsson et al. 2008; Richards et al. 2010) proteins. HA-reactive CD4 T cells have also been reported to be abundant in the memory compartment of many individuals (Babon et al. 2009; Richards et al. 2010, 2015; Hayward et al. 2015; Leddin et al. 2015), and these may be particularly important for provision of help for neutralizing antibody responses (Sant and McMichael 2012; Nayak et al. 2013b, 2015; Alam et al. 2014, 2017). In humans, reactivity to HA is enriched for specificities in the more highly conserved HA2 domain (Richards et al. 2015), allowing candidate epitopes in this region to be used in universal vaccine efforts. CD4 T cells specific for M1, NP, polymerase proteins, and highly conserved regions of HA are likely to be the major specificities elicited in response to infection with heterologous or novel potentially pandemic strains (Roti et al. 2008; Babon et al. 2009; Richards et al. 2009; Duvvuri et al. 2010; Alam and Sant 2011; Weinfurter et al. 2011; Bethell et al. 2013; Duvvuri et al. 2013) and may contribute to attenuating the course of infection. The degree of cross reactivity in CD4 T cells elicited in response to the novel 2009 potentially pandemic strain is likely to have contributed to the mild course of disease observed in many subjects, despite a lack of cross-reactive neutralizing antibody.

A Major Challenge: Identification of the CD4 T-Cell Subset(s) That Are a Limiting Factor for Human Protective Immunity to Influenza Virus

It is clear that the presence of high-affinity neutralizing antibody to influenza is the best correlate of protection from natural infection.

However, the ability of influenza virus to mutate and to deviate from serum-mediated protection and the resulting high burden of seasonal influenza infection indicates that current strategies to induce sterilizing immunity are inadequate. Thus, there is a need to enhance protective immunity by more effectively engaging the cellular arm of the immune response. Despite this need, a significant challenge is defining which CD4 T cells are deficient and need boosting by vaccination. In studies of human immunity to influenza, one of the most difficult issues is identification of cells that will participate in the response to infection or vaccination. For example, the vast majority of studies that have identified correlates of influenza-specific CD4 T cells with the elicited antibody responses have performed so only through examination of cells that have increased their representation after vaccination (Bentebibel et al. 2013; Nayak et al. 2013b, 2015; Spensieri et al. 2013, 2016; Herati et al. 2014; Koutsakos et al. 2018). Very few correlates have been identified within the circulating CD4 T-cell repertoire before vaccination or infection. One human influenza challenge study did correlate levels of preexisting cytotoxic CD4 T cells with protection, but this subset was identified after *in vitro* expansion (Wilkinson et al. 2012). More sensitive detection of additional markers such as chemokine receptors expressed by influenza-specific CD4 T cells, coupled with early sampling times (e.g., 1–3 d postvaccination or -infection) and human challenge studies (Lillie et al. 2012; Oxford 2013; Darton et al. 2015; Pleguezuelos et al. 2015) would likely provide insight into the circulating memory CD4 T-cell subsets that are recruited into the response and whose presence in peripheral blood of humans either positively or negatively correlates with protective immunity to influenza. As knowledge is gained in these issues, we will have more of the needed insight into the most critical deficiencies in the influenza-specific CD4 T-cell repertoire and, with this, the needed framework for improved vaccine strategies. Studies in both animal models and more recent studies in humans have provided evidence that influenza-specific T cells can establish long-term tissue resident memory (Zens et al. 2016, 2017; Oja

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et al. 2018; Chu et al. 2019; Snyder et al. 2019) that may provide protective immunity very early after infection.

In considering what subsets of CD4 T cells are most critical to protection from influenza in humans, it is important to understand that many of the in vivo studies that have implicated particular effector functions of CD4 T cells to protection from influenza challenge have used animal models. In these studies, the contribution of CD4 T cells to immunity has been shown either using adoptive transfer strategies to introduce a CD4 T cell with dominant function into a naive host or where functionality of the CD4 T cells can be eliminated or promoted by genetic means (Teijaro et al. 2011; Brown et al. 2012; McKinstry et al. 2012). Extrapolating these studies to humans is difficult because of the tremendously complex preexisting immunity to influenza that accumulates in humans over a lifetime of exposure via infection and vaccination. To be able to understand the contribution of CD4 T cells in humans toward protective immunity or more robust responses to vaccination, it is essential to quantify the functional activities of CD4 T cells that are limiting or that constitute bottlenecks in protective immunity to influenza: Do most people need more cytotoxic CD4 T cells or more tissue resident memory? Is CD4 T-cell help for rapid antibody responses to infection limiting for most people? Do only a small number of influenza-specific CD4 T cells in humans have the capacity to be recruited to the lung early after infection and quickly mobilize the innate response? Does initial contact of the human host to influenza via peripheral vaccination rather than infection or intranasal vaccination diminish establishment of memory CD4 T cells that can be recalled into the lung after infection or that will establish long-term tissue resident memory (Zens et al. 2016; Calzas and Chevalier 2019; Jansen et al. 2019; Liu et al. 2019). Addressing these issues in humans is difficult because of the challenges associated with sampling of the respiratory tract after infection, but these are critically important issues to gain better insight into. A more comprehensive understanding of these issues will help direct the priorities in design and composition of more

effective vaccines that most effectively establish protective immunity.

From the studies of human responses to vaccination and animal models of infection, it is clear that CD4 T cells of the correct functional subset (Bentebibel et al. 2013, 2016; He et al. 2013; Spensieri et al. 2016) and specificity (Nayak et al. 2013a,b; Alam et al. 2014, 2017; He et al. 2017) can be a limiting factor in eliciting neutralizing antibody. Evidence from animal models has supported the possibility that more abundant CD4 T cells of the correct specificity can enhance antibody production during the course of infection (Alam et al. 2014). Such early antibody responses, even of nonneutralizing HA or NA specificities, could blunt the course of infection, through cellular and Fc-receptor-based mechanisms (Wohlbold et al. 2015, 2017; DiLillo et al. 2016; Tan et al. 2016; He et al. 2017; Sicca et al. 2018; Boudreau and Alter 2019). Vaccine strategies that populate the host with CD4 T cells that are poised to become Tfh cells of the desired protein specificity, matching the B-cell specificity, may thus lead to antibody responses that can lead to either sterilizing immunity and/or attenuated disease. Greater peripheral Tfh following adjuvanted vaccination (Mastelic Gavillet et al. 2015; Harandi 2018), use of high-dose influenza vaccination in the elderly (Pilkinton et al. 2017), and strategies that target protein vaccines to cell surface proteins such as Clec9a (Kato et al. 2015) or particular DC subsets (Yamasaki et al. 2016) suggest that it may be possible to use alternative vaccination strategies to optimize Tfh priming, which may foster stable Tfh memory that can promote future protective antibody responses.

The possibility of enhancing protection from influenza infection through amplification of T-cell responses has been supported by recent vaccine and challenge studies in humans (Lillie et al. 2012; Pleguezuelos et al. 2015). Use of virus vectors to expand T cells specific for M1 and NP, without elicitation of antibodies, was associated with a lower infection frequency, virus shedding, and diminished symptomology when a challenge virus was administered (Lillie et al. 2012). Consistent findings were observed when multi-epitope vaccines were similarly tested

(Pleguezuelos et al. 2015). It is likely that these types of experimental challenge models (Oxford 2013; Darton et al. 2015), coupled with strategies to selectively promote expansion of CD4 T cells with the desired functionality and specificity, could provide critical new insight into the most limiting protective functions of CD4 T cells.

Collectively, recent advances have provided much of the insight needed to initiate cross protective immunity at the systemic level, as well as the site of influenza infection. The control of CD4 T-cell fate decisions that regulate generation and expansion of discrete functional subsets of CD4 T cells that can contribute to protective immunity to influenza in humans are complex and multilayered. The major challenge will be to design the appropriate vaccination and challenge studies to reveal and ultimately correct the most critical deficits in CD4 T-cell functions in human populations. The roles of specific viral antigens as targets of vaccination, different innate activators, and antigen persistence are increasingly better understood (Bautista et al. 2016; Zens et al. 2016; Allie and Randall 2017; Nish et al. 2017; Strutt et al. 2017), which can also better inform vaccine designs that populate the host with the most broadly protective CD4 T cells.

B-CELL RESPONSE TO SEASONAL INFLUENZA INFECTION

The B-cell response to IAV infection has recently been comprehensively reviewed (Krammer 2019) and is also discussed extensively in this volume by Wilson and colleagues (Wilson et al. 2019). Here, we emphasize the role of MBCs in the B-cell response to seasonal IAV infection in influenza-immune adults (Sangster et al. 2019). All proteins expressed by an infecting IAV can potentially activate B cells and generate specific antibodies (Abs) and MBCs, but the response is typically strongest against the more abundant structural proteins. Circulating Abs generated by IAV infection can be maintained for long periods. Long-term maintenance of circulating virus-specific IgG levels primarily reflects seeding of the bone marrow by activated

precursor cells that give rise to long-lived plasma cell populations (Halliley et al. 2015; Lau et al. 2017). Infection also generates IgA antibody-secreting cells (ASCs) that migrate to the submucosa of the respiratory tract and maintain a protective IgA barrier at mucosal surfaces (Kato et al. 2013). Abs against the virus's surface glycoproteins, the HA and NA, have direct antiviral activity and provide the most effective protection against the initiation or progression of infection. Most Abs generated by infection are likely to target the viral HA, the viral attachment protein that initiates cell infection by binding to sialylated receptors.

Molecular features of the HA are important determinants of the fine specificity and function of anti-HA Abs. The HA consists of two structurally distinct domains: an antigenically variable membrane-distal head domain containing the receptor binding site and a relatively conserved membrane-proximal stalk domain (Wu and Wilson 2018). The head domain is immunodominant over the stalk domain and is the target of the vast bulk of HA-reactive Abs produced by IAV infection (Zost et al. 2019). Abs against the HA head that block binding of virus to host cells have the most potent virus neutralizing activity; Abs against the stalk protect via other mechanisms and are less potently neutralizing, but are more broadly reactive across HA variants and subtypes (Krammer and Palese 2013). Modification of antigenic sites in the head domain by antigenic drift generates variant influenza strains that are less well controlled by previously formed anti-HA Abs. Periodically, HA variant viruses emerge and cause influenza outbreaks in communities in which diminished protection by preexisting Abs is widespread (Petrova and Russell 2018).

Abs against the HA are also the basis of "original antigenic sin" (OAS) as originally termed (Francis 1960) and more recently designated "antigenic seniority" (Lessler et al. 2012). Based on Ab titers measured by hemagglutination inhibition assay, influenza infection of adults typically generates an Ab response that is broadly cross-reactive. Frequently, this response is of the OAS type and characterized by preferential boosting of Abs against sets of HAs

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related to those of viruses that circulated early in an individual's life (Fonville et al. 2014; Tesini et al. 2019). The term OAS has also been applied to circulating HA-reactive Ab levels that are highest against "older" HAs, a pattern that is largely maintained by OAS responses to IAV infection. It is postulated that OAS reflects a pattern of expansion of HA-reactive MBCs "imprinted" by early-life HA exposure, probably in the form of significant IAV infection (Cobey and Hensley 2017; Henry et al. 2018).

If infection is not blocked or quickly terminated by preexisting Abs, perhaps because of waning levels or the emergence of an "escape" variant, the activation of MBCs results in rapid and vigorous secondary Ab production that acts in concert with other forms of adaptive responses to clear infectious virus (Inoue et al. 2018). Typically, MBCs are the isotype-switched products of GC reactions in which they have undergone somatic hypermutation and selection for expression of B-cell receptors (BCRs) with high affinity for the activating antigen. After formation, MBCs generally disperse to lymphoid tissues throughout the body. However, MBCs generated by IAV infection are likely to maintain highest frequencies at sites of formation in the respiratory tract (Pichyangkul et al. 2015; Jegaskanda et al. 2018; Koutsakos et al. 2018). MBCs are more readily activated than naive B cells because of cell-intrinsic factors that include epigenetic modifications, altered transcriptional networks, and greater signaling capacity of the IgG BCR (Good-Jacobson 2018). Furthermore, MBCs localize around the periphery of lymphoid tissues and are better positioned to capture antigen draining from a site of infection than are B-cell follicle-associated naive B cells (Aiba et al. 2010; Moran et al. 2018; Zhao et al. 2018). Antigen uptake is the first step in establishing cognate interactions with CD4 T cells that are essential for MBC (and naive B cell) activation (Cyster and Allen 2019). In many cases, it is likely that the strong Ab response generated by MBC activation after infection is sufficient to terminate infection with little, if any, contribution by naive B cells.

Evidence that early production of antiviral Abs following IAV infection in immune adults

results from activation of preexisting MBCs comes from analysis of Ab-secreting plasmablasts (PBs) that appear in the circulation at about the same time as the first virus-induced Abs. These PBs generally reach peak frequencies 4–6 d post-symptom onset, but remain detectable for another 1–2 wk (Tesini et al. 2019). Notably, up to 50% of virus-specific PBs bind the viral HA. Analysis of HA-reactive PBs has identified recently proliferated clonal lineages that express Abs with highly mutated immunoglobulin variable genes, indicating derivation from recently activated MBCs that had previously undergone affinity maturation (Wrammert et al. 2011).

In responding lymph nodes, MBC activation that produces the first Abs (and circulating PBs) following IAV infection occurs extrafollicularly in the outer lymph node cortex (Moran et al. 2018). It is unclear whether MBCs are also generated via this extra-GC pathway or whether MBCs are only generated in GCs after some level of remodeling of antigen reactivity. Notably, the recent analysis by Tesini et al. (2019) showed a close relationship between patterns of early Ab production after IAV infection measured against a range of HA variants (including OAS patterns) and patterns of early expansion of MBC populations reactive to the same set of HAs. This result is consistent with Ab production and MBC formation stemming from activation of the same precursor MBC. B-cell clonal lineage analysis by Ellebedy et al. (2016) also indicated that PB and MBC lineages could have originated from the same activated MBC, but does not exclude a contribution of GCs to at least part of the MBC lineage.

It is generally accepted that OAS patterns of HA-reactive Ab production after IAV infection reflect the competitive dominance of MBCs reactive to conserved epitopes on the HA head domain (Cobey and Hensley 2017; Henry et al. 2018). The reactivity profiles of Abs expressed by individual PBs generated early in the response to IAV infection have not been extensively characterized. However, limited studies indicate that at least some of the PB-expressed Abs bind to one or more older HAs, sometimes with higher affinity than to the infecting virus

HA (Moody et al. 2011; Wrammert et al. 2011). These findings are consistent with PB derivation from MBCs that were generated by exposure to older HAs and respond to sufficiently conserved epitopes on the HA of the infecting virus. High-affinity MBCs would be expected to outcompete lower affinity MBCs for available antigen and thus obtain the strong level of T-cell help associated with differentiation of activated B cells into Ab-secreting PBs. However, the importance of MBC affinity might be diminished in the context of IAV infection, which is associated with increased levels of cytokines and TLR agonists that help drive B-cell activation (Lam and Baumgarth 2019).

In addition to the broad HA head-reactive Ab response, ~10%–20% of anti-HA Abs generated by seasonal IAV infection bind to the stalk domain. The response to the stalk follows the same kinetics as the antihead response and includes early induction of stalk-specific PBs with high numbers of mutations in immunoglobulin variable genes, indicating formation from activated MBCs (Wrammert et al. 2011; Margine et al. 2013; Tesini et al. 2019). A key determinant of the magnitude of the Ab response to the stalk is the dominance of head-reactive over stalk-reactive MBCs in competition for antigen. An individual's exposure to HA through vaccination and infection would be expected to progressively increase the ratio of head- versus stalk-reactive MBCs, primarily because of immunodominance of the head. Notably, few stalk-reactive Abs are produced after seasonal inactivated influenza vaccination (Moody et al. 2011; Wrammert et al. 2011), suggesting that factors associated with IAV infection facilitate stalk-reactive MBC activation.

A subset of B cells activated by IAV infection enter GC reactions in which they undergo affinity maturation (Mesin et al. 2016). A proportion of the preexisting MBCs activated by infection become GC B cells as an alternative to extra-GC differentiation into PBs. Activated naive B cells might also enter GCs, especially when infection is severe and the response is prolonged (Wrammert et al. 2011). Experiments in mice indicate that GCs initially generate mostly MBCs and then switch after 2 or more weeks to plasma

cell formation (Weisel et al. 2016). MBC formation after fewer rounds of selection would limit the extent of affinity maturation or “adaptation” to the HA of the infecting virus, but might provide the advantage of expanding MBCs able to respond to the infecting virus HA as well as to emerging HA variants. There is nevertheless a degree of affinity maturation of de novo-generated MBCs. Tesini et al. (2019) analyzed responses to seasonal H3N2 infection in the 2012–2013 season when infecting viruses were HA drift variants (DeDiego et al. 2016a). Circulating MBCs adapted to the H3 head domain were detected within ~4 wk of symptom onset. Evidence that HA-reactive MBCs with increased levels of somatic hypermutation are formed many months after HA vaccination in humans indicates that affinity maturation of MBCs continues long after any early switch by GCs to predominantly plasma cell production (Andrews et al. 2019; Matsuda et al. 2019).

In summary, a critical determinant of the B-cell response to seasonal IAV infection in immune adults is the responsive MBC pool generated by infection and vaccination over many years. The composition of an individual's HA-reactive MBC pool in particular reflects the imprint of early-life HA exposure and is responsible for OAS patterns of Ab production. Early PB formation from activated high-affinity MBCs optimizes the protective efficacy of the initial wave of Abs. Competition for antigen between MBCs is a key determinant of the specificities of MBCs that receive activation and differentiation signals. The outcome of MBC competition depends on the numbers and affinities of specific MBCs, immunodominance features of particular IAV proteins, and the forms of antigens available for recognition by B cells. Intramolecular competition between HA-reactive MBCs is the basis for OAS response patterns, as well as the strength of responses to the HA head versus the stalk. Intermolecular competition, as might occur when proteins are present on intact virions or envelope fragments, could determine the strength of early Ab responses to the HA versus the NA. Activated preexisting MBCs form not only PBs, but also generate cells that, along with activated naive B cells, seed GCs

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for generation of MBCs and long-lived plasma cells adapted to variant IAV proteins. As part of the B-cell response, MBCs that maintain OAS responses are replenished, perhaps via both extra-GC and GC pathways. Our increasing understanding of B-cell response processes continue to guide development of vaccine formulations and administration strategies that optimize protective efficacy.

CD8⁺ T-CELL RESPONSES TO SEASONAL INFLUENZA INFECTION

The human immune system is exposed to influenza in most people during their childhood. Until recently, fewer children were vaccinated, suggesting these early exposures are most likely to be from infections. The field has also recently realized that these early life exposures result in lifelong “imprinting” of the immune system, particularly in regard to antibody and memory B-cell specificities (for review, see Guthmiller and Wilson 2018; Lewnard and Cobey 2018; Francis et al. 2019; Sangster et al. 2019). The early infections also generate long-lived memory T cells, although whether a pattern of antigenic imprinting is created has not yet been shown.

In recent decades, much insight has been gained on the elicitation of CD8 T cells in the response to influenza, most of which is derived from animal models of infection. The CD8 T-cell response to influenza begins ~2–3 d after a primary infection, when virus antigen-bearing, activated DCs arrive in the lymph nodes draining the site(s) of infection, which are typically the nasopharynx, trachea, and upper airways in the case of seasonal influenza viruses (Legge and Braciale 2003; Belz et al. 2004; Yoon et al. 2007). Specialized APCs that carry influenza antigens from the lung to the draining lymph nodes are derived from CD103⁺ DCs that patrol the respiratory epithelium (Ho et al. 2011; Beauchamp et al. 2012). Naive CD8 T cells specific for peptides derived from viral proteins encounter these professional APC or that access viral antigens via cross presentation (den Haan and Bevan 2001; Di Pucchio et al. 2008). After processing the protein antigens of the virus into short peptides, APCs display these peptides on their sur-

face in the context of self-derived class I histocompatibility leukocyte antigens (HLA). Naive T cells preferentially migrate through secondary lymphoid organs (SLOs) such as lymph node and spleen where they encounter antigen-bearing DCs. Naive T cells are efficient in trafficking to SLOs by expressing adhesion molecules (L-selectin/CD62L) and chemokine receptors (CCR7) responsive to ligands expressed on high endothelial venules (HEVs) supplying blood to SLOs (Sallusto et al. 1999; Geginat et al. 2001). During the 1–3 d within the draining lymph node, the CD8 T cells receive signals through their TCR and costimulatory molecules, CD28 on the T cells, and CD80 and CD86 expressed by the activated DC. Additional stimulatory signals include cytokines like IL2, released in autocrine and paracrine fashions, the latter likely from activated virus-specific CD4⁺ T cells interacting with the same or adjacent DC. These signals drive proliferation and differentiation of the naive CD8⁺ T cells into activated effector T cells and memory cells. It is worth noting that the lymph nodes may not be the only SLOs in which effector T cells are generated. Mathematical modeling suggested the lymph nodes cannot numerically account for the number of effector T cells found in the lung during experimental infection of mice with influenza (Wu et al. 2011). Later experimental confirmation showed that the spleen is a major source of effector CD8⁺ T cells specific for influenza (Turner et al. 2013), although this may depend on the virus strain (Keating et al. 2018). Splenic priming may occur because activated DC from the site(s) of infection enter circulation and migrate to the spleen or because some of the abundant viral antigens in the lung drains to the spleen. Within the SLOs, the elicited CD8 T cells down-regulate SLO homing receptors and begin expressing molecules that further antagonize retention in the SLOs. Instead, they acquire expression of adhesion molecules like CD44 and increased LFA-1 and CD49d integrins before leaving the SLOs and entering the bloodstream (Siegelman et al. 2000). LFA-1 and CD49d recognize coreceptors up-regulated by the innate response on blood vessels within inflamed tissues such as infected mucosal surfaces (Siegel-

man et al. 2000). These interactions along with chemokine signals on the endothelium cause the T cells to arrest and undergo transendothelial migration into the infected tissue. CD8 T cells migrate through the space between the blood vessel and airway epithelium and cross the basement membrane of the epithelium to reach the infected epithelial cells (Lambert Emo et al. 2016). Mouse studies suggest the CD8 T cells must also have an encounter with APC within the tissue to be retained and exert effector functions (McGill et al. 2008).

Role of CD8 T Cells in Protection

CD8⁺ T cells recognize peptides 9–10 amino acids in length (called an “epitope”) presented in the context of HLA class I molecules on the surface of APCs and infected cells (Krangel et al. 1982; Parham 1988). Many of the dominant epitopes identified thus far come from viral proteins that are relatively conserved, such as the internal proteins. CD8 T cells can even recognize epitopes derived from different strains of influenza within the same lineage (e.g., influenza A H1N1 and H3N2), but also across lineages (influenza A, B, or C) (Koutsakos et al. 2019), although these highly cross-lineage-reactive cells are rare. The genetic conservation of CD8 T-cell peptide epitopes makes this subset of T cells attractive candidates for targets of universal flu vaccines that provide broad cross-reactive immunity, also termed heterologous immunity (Bennink et al. 1978). Unlike neutralizing antibodies, this type of immunity does not prevent infection but serves to reduce the severity, magnitude, and duration of the infection (Bennink et al. 1978; Ray et al. 2004). Two studies of natural infection or deliberate challenge of humans with influenza came to different conclusions regarding the type of T cells that reduce disease severity. In the study of natural infection with the 2009 H1N1 virus, subjects with higher frequencies of CD8⁺ CD45RA⁺ CCR7[−] effector memory T cells making IFN- γ and having cytotoxic potential associated with reduced disease (Sridhar et al. 2013). These subjects were seronegative before infection and there was no correlation with CD4 T-cell responses. In a model

of human influenza challenge using either H1N1 or H3N2 viruses, the investigators found an inverse correlation between disease severity and CD4⁺ memory T-cell frequencies before infection (Wilkinson et al. 2012). The reasons for the discrepancy in the conclusions are not clear, but they were very different study designs. In a much earlier classical study performed by McMichael et al. (1983), the frequencies of preexisting cytotoxic cells correlated with reduced disease. Wang and colleagues (2015) found that H7N9 avian influenza-infected subjects who recovered early from infection had higher frequencies of virus-specific CD8⁺ T cells that produced IFN- γ relative to those individuals with longer duration illness or death.

Recent evidence suggests that local T-cell immunity in the respiratory tract may be a particularly effective form of preexisting T cell in the host (Cauley 2016; Takamura 2018; Topham and Reilly 2018). Memory CD8⁺ T cells that reside in the airway tissues for long periods are called tissue resident memory (Ray et al. 2004) or T_{RM} cells. T_{RM} cells express adhesion molecules that allow them to interact with the extracellular matrix (CD49a/ α -1 integrin) and epithelial cells directly (CD103/ α -e) and are found in both mice and humans (Piet et al. 2011; Purwar et al. 2011). They can be found in all tissues, but preferentially within barrier tissues such as the respiratory tract, digestive tract, and reproductive tract and skin (Kumar et al. 2017; Rosato et al. 2017). T_{RM} cells are believed to perform constant surveillance and are unusually effective at providing rapid protection presumably because they are already located at the site of infection (Cauley 2016). They act immediately upon encounter with infected cells during the first few days of the secondary infection, before reactivated memory T cells from SLO reach the tissue, which can take 3–5 d (Woodland et al. 2002; Swain et al. 2004). In a mouse study, the secondary challenge of influenza infection primed mice that lacked lung T_{RM} cells because of antibody blocking or genetic deletion of a key integrin (CD49a/ α -1) that reduced T_{RM} cells led to increased mortality and increased disease (Ray et al. 2004).

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Specificity of CD8 T Cells

Studies of CD8⁺ T-cell antigen specificity in adult humans have shown broad reactivity across the influenza proteome, with a high degree of variability in both frequency and specificity across all HLA types (Rimmelzwaan et al. 2009; Quinones-Parra et al. 2014; Grant et al. 2016; Li et al. 2019). In a study looking for cross reactive CD8 T cells against an H5N1 virus, with cohorts in both Asia and Europe, the influenza proteins with the most cross-reactivity were the matrix protein (MP), nucleoprotein (NP), and polymerase basic subunit 1 (PB1) of the virus, although T cells specific for epitopes from all virus proteins were detected (Lee et al. 2008). The reasons for the relative focus on MP, PB1, and NP are not clear, though it may be the conservation, relative abundance of these proteins after infection, or preferential processing in the APC. There is even evidence of cross-recognition of relatively conserved NP peptides (NP388-346 and NP44-52 restricted by HLA B27 and A*01:01, respectively) even though there are point mutations in those epitopes in some viruses (Grant et al. 2018). The same group identified conserved epitopes across influenza A, B, and C viruses, potentially providing strategies for developing universal vaccines (Koutsakos et al. 2019). It is important to define shared epitope specificities to successfully design vaccines that promote cross-reactive immunity. Repeated exposures to influenza during one's lifetime may reinforce particular specificities for more conserved epitopes (Van Braeckel-Budimir et al. 2017, 2018).

Effector Mechanisms of CD8 T Cells

In contrast to CD4 T cells, CD8⁺ T cells memory subsets are far less diverse. T cytotoxic 1 (Tc1) and T cytotoxic 2 (Tc2) cells defined by the ability to make IFN- γ or IL4, respectively, have been described. Tc2 cells are rare and have been most frequently studied and observed in experimental mice, in a cancer setting or in vitro (Dobrzanski et al. 2004). By far, human CD8⁺ T cells specific for influenza are most commonly cytotoxic, and make IL2, IFN- γ , and/or TNF- α

in different combinations (La Gruta and Turner 2014). Studies in mice have indicated that cytotoxicity is due to the targeted secretion of perforin and granzymes to infected cells, although less well-targeted cell death pathways such as Fas/Fas ligand (Topham et al. 1997) and TRAIL (Brincks et al. 2008) have been implicated in augmenting cytotoxicity. Studies involving HIV or CMV (Betts et al. 2006; Kannanganat et al. 2007) infected subjects suggested the cells that produce multiple cytokines (IL2, IFN- γ , and TNF- α) are the most potent effectors, correlating with decreased viral load. Whether the same is true in influenza is not well-established.

Age

Age has a strong influence on T-cell responses to influenza. Of course, during infancy, influenza infection is confronted by a naive immune system with no preexisting T-cell or B-cell adaptive immunity to the virus. However, recent work has shown that infants possess a unique non-conventional population of CD8⁺ T cells at birth that express CD31 and secrete IL8 upon TCR and CD28 costimulation (Scheible et al. 2015, 2018). Furthermore, the frequencies of these CD31⁺ CD8⁺ naive T cells at discharge from the hospital positively correlate with reduced frequency of respiratory virus infections, and reduced severity when infected, during the first year of life (Scheible et al. 2018). This was true for all the respiratory pathogens tested. The mechanism of protection in the respiratory tract is not known, but unlike T cells from older children and adults, these T cells respond to Toll-like receptor ligands (Kollmann et al. 2009) and may have dual lymphatic and mucosal homing capacity. They express CD62L and the α -4/ β -7 heterodimer that in older children and adults direct activated and memory T cells to mucosal sites, and the gut in particular. Interestingly, the neonatal lung expresses the coreceptor mucosal addressin cell adhesion molecule (MAdCAM) (Salmi et al. 2001). This homing molecule may convey the potential to migrate directly into the airway tissues, although this remains to be confirmed. The CD31⁺ CD8⁺ T cells disappear over the first year of life, and MAdCAM expression in

the lung is also reduced (Scheible et al. 2018). Together, these suggest there is a mechanism to provide protective immunity to respiratory pathogens during infancy, giving time for adaptive immune responses to develop.

The other end of the spectrum, in old age, also affects CD8⁺ T-cell responses to influenza. The elderly are generally considered to be less immunologically competent in many aspects of their immune compartments (Gustafson et al. 2018; Lorenzo et al. 2018; Crooke et al. 2019). This feature of aging is most apparent in the B-cell and immunoglobulin responses to vaccination (Henry et al. 2019). Part of the deficiency is reduced diversity in the B-cell repertoire, as well as lower degrees of affinity maturation, which together affect the ability to respond to new or drifted antigens. The CD8⁺ T-cell repertoire is also known to become less diverse and more restricted with age. For example, in an analysis of TCR repertoire diversity against an immunodominant M1 peptide antigen comparing young (>18 yoa) and elderly (>61 yoa) adults, the young adults had more TCRs with public TCRs (those shared among different individuals) and fewer private TCRs (those unique to an individual). Elderly adults had low percentages of public TCRs and higher proportions of expanded private clonal populations (Sant et al. 2018b). Other features of TCRs from older individuals included relatively long complementarity determining 3 regions (CDR3), with increased G/A runs (Gil et al. 2015). The investigators suggest these features convey molecular flexibility in recognizing different peptides, theoretically making the T-cell recognition of antigens more degenerate (Gil et al. 2015). Greater TCR diversity was observed in elderly subjects with severe H7N9 infection, suggesting the capacity to recruit and expand a more diverse repertoire from low frequency T-cell clones (Sant et al. 2018b). Various publications suggest increasing defects in CD8⁺ T-cell effector functions with age (McElhaney et al. 2016; Goronzy and Weyand 2017). However, an exhaustive study of CD8⁺ T-cell responses to respiratory syncytial virus (RSV) comparing younger and older adults in their ability to produce multiple effector cytokines, there were no numerical or effector func-

tion differences detected (Roumanes et al. 2018). Despite reductions in receptor diversity, CD8⁺ T cells from older subjects nevertheless appear to retain their ability to respond to their cognate antigens. The reduced diversity may affect responses to newly emerged viruses, but the degree of conservation among the internal proteins should preserve heterologous immunity.

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