RESPIRATORY VIRUSES PROMOTE GLYCOLYSIS: THE CRITICAL ROLE OF METABOLISM IN HUMAN PLASMACYTOID DENDRITIC CELL ANTIVIRAL RESPONSES

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I am submitting herewith a dissertation written by Gagan Bajwa entitled "Respiratory Viruses Promote Glycolysis: The Critical Role of Metabolism in Human Plasmacytoid Dendritic Cell Antiviral Responses". I have examined this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Molecular Biology.

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ABSTRACT

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RESPIRATORY VIRUSES PROMOTE GLYCOLYSIS: THE CRITICAL ROLE OF METABOLISM IN HUMAN PLASMACYTOID DENDRITIC CELL ANTIVIRAL RESPONSES

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Respiratory viruses, including influenza and human rhinoviruses (RV), pose a major threat to global public health. Plasmacytoid dendritic cells (pDCs) are key players in directing immune responses to viral pathogens via secretion of interferon-α (IFN-α) and by presenting antigen to naïve T cells. Recent evidence indicates that many immune cell functions are coupled and dependent on cellular metabolism, but no information about metabolism in pDCs is available. Metabolism is known to regulate Toll-like receptor (TLR)-induced DC activation in mice, but data in humans is lacking. Here we studied the effects of influenza and RV on human pDC metabolic pathways and key pDC functions: IFN-α production and pDC maturation. Purified pDCs from healthy donor blood samples were exposed *ex vivo* to media controls, influenza, RV or gardiquimod (a TLR7 agonist). IFN-α concentrations and pDC maturation status was determined by ELISA and flow cytometry respectively. The ATP level was measured using an ATP luciferase assay. Microarray analysis was performed to determine differentially regulated metabolic genes, followed by confirmation using qRT-PCR. Extracellular acidification rate (ECAR, an indicator of glycolysis) and lactate efflux were measured using the Seahorse extracellular

flux analyzer and mass spectrometry. We demonstrated that influenza, RV and gardiquimod induced glycolysis in human pDCs. Higher ATP content, up-regulation of glycolytic genes (hexokinase 2, *HK2* and Lactate dehydrogenase A, *LDHA*), higher ECAR and elevated glucose-derived lactate production characterized this induction. Inhibition of glycolysis by 2-deoxyglucose (2-DG) significantly impaired key pDC functions including viral-induced pDC IFN-α production and phenotypic maturation. The findings that live attenuated influenza virus vaccination promoted higher glycolytic rates in circulating pDCs highlighted the significance of glycolysis in pDC responses to *in vivo* viral infections. Influenza exposure increased pDC hypoxia inducible factor-1α (HIF-1α) expression and promoted its nuclear translocation, indicating that this induction is likely mediated by HIF-1 activation. In conclusion, our data emphasize an important role of glycolysis in pDC antiviral responses. Targeting metabolic pathways may provide potential therapeutic strategies for modulating pDC antiviral and vaccine responses to clinically relevant viral pathogens.

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LIST OF ABBREVIATIONS

Ab – Antibody

ANOVA – Analysis of variance

AP-1 – Activator protein 1

APC – Antigen presenting cells

APC – Allophycocyanin

ATP – Adenosine triphosphate

CD – Cluster of differentiation

DAPI – 4',6'-diamidino-2-phenylindole

DAVID – Database for Annotation, Visualization and Integrated Discovery

EBV – Epstein-barr virus

ECAR – Extracellular acidification rate

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

FCS – Fetal calf serum

FITC – Fluorescein isothiocyanate

Flu – Influenza virus

g – g-force or Relative centrifugal force

h – Hour

HA – Hemagglutinin

HCMV – Human cytomegalovirus

HCV – Hepatitis C virus

HIF – Hypoxia inducible factor

HK – Hexokinase

HPV – Human papillomaviruses

HSV – Herpes simplex virus

IFN – Interferon

IFNAR – IFN-α receptor

Ig – Immunoglobulin

IL – Interleukin

IRF7 – Interferon regulatory factor 7

KSHV – Kaposi's sarcoma herpesvirus

LAIV – Live attenuated influenza vaccine

LDH – Lactate dehydrogenase

MAMPs – Microbe associated molecular patterns

mDC – Myeloid dendritic cell

MESF – Molecules of Equivalent Soluble Fluorochrome

MFI – Mean fluorescence intensities

MHC – Major histocompatibility complex

ml – Milliliter

MOI – Multiplicity of infection (infectious units per cell)

MyD88 – Myeloid differentiating factor 88

NA – Neuraminidase

NF-κB – Nuclear factor κB

^{0}C – Degrees Celsius

OCR – Oxygen consumption rate

OXPHOS – Oxidative phosphorylation

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate buffer saline

pDC – Plasmacytoid dendritic cell

PE - Phycoerythrin

PFK – Phosphofructose kinase

PFU – Plaque forming unit

PGAM – Phosphoglycerate mutase

PI3K – Phosphatidylinositol 3-kinase

PRR – Pattern-recognition receptors

RNA – Ribonucleic acid

rpm – Revolutions per minute

RPMI – Roswell Park Memorial Institute 1640 media

RSV – Respiratory syncytial virus

RT-PCR – Real time polymerase chain reaction

RV – Human rhinovirus

STAT – Signal transducer and activator of transcription

Th1 – Type 1 T helper cell

Th2 – Type 2 T helper cell

TLR – Toll-like receptor

TNF – Tumor necrosis factor

μg – Micrograms

μl – Microliter

μM – Micromolar

VEGF – Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Respiratory Virus Infections

Respiratory tract infections impose a great disease burden on public health, causing an estimated 4 million deaths worldwide per year (1-3). About half of these respiratory infections are caused by viruses, and efforts to reduce mortality associated with these infections are challenging due to limited antiviral treatments and availability of effective vaccines (4, 5). Influenza and rhinoviruses are two of the most common viruses contributing to this burden of respiratory viral diseases (6, 7). Influenza viruses – segmented, negative-sense single stranded RNA (ssRNA) viruses in the family *Orthomyxoviridae*, are an important cause of morbidity and mortality worldwide. Each year, more than 35,000 deaths and 200,000 hospitalizations associated with influenza infections are reported in the United States alone (8-10). Influenza viruses are classified into type A, B and C, but the majority of human influenza diseases are caused by types A and B. Influenza viruses are divided into subtypes based on the antigenic nature of two surface glycoproteins, hemagglutinin (H or HA) and neuraminidase (N or NA). Influenza A subtypes include 17 HA and 10 NA, and different strains affect not only humans but also pigs, birds, horses, seals and whales (11). Influenza B has a more restricted host range and is divided into only 2 lineages (Yamagata 88-like and Victoria 87-like) (12, 13). Influenza C viruses are known to cause mild illness in humans. Due to the higher

rates of antigenic mutation and wider host range leading to antigenic shifts (a dramatic shift in the antigenicity of a virus resulting from recombination of the genes from mixing segments from different strains), type A is the most virulent of all and is responsible for seasonal epidemics and potentially devastating pandemics (7-10, 14). In humans, influenza virus infects and replicates in alveolar epithelium cells and macrophages (15-17). In a typical infection, influenza virus enters the host cell by binding of viral HA molecules to sialic acid residues on the host cell surface and the infectious virus begins to be released within 12 hours after virus entry. Seasonal influenza vaccination (intramuscular trivalent inactivated influenza vaccine and intranasal live attenuated influenza vaccine) is available and is the primary strategy to prevent influenza infection (18, 19).

Human rhinoviruses (RV) are positive-sense non-segmented ssRNA viruses and belong to the family of *Picornaviridae.* Human RVs are classified into type A, B and C based on the phylogenetic sequence analysis and susceptibility to antiviral agents (20). Because RV genome consists of one segment of ssRNA, antigenic shift does not occur in RV. Instead, RV undergoes antigenic drift due to the error-prone RNA polymerase, resulting in generation of over 100 strains of circulating virus (21). Rhinoviruses (RV) are recognized as frequent contributors to respiratory viral disease in both children and adults. While some studies attribute up to 50% of all cold and flu-like illnesses to RV alone (6, 22), others highlight more severe complications of RV infection including, sinusitis, pneumonia and exacerbations of pulmonary diseases including asthma, chronic

obstructive pulmonary disease (COPD) and cystic fibrosis (6, 23-27). The majority of RV-A and RV-B (about 90%) gains entry into the host cell by binding to intracellular adhesion molecule-1 (ICAM-1) that includes RV-16, while the remaining minor group (about 10%) binds low-density lipoprotein receptor (LDLR), very-LDLR and LDLR-related protein 1 for cell entry (28-30). Some RVs in the majority group (RV-54, RV-8) also bind heparan sulfate proteoglycans (HSPG) in addition to ICAM-1 (31, 32). However, the receptor for RV-C is unknown (33). RV replicate efficiently in the upper and lower epithelium of airway lumen (24, 29). Due to the existence of more than 100 serotypes with the high level of sequence variability at the antigenic sites, the efforts to develop vaccines for RV remains challenging (6).

Innate and adaptive immune responses are essential in controlling respiratory viral infections (34, 35). Dendritic cells (DCs) play an important role in initiating and regulating immune responses to pathogens. DCs are uniquely equipped i) to sense invading pathogens, ii) to capture and process antigens and iii) to prime T cells to initiate adaptive immune responses (36-38). DCs are thus crucial in linking innate and adaptive immune responses (39, 40).

Plasmacytoid Dendritic Cells (pDCs) Development, Localization and Migration

In humans and mice, DCs comprise a heterogeneous population of cells that has been categorized into various subsets based on development, surface receptor expression and function (41). The development of DC subsets is controlled by transcription factors and cytokines (39). The various subsets of DCs include conventional/myeloid DCs

(mDCs), plasmacytoid DCs (pDCs), langerhans DC, dermal DCs and monocyte-derived DCs (moDCs). Myeloid DCs are specialized for antigen processing and presentation, exhibit high expression of MHC class II molecules, and are located in the lymphoid and peripheral tissues. Langerhans and dermal DCs are located in the epidermal and dermal layer of skin respectively and migrate to the skin-draining lymphoid tissues after antigen exposure. Monocyte-derived DCs are circulating monocytes that differentiate into DCs at the site of inflammation (39, 42). Plasmacytoid dendritic cell (pDCs) represent a unique subset of DCs with plasma cell-like morphology and comprise about 0.1% - 0.8% of peripheral blood mononuclear cells in both mice and humans (43). These cells exhibit unique surface receptor phenotype and are characterized as $CD4^{+}DR^{+}Lin^{-}$(CD3, CD14, CD16, CD19 and CD56) $CD11c^{-}CD123^{high+}$ (43). The developmental pathway of pDCs still remains illusive. Earlier data suggested that, in humans, pDCs were derived from lymphoid progenitors of hematopoietic stem cells (44, 45), but recent studies have demonstrated that pDCs can be efficiently produced from myeloid as well as lymphoid progenitors (46). The development and differentiation of pDCs from hematopoietic stem cells is dependent on fms-like kinase 3 ligand (Flt-3L), STAT3, IRF8 and transcription factor, E2-2 (47, 48). In both mice and humans, E2-2 specifically regulates differentiation of pDCs. E2-2 binds to the promoter of several pDC selective genes involved in pDC development and function such as *BDCA2*, *LILRA4, SPIB, IRF8* and *IRF7*. In mice, deletion of E2-2 results in lack of pDCs, suggesting a specific role of this transcription

factor in the development of these cells (48). The mobilization of pDCs from bone marrow is promoted by granulocyte colony-stimulating factor (49, 50).

In homeostatic conditions, immature pDCs circulate in blood and can be identified in lymphoid tissues (lymph nodes, tonsils, spleen, thymus, bone marrow and Peyer's patches) and certain peripheral tissues including lung and fetal liver (51, 52). However, during pathological conditions such as inflammation, injury and viral infections, pDCs are recruited to the site of infection (53). For example in humans, pDCs accumulate in the following: the upper respiratory tract in children with acute viral infections (54); the bronchial airway lumen of asthma patients upon allergen challenge (55); the nasal mucosa of allergic subjects after allergen exposure (56); the skin of patients affected by psoriasis (57); brain lesions of patients with multiple sclerosis (58); the inflamed tissues of people with rheumatoid arthritis (59); and the salivary glands of people with Sjogren's syndrome (60). In addition, the accumulation of pDCs has been demonstrated in various animal models of disease. In mice models, pDCs are recruited to the vaginal mucosa during HSV-2 infection (61) and to the lungs during respiratory syncytial virus infection (62).

Plasmacytoid DCs migrate from the bloodstream to the site of infection where they can secrete type I interferons (IFNs), capture antigens and migrate to the draining lymph nodes to present antigen to T cells (63). Plasmacytoid DCs express various chemokine receptors and adhesion molecules that facilitate their transmigration from blood via high endothelial venules (HEV) to the site of infection (43). Homing of pDCs

to HEV is mediated by the expression of L-selectin, P-selectin glycoprotein ligand-1 (PSGL1) and C-X-C motif receptor 4 (CXCR4) on pDCs, which are the ligands for E selectins, P selectins and C-X-C motif ligand (CXCL12) respectively produced by the endothelial cells lining of HEV (64). In addition, endothelial cells also express IL3 and VEGF that interacts with pDCs IL3R (CD123) and BDCA4 respectively, and promotes pDC survival and migration. In mice, under homeostatic and inflammatory conditions, interaction of C-C chemokine receptor type 9 (CCR9) on pDCs with its ligand C-C motif ligand 25 (CCL25) facilitates its migration to the small intestine (65). Trafficking of pDCs to the skin is known to be mediated by the expression of ChemR23, a G-protein-coupled receptor that interacts with chemerin, a chemoattractant produced by skin during injury (53). Dermal endothelial cells, fibroblasts and keratinocytes produce prochemerin, an inactive precursor, which is cleaved to chemerin by serine proteases released during tissue injury (66). During viral infection, the nasal and bronchial epithelial cells produce various chemokines such as CCL8 (IL-8), CCL10 (IP-10), CCL3 (macrophage inflammatory protein-1α, MIP-1α), CCL2 (monocyte chemoattractant protein-1, MCP-1), CCL5 (RANTES) and cytokines such as IFN, IL-6, IL-β and TNF-α. These cytokines facilitate migration and infiltration of several immune cells including pDCs (54, 67, 68). After activation, pDCs express additional molecules such as CXCR3, CCR5 and CCR7 that mediate their homing to the lymph nodes (via secretion of CXCL10, CCL3/CCL4/CCL5 and CCL19/CCL21), where they can prime T cells (69, 70). A model

of pDC migration upon viral infection and homing to the lymph node is illustrated in Figure 1.

Plasmacytoid DCs as Professional Producers of Type I IFN, Potent Antiviral Cytokines

Plasmacytoid DCs play a critical role in innate antiviral immunity by rapidly generating massive amounts of type I IFN in response to various RNA and DNA viruses, including influenza and RV (71-73). pDCs are thus designated as "professional type I IFN producing cells" (43). IFNs were originally named for their ability to "interfere" with the influenza virus replication (74). The IFN family of cytokines is recognized as a key component of the innate immune response and the first line of defense against viral infection (75). Other types of IFNs distinct from type I include type II and III, and the classification is based on the receptor complex these IFNs signal through. In humans, type I IFNs include 14 IFN-α subtypes, IFN-β, IFN-τ, IFN-ε, IFN-κ and IFN-δ (75). All type I IFNs engage IFNAR (IFN-α receptor, composed of IFNAR1 and IFNAR2) and regulate antiviral immune response by inducing expression of various IFN stimulatory genes (ISGs). Type II IFN comprises IFN-γ, which is secreted by CD4+ T helper cell type 1 (Th1), CD8+ cytotoxic lymphocytes, NK cells, macrophages, DCs and B cells (76-78). IFN-γ binds to IFNGR (IFN-γ receptor, composed of IFNGR1 and IFNGR2) and mediates immune response to pathogens besides viruses. However, the most recently discovered type III IFNs comprise 3 IFN-λ types that signal through IFNLR (IFN-λ receptor, form complex with IL10R2) and mediate antiviral responses (75).

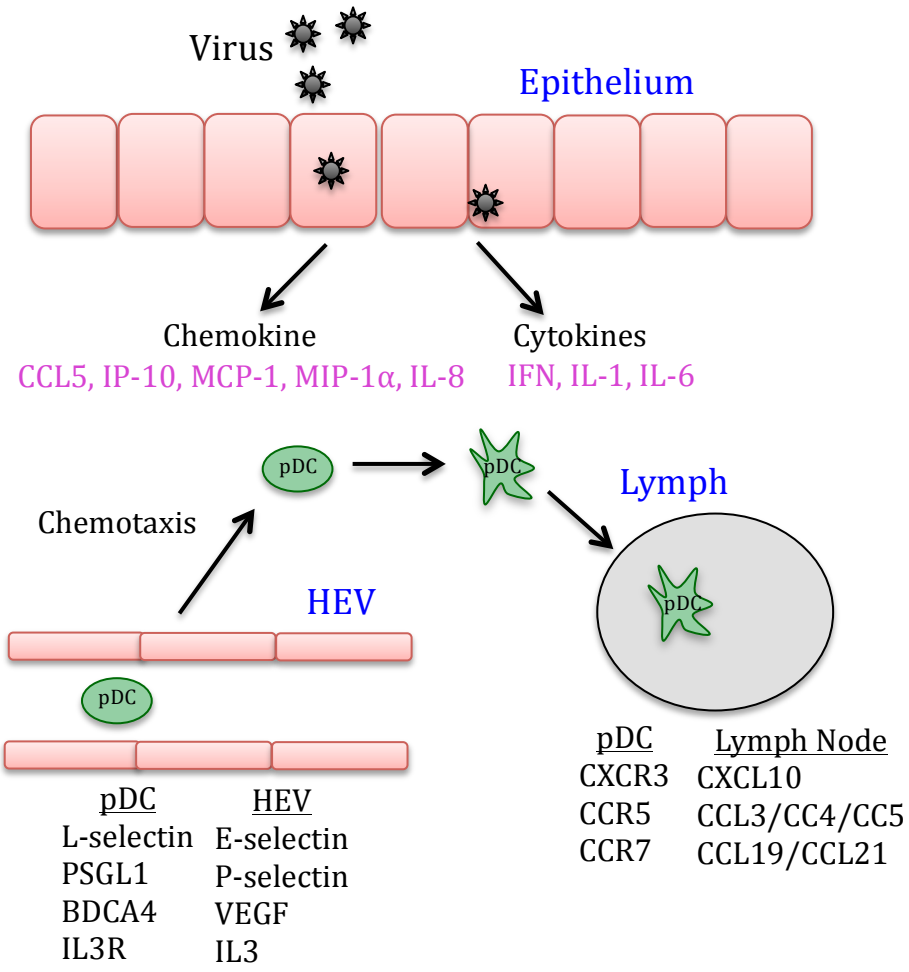

Figure 1. Plasmacytoid DC migration. Upon viral infection, the nasal and bronchial epithelial cells secrete various chemokines (CCL5, CXCL10, MIP-1, IL-8) and cytokines (IFN, IL-1 and IL6), which attract and recruit pDCs and other inflammatory cells. Transmigration of pDCs from blood is mediated by interaction of adhesion molecules on pDCs (L-selectin, PSGL-1, BDCA4 and IL3R) with the molecules on cells lining the high endothelial venule, HEV (E-selectin. P-selectin, VEGF and IL3). After activation at the site of infection, pDCs acquire additional markers such as CXCR3, CCR5 and CCR7, which facilitate their homing to lymph nodes (via CXCL10, CCL3/CCL4/CCL5 and CCL19/CCL21). Illustration by Bajwa (54, 64, 67-70).

Secretion of type I IFNs by pDCs constitutes a critical component of antiviral defense that makes host cells more resistant to viral infection. The unique ability of pDCs to sense and respond rapidly to microbes by producing large amounts of type I IFNs (~10 pg/cell) depends on the presence of a selective subset of Toll-like receptors (TLRs) (79). The TLRs are pattern-recognition receptors (PRRs) that are expressed on immune cells that recognize signature molecules of microorganisms, known as microbe associated molecular patterns (MAMPs). In humans, so far, 12 TLRs have been identified that are localized in distinct cellular components. The TLRs 1, 2, 4, 5 and 6 are expressed on the surface of the cell and sense lipids and proteins, while TLRs 3, 7, 8 and 9 are expressed in ER and intracellular vesicles like endosomes, and their ligands are mainly nucleic acids (80). The TLRs 3, 4, 7, 8 and 9 regulate antiviral immune response by inducing type I IFNs and other proinflammatory effector molecules (81). Plasmacytoid DCs express TLR7 and TLR9 in their endosomal compartments. TLR7 can detect single stranded RNA (ssRNA) from viruses and TLR9 can recognize DNA viruses and unmethylated-CpG oligonucleotides from various bacteria (82, 83). Various viruses including influenza are known to induce IFN-α production in pDCs via activation of the TLR7 pathway (Fig. 2) (72). In pDCs, the TLR7 signaling cascade is induced in response to ssRNA molecules such as the genome of influenza (Fig. 2). This pathway involves recruitment of the adapter protein myeloid differentiation primary response 88 (MyD88) to the cytoplasmic portion of TLR, which further activates and recruits other factors including IL-1R-associated kinase I (IRAK1) and IRAK4 leading to the activation of

constitutively expressed interferon regulatory factor 7 (IRF7). In addition, MyD88 also promotes the activation of activator protein 1 (AP-1) and nuclear factor-κB (NF-κB), which, along with phosphorylated IRF7, get translocated into the nucleus and induce the transcription of type I IFN genes, including IFN-α and IFN-β (84). Induction of IFNs not only inhibits viral replication directly but also activates various effector cells of the adaptive immune system. In a canonical type I IFN signaling pathway, the binding of type I IFNs to the IFNAR triggers a signaling cascade via activating signal transducer and activator of transcription 1 (STAT1) and STAT2 that leads to the induction of more than 300 ISGs, many of which exhibit antiviral effector functions (85). In addition to these innate antiviral defenses, type I IFNs produced by pDCs link innate and adaptive immune responses by promoting antigen presentation and development of high affinity antigen-specific T and B cell responses (86). Type I IFNs induce $CD4^+$ Th cell responses, enhance the survival of T cells, facilitate the proliferation of memory $CD8^+$ T cells through the induction of IL-15 and enhance B cell receptor mediated B cell responses such as antibody production (87-90). In mouse models, pDC depletion studies have indicated that pDCs promote viral clearance and improve survival during influenza, respiratory syncytial virus and HSV infections (62, 91, 92). Plasmacytoid DCs are thus important for defense against respiratory viruses (62, 93, 94).

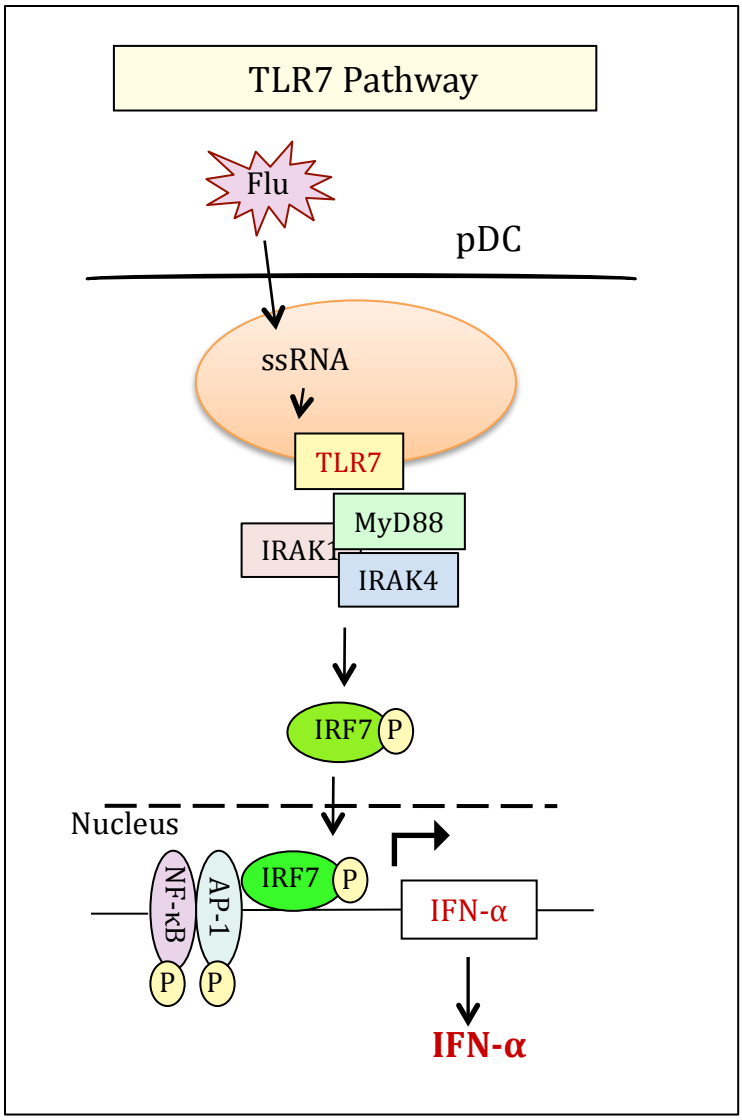

Figure 2. TLR7 pathway in pDCs. In pDCs, ssRNA molecules such as influenza (Flu) genome is sensed by the endosomal TLR7. Stimulation of TLR7 leads to the induction of MyD88-dependent pathway resulting in the activation of IRAK1, IRAK4 and constitutively expressed IRF7. Phosphorylated IRF7 translocates to the nucleus and together with activated AP-1 and NF-κβ initiates transcription of IFN-α/β genes {Adapted from (84)}.

Studies have highlighted that type I IFNs also suppress the development of other subsets of cells and their associated effector functions (95, 96). For example, in humans, type I IFNs negatively regulate the differentiation and stability of type 2 T helper (Th2) cells through the inhibition of GATA3 transcription factor (96, 97). T-helper (Th) cells have the ability to secrete many cytokines and, upon stimulation, two major kinds of polarized pattern of cytokine production can emerge, Th1-type and Th2-type. Th1 cells produce high levels of proinflammatory cytokines, including IFN-γ, interleukin-2 (IL-2) and TNF-β, that promote phagocytosis and destruction of microbial pathogens (98). Th2 cells secrete IL-4, IL-5 and IL-13 which promote mast cell and eosinophil functions but are also known to mediate allergic inflammatory responses such as asthma (99). Therefore, the ability of type I IFNs to restrict Th2 development suggests a potential role for this group of antiviral proteins in controlling IgE-mediated-type I hypersensitivities (96).

Plasmacytoid DCs are also known to play an important role during viral vaccine responses. Several studies indicate that pDCs participate in viral vaccine responses, including immune responses to influenza vaccine (100, 101). For example, in mice, type I IFNs produced by pDCs were essential to generate an initial response to whole inactivated influenza vaccine (100). Depletion of pDCs from human blood mononuclear cells results in impaired secretion of influenza-specific IgG production, suggesting their important role in generation of plasma cells and antibody responses against influenza virus (102). In mice, IFN-α secreted by pDCs induces B-cell activation and strengthens

humoral response by stimulating the production of IgG in response to influenza vaccine (101, 103). A recent study has demonstrated that pDCs can be safely utilized in vaccine therapy with minimal toxicity (104). In melanoma patients, intranodal vaccination with autologous pDCs loaded with tumor peptides induces a favorable antigen-specific CD8+ T cell responses *in vivo* (104).

Plasmacytoid DCs as Antigen Presenting Cells

In addition to secreting type I IFNs upon viral exposure, another major function of pDCs is to present viral antigens to $CD4^+$ and $CD8^+$ T cells. Plasmacytoid DCs are therefore designated as antigen-presenting cells (APCs) and play critical roles in initiating adaptive immune responses. Upon exposure to antigen, pDCs undergo a developmental program called maturation, a process in which they lose their ability to take up antigen and obtain a phenotype of professional APCs. This involves up-regulation of costimulatory molecules including major histocompatibility complex II (MHC II), CD80, CD86, CD83 and chemokines necessary to stimulate naïve T cells (105). After degrading the antigen into short immunogenic peptides and loading the peptides on MHC II molecules, pDCs migrate from mucosa to the draining lymph nodes of the lung and induce effective T cell responses (106). Th cell stimulation requires two signals: the interaction of MHC II plus an antigen on a pDC with CD4 receptor on a Th cell, and the interaction of CD80/CD86 on pDCs with CD28 molecules on a Th cell. Plasmacytoid DCs are capable of cross-presenting endogenous viral antigens via MHC I to $CD8^+$ T cells. Dipucchio and group have demonstrated that influenza exposure leads to

the routing of MHC I to the pDC surface within 30 min and cross-presentation of influenza antigens to $CD8^+$ T cells within 4 hours. This process does not require viral replication, as cross-presentation is dependent on endocytic recycling and is independent of the proteasomal pathway (107). Plasmacytoid DCs are thus important for the development of antiviral T lymphocyte responses.

Role of Metabolism in Immune Responses

Immune cells face a considerable bioenergetic challenge and rely on appropriate metabolic pathway(s) to generate immune responses to invading pathogens (108). Metabolism is defined as a complex network of biochemical and physical reactions leading to energy production and biosynthesis of molecules. During activation, lymphocytes undergo metabolic reprogramming in order to (i) meet the bioenergetics and biosynthetic demands of increased cell proliferation and maturation, and (ii) adapt to the changing environment where oxygen and nutrients may be limited (109). Glycolysis is a central energy-producing pathway in which one glucose molecule is broken down into two molecules of pyruvate and yields two reduced nicotinamide adenine dinucleotide (NADH) and two ATP molecules. Pyruvate can either enter mitochondria and be completely oxidized via the tricarboxylic acid (TCA) cycle (OXPHOS) or can be fermented to lactate by lactate dehydrogenase (LDH) under anaerobic conditions, regenerating NAD^+ for feeding back into glycolysis (110). However, glucose can also be metabolized to lactate in oxygen-rich conditions, a phenomenon termed aerobic glycolysis (111). Under physiological conditions such as stress and inflammation, the

metabolic switch to aerobic glycolysis and higher glycolytic flux allows a cell to generate ATP more quickly yet less efficiently than OXPHOS (112, 113).

Aerobic glycolysis is a metabolic hallmark of cancerous cells (114) and has also been shown to be induced by several oncogenic and non-oncogenic viruses including human cytomegalovirus (HCMV), herpes simplex virus I (HSV 1) and Kaposi's sarcoma-associated herpes virus (KSHV) in mammalian cells (115-117). Research in recent years has provided evidence that the regulation of immune cell function is coupled to cellular metabolism. Several of these studies evaluated mouse myeloid DCs and human T lymphocytes. Krawczyk and group demonstrated that exposure to TLR agonists induces bone marrow-derived mouse DCs to undergo a metabolic transition from OXPHOS to aerobic glycolysis to meet the energy requirement of the cells (118). This metabolic reprogramming is promoted by PI3K/Akt signaling and is essential for DC maturation (118). DC maturation is accompanied by increases in transcriptional and translational activities, promoting the expression of genes encoding cytokines, chemokines and costimulatory molecules. Everts and group have demonstrated that in mice moDCs, TLR agonists induce early induction of glycolysis that is essential to support the anabolic demands of the cell (119). In addition, this study highlights the requirement of glycolysis-driven pentose phosphate pathway for the activation of DCs (119). In contrast to mature DCs, tolerogenic moDCs exhibit metabolic signatures of increased OXPHOS and high mitochondrial function activity (120).

Several studies have pinpointed the importance of metabolic reprogramming in T lymphocytes, demonstrating that antigen exposure leads to metabolic profile changes in these cells during the course of an immune response. Naïve and memory T cells are less active and metabolically quiescent, and utilize basal uptake of nutrients and rely on OXPHOS for their energy needs. However, upon activation, naïve T lymphocytes undergo a metabolic transition characterized by increased nutrient uptake, enhanced aerobic glycolysis and reduced OXPHOS for ATP production to support their growth, proliferation and effector functions (109, 121).

Data on how viral exposure impacts cellular metabolism and the role of metabolism in regulating immune responses to respiratory viruses in primary human pDCs is lacking. We thus investigated the *ex vivo* effects of influenza and RV, two common respiratory viral pathogens, on human pDC metabolic pathways and pDC functions including IFN-α production and pDC maturation. We hypothesized that influenza and RV would induce metabolic changes in pDCs and that the inhibition of those metabolic pathway(s) would affect pDC antiviral function(s). In this study, we addressed this hypothesis with three aims; i) assessing IFN-α production and surface expression of pDC maturation markers in response to influenza and RV infection to determine the effects on activation; ii) determining if influenza, RV and the TLR7 agonist, gardiquimod induce metabolic changes in pDCs; and iii) investigating if inhibition of glycolysis impairs pDC antiviral responses. This study will provide insight

on the role of metabolism in directing the innate immune responses in this rare but crucial immune cell.

CHAPTER II

MATERIALS AND METHODS

Human Subjects

For all *ex vivo* studies, enriched leucocyte packs (buffy coat) from anonymous blood donors were obtained from a local blood bank. For *in vivo* studies, informed consent was obtained from 10 healthy volunteers (Table 1) with no recent history of influenza infection or vaccination. Blood was obtained by venipuncture (using vacutainer tubes with Acid Citrate Dextrose Solution A, BD Biosciences, Franklin Lakes, NJ) before, day 1 and/or day 3 after administering LAIV. All the procedures were performed according to an IRB-approved protocol.

Purification of pDCs

Blood was diluted 1:1 (vol/vol) with sterile PBS (supplemented with 2% FBS and 2 mM EDTA, pH=7.4). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation followed by purification of pDCs by negative selection using antibody-coated magnetic beads (Human plasmacytoid DC enrichment kit, # 19062, Stem Cell Technologies, Vancouver, BC), according to the manufacturer's recommendations (Fig. 3). The purity of pDCs was tested by flow cytometry using the following fluorochrome-conjugated anti-human Abs: lineage-FITC, HLA-DR APC-Cy7, CD123-PE-Cy5, CD11c-APC and

CD14-V450 (Cat # 335796, 551065, 559877 and 560349, BD Biosciences). The pDC purity (gated on lineage^{-}DR^{+}CD123^{+}) was greater than 95%.

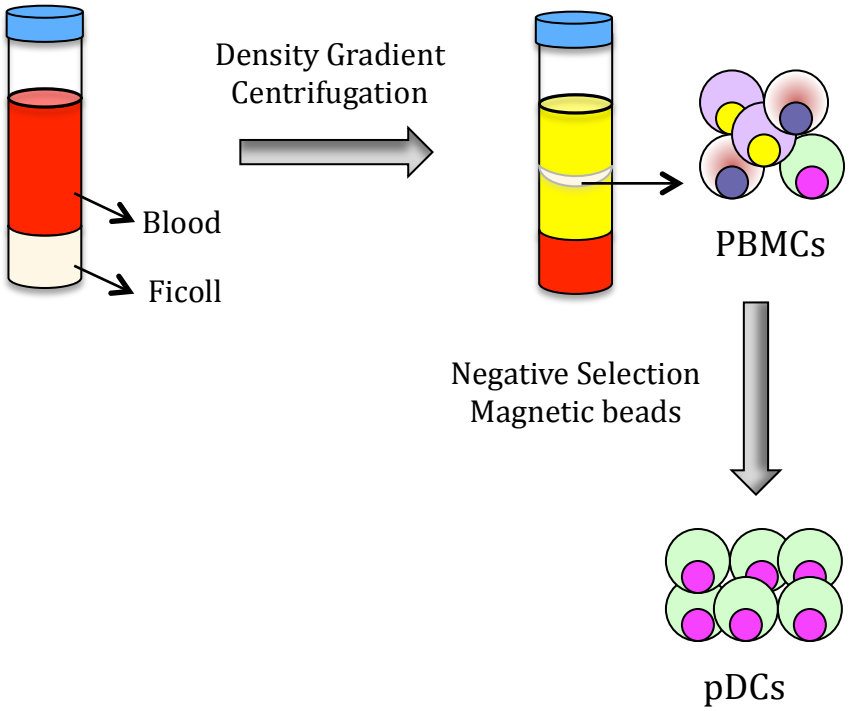

Figure 3. Purification of blood pDCs. Blood was diluted 1:1 (vol/vol) with complete PBS and was layered on Ficoll followed by density gradient centrifugation. pDCs were purified from PBMCs by negative selection using antibody coated magnetic beads (Stem Cell Technologies).

Viruses

Influenza A/PR/8/34 (H1N1) was obtained from Charles River Laboratories, Wilmington, MA. The purchased virus was propagated in specific-pathogen free (SPF) eggs in the allantoic cavity, purified using sucrose density centrifugation and quantified. The stock was diluted in our laboratory to 0.7 x 10^7 PFU/ml and was used at the final concentration of 0.08 PFU/cell in most of the experiments and 0.2 PFU/cell for the microarray experiment.

Human rhinovirus-16 (RV-16) was obtained as a gift from James Gern and Yury Bochkov, University of Wisconsin. The obtained virus was propagated on HeLa cells, purified using sucrose gradient centrifugation and quantified in Dr. Gern's laboratory. The final concentration of RV used was 1 PFU/cell in most of the experiments except in a few seahorse metabolic flux experiments where 10 PFU/cell was used.

Plasmacytoid DC Culture Conditions and Reagents

Plasmacytoid DCs were cultured at the concentration of 2 x 10^4 to 5 x 10^5/ 0.2 ml in complete RPMI 1640 media (10% heat-inactivated FBS, 1% penicillin-streptomycin, 1% sodium pyruvate, 1% HEPES buffer solution, 1% non-essential amino acids, 1% glutamate and 100 μM β-mercaptoethanol) in 96-well plates. IL-3 (10 ng/ml; R&D Systems, Minneapolis, MN) was added to all the cultures to maintain the viability of the cells. The pDC culture conditions were evaluated with various treatments including: media control, influenza A, RV and gardiquimod (a TLR7 agonist, final concentration 1 μg/ml; InvivoGen, San Diego, CA). Cell viability was evaluated using trypan blue dye

(Invitrogen) exclusion staining. pDC cultures were harvested by centrifugation at 240 g for 5 min and supernatants were stored at -80^{o}C for ELISA and lactate assays. In select experiments, pDC pellets were harvested for RNA isolation or flow cytometry.

For type I IFN signaling experiments, pDCs were treated with human IFN-α (1000 U/ml; PBL Assay Science), or influenza with or without anti-human IFN- α/β receptor antibody (5 μg/ml; PBL Assay Science, Piscataway, NJ) or media control, followed by lactate measurements. Gardiquimod- or influenza-activated pDCs with or without chloroquine (2 μg/ml; Sigma-Aldrich) were evaluated for IFN-α and lactate quantification at 24 h.

To determine the effects of heat-inactivated influenza, purified pDCs were cultured in the presence or absence of equivalent amount of live or heat-inactivated (exposed to 56^{0}C for 30 min, from the same lot of virus) influenza for 24 h and 48 h. Supernatants were harvested and total ^{13}C-lactate and IFN-α was measured.

RNA Extraction, Microarray Analysis & Quantitative Real-Time PCR

For microarray analysis, purified pDCs (5×10^5) from blood of 5 healthy human donors were treated independently with or without influenza (MOI of 0.2 PFU/cell) for 8 h. Cells were harvested and total RNA was isolated from cell pellets using the Arcturus® PicoPure® RNA isolation kit (Life Technologies, Grand Island, NY) according to the manufacturer's protocols. RNA was submitted to the University of Texas Southwestern Medical Center microarray core facility for microarray analysis. The quality of RNA was checked using a Bioanalyzer Chip and DNA Microarray was performed using HumanHT-

12 v4 Expression BeadChip (Agilent Technologies, Santa Clara, CA). The raw image files were converted into signal intensity values without normalization using GenomeStudio Data Analysis Software and data was analyzed using GeneSpring GX 11 Version 4.0 software (Agilent Technologies). Raw data was normalized using quantile normalization and paired t-test ($p \leq 0.05$) was performed to identify genes that were differentially expressed with the cut off value of ±1.25 in response to influenza. The differentially altered gene list was further subjected to pathway analysis using DAVID - Database for Annotation, Visualization and Integrated Discovery (DAVID Bioinformatics Resources 6.7).

Quantitative RT-PCR was performed using Taqman probes/primers, both to confirm differences from the microarray results and to measure the expression of *TLR7* and *IRF7*. Replicate samples of purified pDCs were cultured i) in the presence or absence of influenza or RV for 8 h to quantitate the *TLR7* and *IRF7* expression and, ii) in the presence or absence of influenza, RV or gardiquimod for 8 h and 24 h time points to confirm the microarray results. Briefly, total RNA was purified as described above and cDNA was synthesized using High Capacity cDNA Reverse Transcriptase Kit (Life Technologies, Grand Island, NY). The relative expression of *TLR7*, *IRF7*, hexokinase 2 *(HK2*), lactate dehydrogenase A *(LDHA)*, phosphofructose kinase P *(PFKP)* and hypoxia inducible factor (*HIF)-1α* mRNA was quantified using Taqman probe/primer sets (Life Technologies) by 7900HT Fast Real Time PCR system (Applied Biosystems). *HPRT* was used as an endogenous control because the expression of this housekeeping gene was not

altered by influenza exposure based on our microarray data. The results were based on $2^{-\Delta\Delta ct}$ values.

ATP Quantification

For ATP quantification**,** pDCs (10^5) were cultured in the presence or absence of influenza or RV as described, and cell pellets were harvested at 24 h. Cell lysates were prepared by adding 50 µl cell lysis buffer (containing 10 mM Tris, 0.1 M NaCl, 1 mM EDTA and 0.01% Triton X-100, pH 7.5) at 4^{o}C, followed by centrifugation at 16,000 g for 10 min/4^{o}C. ATP concentration was measured in the lysates using Molecular Probes' ATP Determination Kit (Molecular Probes, Grand Island, NY) according to the manufacturer's instructions.

Extracellular Acidification Rate (ECAR) and Oxygen Consumption Rate (OCR) Measurements

pDCs were cultured in complete RPMI media in the presence or absence of influenza, RV or gardiquimod for 24 h. Cells were harvested and seeded on Cell-Tak (BD Biosciences) coated 24-well plate in XF media (non-buffered RPMI 1640 with 2 mM L-glutamine) for 1-2 h at 37^{o}C. The plate was placed on an XF-24 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA) to evaluate the ECAR and OCR in real time. The measurements were done first, at baseline with the addition of 10 mM D-glucose (glycolysis substrate, to assess basal glycolysis), followed by addition of 1 µM oligomycin (an ATP synthase inhibitor), to evaluate the maximum glycolytic capacity

and finally 100 mM 2-deoxyglucose (2-DG, a glycolytic inhibitor), to halt glycolysis and measure the glycolytic reserve.

Lactate Quantification by Gas Chromatography - Mass Spectrometry

For quantification of glucose-derived lactate, pDCs (10^5) were cultured in glucose-free RPMI 1640 media (supplemented with 10% dialyzed FBS, 10 mM D-[1,6-^{13}C]-glucose, 1% penicillin-streptomycin, 1% HEPES buffer solution, 1% non-essential amino acids, 1% glutamate and 100 μM β-mercaptoethanol) with or without influenza, RV or gardiquimod. Supernatants from replicate wells were harvested at 6, 24 or 48 h time points. Lactate was extracted by mixing 25 μl of sample supernatant with 1 ml of methanol followed by chloroform (1 ml) and water (1 ml). Sodium L-[1,2,3-^{13}C]-lactate (5 μl) was added as an internal standard. The samples were centrifuged for 2000 rpm/5 min/RT and the aqueous phase was collected. Samples were evaporated (42^{o}C for 3-4 h) and derivatized by trimethylsilylation (Tri-Sil HTP reagent; Thermo Scientific) at 42^{o}C/ 30 min. An aliquot of 1-3 μl was injected into an Agilent 6970 gas chromatograph networked to an Agilent 5973 mass selective detector (Agilent Technologies). Glucose-derived ^{13}C-lactate concentration in the samples was calculated based on ^{13}C-lactate enrichment and total abundance of lactate. To measure the atom percent access ^{13}C-lactate enrichment, standard curve was prepared for 0, 50 and 100% enrichment of sodium L-[3-^{13}C]-lactate (S0 = 10 μl unlabeled sodium lactate; S50 = 1:1 ratio of unlabeled sodium lactate and sodium L-[3-^{13}C]-lactate and; S100= 10 μl of sodium L-[3-^{13}C]-lactate). To measure the total abundance of lactate, abundance at m/z of 117 (all

unlabeled C), 118 (lactate containing one ^{13}C from glucose) and 119 (internal standard, all 3-^{13}C) was measured, the sum of areas of 117 and 118 was corrected against 119 and multiplied by 17.9 nmoles (amount of internal standard).

***In Vivo* Influenza Immunization Experiments**

For *in vivo* influenza vaccination studies, blood was obtained from the healthy donors before, 1 day and/or 3 days after administration of live attenuated influenza vaccine, LAIV (FluMist Quadrivalent, MedImmune, Gaithersburg, MD) followed by pDC purification. However, we could not get sufficient number of pDCs for all the samples at all the time points. Lactate was quantified in the supernatants of pDCs cultured for 24 h (Table 1 and Figure 10*A*).

Glycolysis Inhibition Assay

For the 2-DG titration assays, pDCs were cultured with influenza in complete RPMI media containing 10 mM D-glucose and 1, 10 or 20 mM 2-DG for 24 h and ^{13}C-lactate and IFN-α was measured in supernatants. Purified pDCs were treated with influenza, RV or gardiquimod, with or without 10 mM 2-DG for 24 or 48 h as described. Supernatants were saved for IFN-α and lactate quantification, and cell pellets for flow cytometry.

IFN-α Quantification

The IFN-α concentration in the pDC culture supernatants was measured by ELISA using the Human IFN-α (pan-specific) ELISA kit (Product code: 3425-1H-6,

MabTech, Cincinnati, OH) according to the manufacturer's directions and analyzed using a DTX 880 Multimode detector at 450 nm (Beckman Coulter, Fullerton, CA).

Flow Cytometry

To evaluate pDC maturation, pDCs were treated with or without influenza or RV for 48 h. Cell pellets were harvested, washed with PBS and incubated with the following fluorochrome-conjugated anti-human Abs: HLA-DR APC-Cy7, CD80-FITC and CD86-PeCy5 (BD Biosciences) for 30 min at 4^{o}C for surface staining. Cells were rinsed with PBS by centrifuging at 240 g for 5 min and fixed with freshly prepared 1% paraformaldehyde (pH= 7.4). Samples were acquired on a BD LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

To determine the effect of glycolysis inhibition on pDC maturation, pDCs were stimulated with or without influenza, RV or gardiquimod in the presence or absence of 10 mM 2-DG for 48 h. Surface staining was performed using the following fluorochrome-conjugated anti-human Abs: HLA-DR APC-Cy7, CD80-FITC, CD86-PeCy5 and CD83-PE (BD Biosciences) and analyzed as described. The mean fluorescence intensity (MFI) of HLA-DR, CD80, CD86 and CD83 on pDCs (gated on total live cells) was measured and subsequently converted to Molecules of Equivalent Soluble Fluorochrome (MESF) using SPHERO^{TM} Rainbow Calibration Particles, 6 peaks (BD Biosciences). For mitochondrial studies, mitotracker staining was performed using MitoTracker Red CMXRos dye (Invitrogen). MitoTracker Red CMXRos is a red fluorescent dye that stains active mitochondria in live cells and its accumulation is dependent on the mitochondrial

membrane potential. Briefly, purified pDCs (10^5) were exposed to influenza or RV for 48 h, cell pellets were washed with PBS and stained with 200 nM of MitoTracker Red CMXRos at 37^{o}C for 30 min. Cells were washed and resuspended in fresh PBS, and subsequently analyzed using flow cytometry.

To determine total HIF-1α intensities and nuclear localization, pDCs were treated with influenza for 24 h. Cells were stained intracellularly with HIF-1α Dyl-488 (ThermoFisher Scientific) or rabbit IgG isotype control-488 Ab (Cell Signaling technologies) and DAPI using the Foxp3 Fix/Perm buffer set (eBiosciences, San Diego, CA) as described by the manufacturer. The ImageStream Mark II Quantitative Imaging Flow Cytometer (Amnis Corporation, Seattle, WA) was used to capture cell images at 60x magnification using excitation lasers set as follows: 10mw of 405, 200mw of 488 and 150mw of 642. About 5000-10,000 cells per sample were collected, spectrally compensated and analyzed using the IDEAS image analysis software (Amnis Corporation). An analysis template was created to identify in-focus nuclei, single cells, HIF-1α DyLight 488 and DAPI double-positive cells and nuclear HIF-1α with similarity values greater than one. Similarity is the log transformed Pearson's Correlation Coefficient and indicates when DAPI and HIF-1α occupy the same pixel space as described previously (122).

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed using Graph Pad Prism version 6, which involved student t-test, one-way ANOVA and two-

way ANOVA followed by multiple comparisons. Pearson's Correlation analysis was performed to measure similarity factor, an indicator of nuclear translocation. $P \leq 0.05$ was taken as the cut off for significant differences.

CHAPTER III

RESULTS

Exposure to Influenza and RV Induces Secretion of IFN-α and Phenotypic Maturation in Human pDCs

To determine whether influenza and RV induce innate immune responses in pDCs, IFN-α was measured in the supernatants of pDCs exposed to influenza and RV. Both influenza and RV exposure resulted in secretion of significant amounts of IFN-α by pDCs (Fig. 4*A*). In addition, there was significant up-regulation of *TLR7* and *IRF7* mRNA levels (Fig. 4*B* and 4*C*) in influenza- and RV-exposed pDCs, suggesting that both the ssRNA viruses activate the TLR7 pathway.

Plasmacytoid DCs function as APCs and play a critical role in initiating the adaptive immune response. To assess the maturation of pDCs on viral exposure, pDCs were treated with influenza or RV for 48 h and the surface expression of maturation markers HLA-DR (a component of the major histocompatibility complex II, MHC II), CD80 and CD86 was measured by flow cytometry. There was significant increase in surface expression of HLA-DR, CD80 and CD86 in pDCs exposed to influenza and RV (Fig. 5*A*-5*C*). Taken together, these results confirm that pDCs elicit innate immune responses and potentially play a role in initiating adaptive immune responses to both these respiratory viruses.

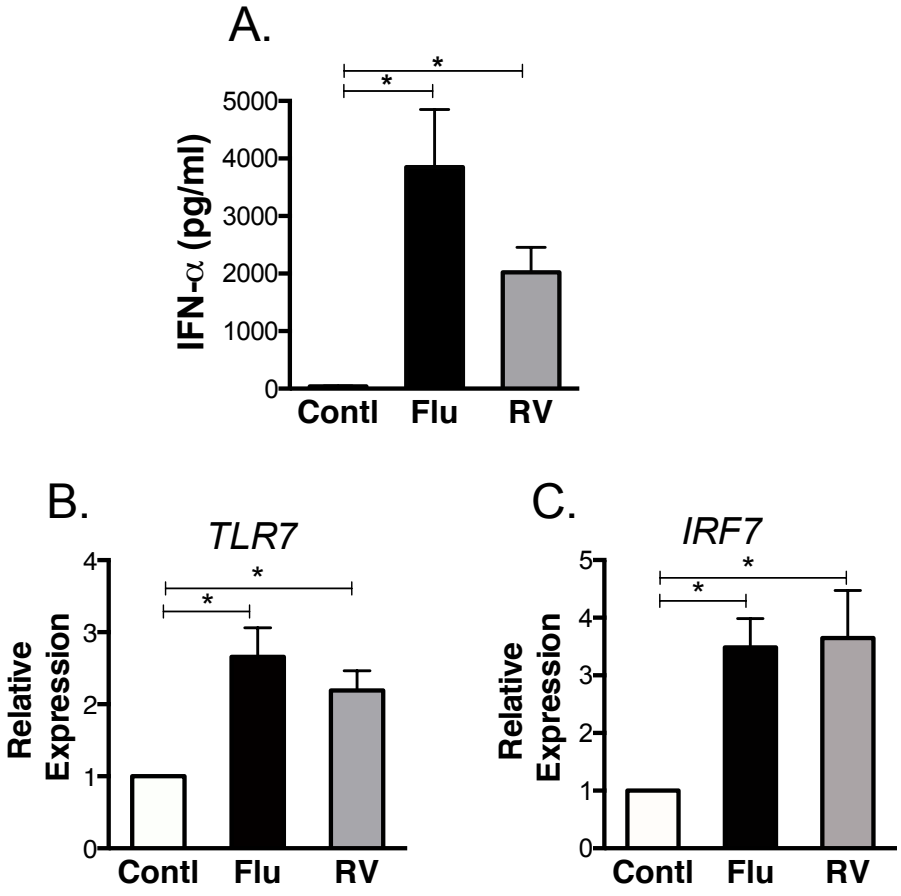

Figure 4. Exposure to influenza (Flu) and human rhinovirus (RV) induces IFN-α secretion and up-regulates *TLR7* and *IRF7* mRNA expression in human pDCs. *A,* Purified pDCs were cultured with IL-3 in the presence or absence of influenza or RV for 24 h and IFN-α concentration was quantified in the supernatants. Total RNA was isolated from influenza or RV-exposed pDCs at 8 h time point, followed by cDNA synthesis and qRT-PCR. Relative mRNA expression of (*B*) *TLR7* and (*C*) *IRF7* are displayed in influenza- and RV-treated pDCs. HPRT was used as an endogenous control and the results were based on $2^{-\Delta\Delta Ct}$. Data represent mean ± SEM of 4-6 independent experiments, * depicts $p \leq 0.05$.

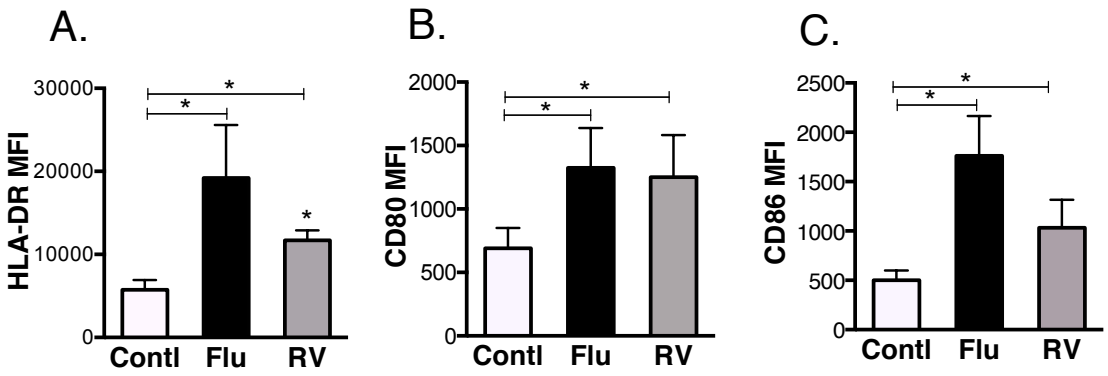

Figure 5. Exposure to influenza and RV induces maturation of human pDCs. Purified pDCs were cultured as described in Figure 1 for 48 h. Cells were harvested and stained with HLA-DR APC-Cy7, CD80-FITC and CD86-PE-Cy5 Abs, and mean fluorescence intensities (MFIs) of (*A*) HLA-DR, (*B*) CD80 and (*C*) CD86 were measured by flow cytometry. Data are expressed as mean ± SEM MFIs of the displayed markers from 4-6 independent experiments, * depicts $p \leq 0.05$.

Influenza and RV Exposure Increases ATP Content of Human pDCs

To determine if viral exposure impacts metabolism in human pDCs, we first compared the ATP levels, a universal energy carrying molecule, in purified pDCs treated *ex vivo* with influenza or RV to untreated control. We found that pDCs exposed to influenza and RV had significantly higher ATP content (~ 2 fold) compared to the control condition (Fig. 6*A* and *6B*). These results support that viral exposure induces metabolic changes in pDCs.

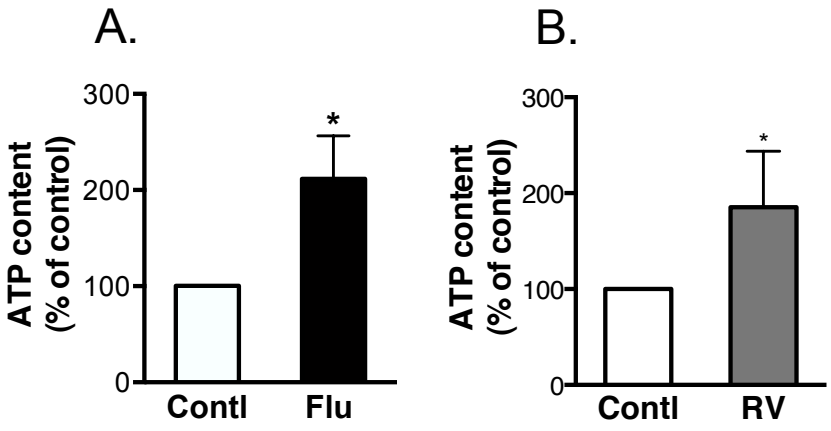

Figure 6. Influenza and RV exposure increases ATP content in human pDCs. Purified pDCs (10^5) were cultured in the presence or absence of influenza, RV or untreated control for 24 h. Cells were lysed and ATP concentration was measured in the lysates using an ATP luciferase assay. The graph displays ATP levels of pDCs treated with (*A*) influenza and (*B*) RV, normalized to the control condition. Data represent mean ± SEM of 3 independent experiments (paired t-test, $p \leq 0.05$).

Exposure to Influenza, RV and TLR7 Agonist Enhances Expression of Key Glycolytic Genes in Human pDCs

Glycolysis and OXPHOS are two major pathways that cells rely on for their energy needs. The higher ATP content of pDCs exposed to viruses led us to investigate which metabolic pathway(s) were induced in these cells during viral infection. To address this, we performed microarray analysis in pDCs exposed to influenza. We found that influenza exposure led to a differential regulation of about 3000 genes in pDCs and in order to extract biological meaning associated with these up and down regulated genes, functional annotation analysis was performed using bioinformatics resource, DAVID (Database for Annotation, Visualization and Integrated Discovery) (123). Plasmacytoid DCs play an important role in antiviral defense by sensing viral nucleic acids via TLR7/9 and by producing massive amounts of type I IFNs including IFN-α and IFN-β (124). As expected, we found significant up-regulation of type I IFN genes and viral-induced genes that were clustered in functional groups including - 'response to virus,' 'IFN- α/β receptor binding' with influenza treatment (Fig. 7*A*). Interestingly, we observed differential regulation of metabolic genes clustered under 'positive regulation of macromolecular biosynthetic process,' 'glucose metabolic process,' 'glycolysis,' 'mitochondrial respiratory chain' and 'OXPHOS' in influenza-exposed pDCs (Fig. 7*A*). While all genes within the glycolysis cluster were up regulated, those in the OXPHOS and mitochondrial respiratory chain categories were not, suggesting that influenza selectively induces glycolytic pathway in pDCs. There was significant up-regulation of

expression of key glycolytic genes including *HK2*, *LDHA*, phosphoglycerate mutase 1 (*PGAM1*) and *PGAM4* in influenza-treated pDCs (Fig. 7*B*). Hexokinase is the first key enzyme in glycolysis that irreversibly phosphorylates glucose to glucose-6-phosphate and lactate dehydrogenase catalyzes the reduction of pyruvate to lactic acid. Induction of aerobic glycolysis is a hallmark of cancerous cells and most cancer cells exhibit increase in expression of specific glycolytic enzymes including HK2 and LDHA (111, 125). Therefore, our microarray data provided evidence for the first time that pDCs up regulate glycolysis during viral infection.

The microarray data were confirmed by quantitative RT-PCR. Exposure of pDCs to influenza, RV and TLR7 agonist, gardiquimod resulted in significant up regulation of *HK2*, *LDHA* and *PFKP* mRNA levels (Fig. 7*C*) compared to control at 8 h and 24 h time points. Taken together, our data confirm that these ssRNA viruses and TLR7 agonist, gardiquimod induce expression of key glycolytic genes in pDCs.

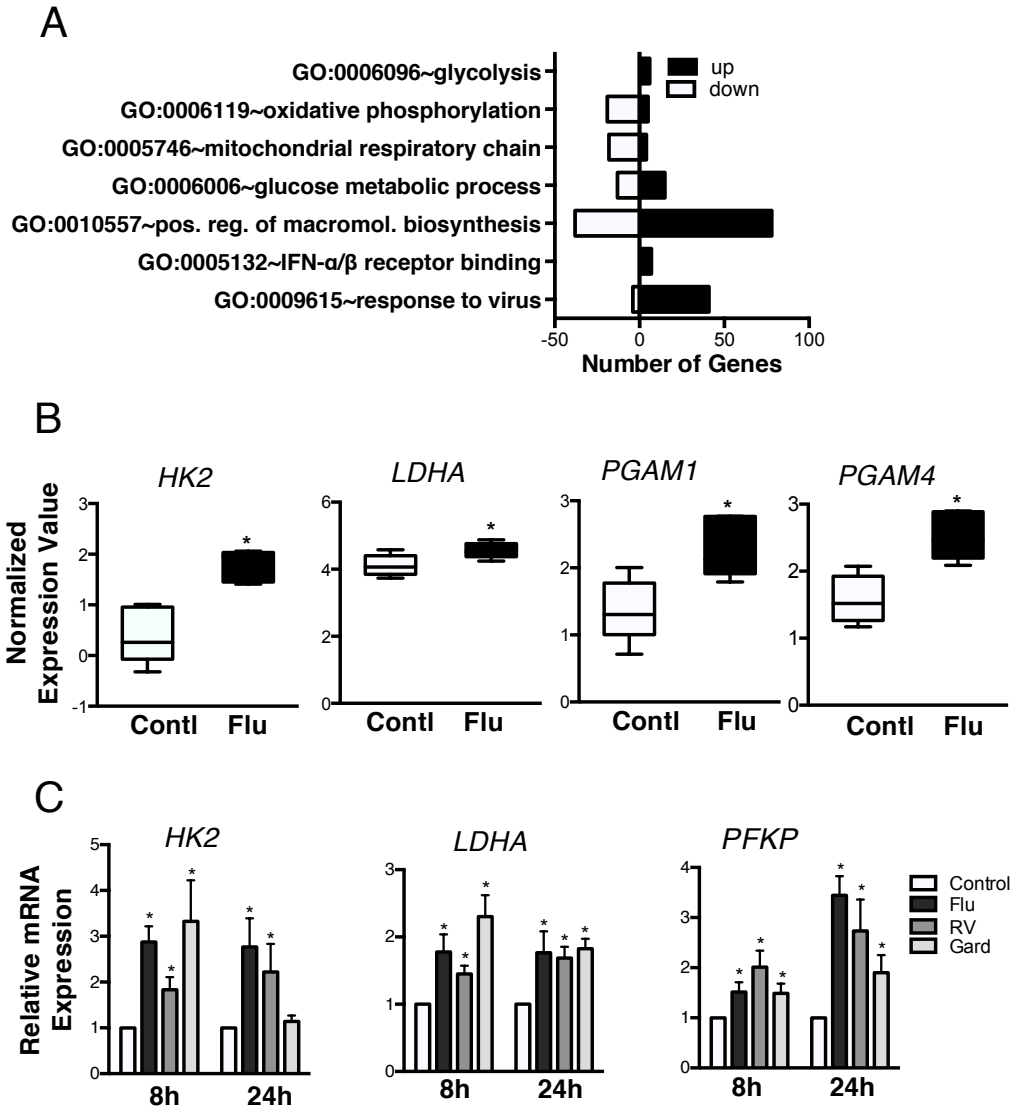

Figure 7. Influenza, RV or TLR7 agonist, gardiquimod induces expression of key glycolytic genes in human pDCs. RNA was purified from pDCs treated with or without influenza for 8 h and DNA microarray analysis was performed using HumanHT-12 v4 Expression BeadChip. *A*, Functional clusters of genes affected by influenza exposure based on annotation analysis are shown. *B*, Normalized expression values of *HK2*, *LDHA*, *PGAM1* and *PGAM4* genes are displayed in control versus influenza-treated pDCs. *C*, Induction of *HK2*, *LDHA* and *PFKP* was confirmed by quantitative RT-PCR in pDCs exposed to influenza, RV or TLR7-agonist, gardiquimod for 8 and 24 h. *HPRT* was used as an endogenous control and the results were based on $2^{-\Delta\Delta ct}$ values. Data represent mean ± SEM of 4-6 independent experiments; *$p \leq 0.05$.

Influenza, RV and Gardiquimod Induce Glycolysis in Human pDCs

We next investigated the impact of viral and TLR7 agonist stimulation on pDC glycolytic flux in real time, measured as extracellular acidification rate (ECAR) using the XF24 extracellular flux analyzer (Seahorse Bioscience). For these experiments, pDCs were first exposed to influenza, RV or gardiquimod for 24 h followed by assessment of ECAR as depicted in Figure 8*A*. We found that at baseline, with the addition of glucose, pDCs exposed to influenza, RV and gardiquimod showed elevated ECAR compared to the untreated control (Fig. 8*A*-8*C*). With the addition of oligomycin, an ATP synthase inhibitor, the increase in ECAR persisted with all 3 stimulants compared to control (Fig. 8*A*-8*C*). The data showing significant increase in ECAR at baseline with the addition of glucose on viral exposure are summarized in Figure 9*A*. These data confirm that glycolytic flux increases in pDCs treated with influenza, RV and gardiquimod. Next, to assess the mitochondrial function in pDCs exposed to viruses, we measured the mitochondrial rate of oxygen consumption, OCR. We found that at baseline, OCR was reduced in response to influenza and RV (Fig. 9*B*). There was no significant difference in the mitochondrial membrane potential of pDC exposed to influenza and RV compared to control (Fig. 9*C*). Taken together, our data suggested that OXPHOS is not elevated in pDCs in response to influenza and RV and these cells preferentially utilize glycolysis for their biosynthetic needs during respiratory viral infection.

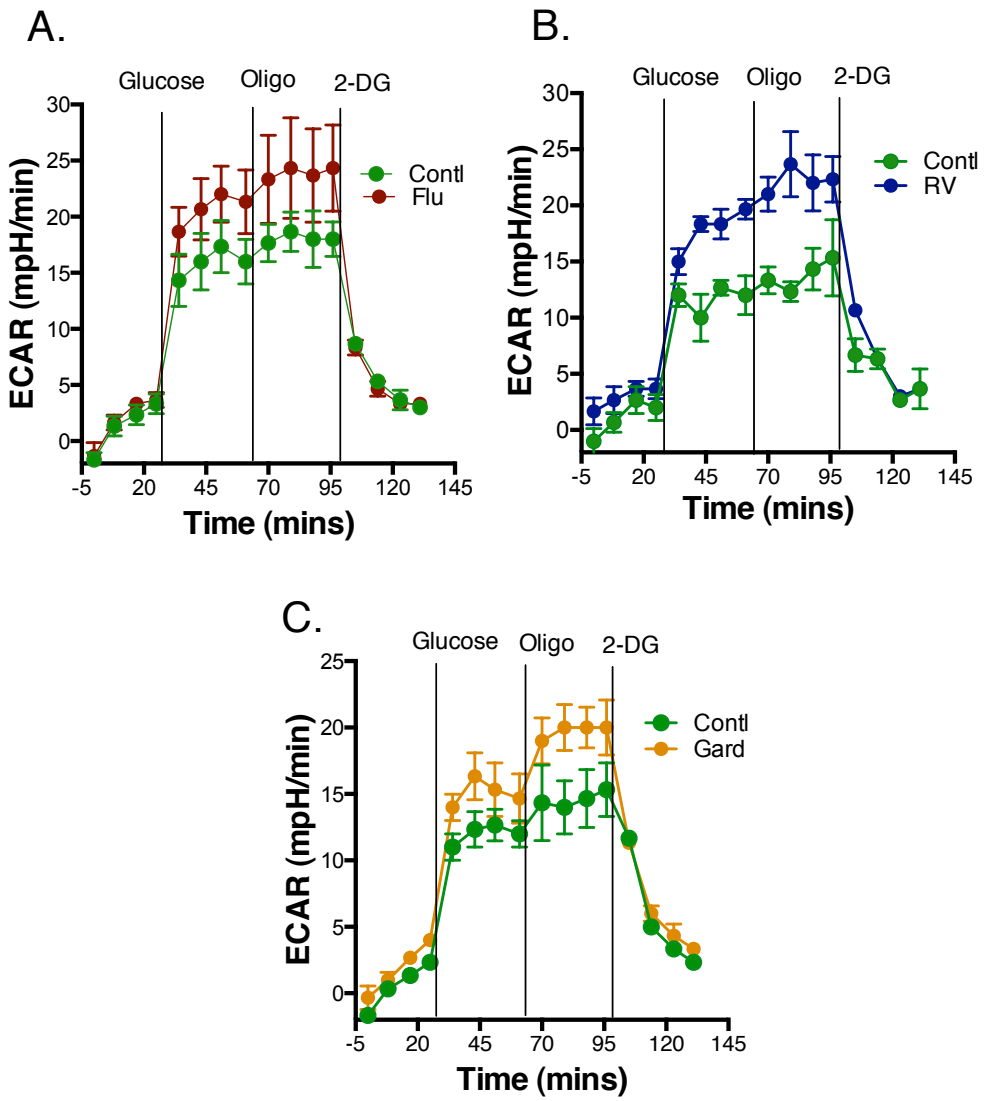

Figure 8. Exposure to viruses and TLR7 agonist increases aerobic glycolysis in human pDC. Purified pDCs were treated *ex vivo* with influenza, RV, gardiquimod or untreated control for 24 h. Cells were washed and were allowed to attach on a 24-well plate in non-buffered media for 2 h and Extracellular Acidification Rate (ECAR) was measured in real time using seahorse extracellular flux analyzer. Vertical lines in each graph indicate time of addition of glucose (glycolysis substrate), oligomycin (ATP synthase inhibitor) and 2-deoxyglucose (2-DG; glycolysis inhibitor). The graph displays ECAR values in pDCs exposed to (*A*) influenza, (*B*) RV or (*C*) gardiquimod versus the control condition. Data are from one experiment representative of 3 individual experiments (*A*-*C*).

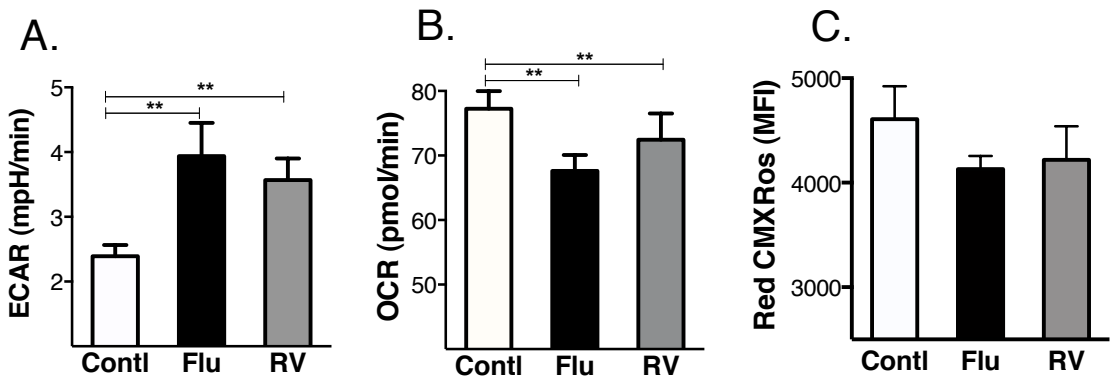

Figure 9. Exposure to viruses and TLR7 agonist in human pDC induce glycolysis but not OXPHOS. Purified pDCs were treated with influenza, RV or untreated control for 24 h. (*A*) ECAR and (*B*) Oxygen Consumption Rate, OCR from the same set of experiments at baseline with glucose are displayed. *C*, Red CMXRos MFI, a measure of mitochondrial function, is shown in pDCs treated with influenza, RV or control for 48 h. **P values ≤ 0.01, data represent mean ± SEM from three independent experiments (*A*-*C*).

We next assessed glycolysis in pDCs treated with viruses and gardiquimod by quantifying extracellular lactate, an indicator of glycolysis. To achieve this, pDCs were cultured in media containing 1,6-^{13}C-glucose with influenza, RV or gardiquimod and the glucose-derived ^{13}C-lactate was assayed in the supernatants using gas chromatography-mass spectrometry (GC-MS). As depicted in figure 10*A*, we observed increased lactate levels in the supernatants of pDCs exposed to influenza, RV and gardiquimod at 24 h and 48 h compared to control. Pooled results of multiple experiments depicting significant higher levels of lactate in the supernatants of influenza-, RV- and gardiquimod-treated pDCs compared to control at both 24 h and 48 h time are summarized in figure 10*B*. The rate of lactate efflux was 2-fold higher in the supernatants of influenza, RV and gardiquimod-activated pDCs compared to control (Fig. 10*C*). Collectively, our ECAR and lactate data confirm that exposure to influenza, RV and gardiquimod results in elevation of glycolysis in human pDCs.

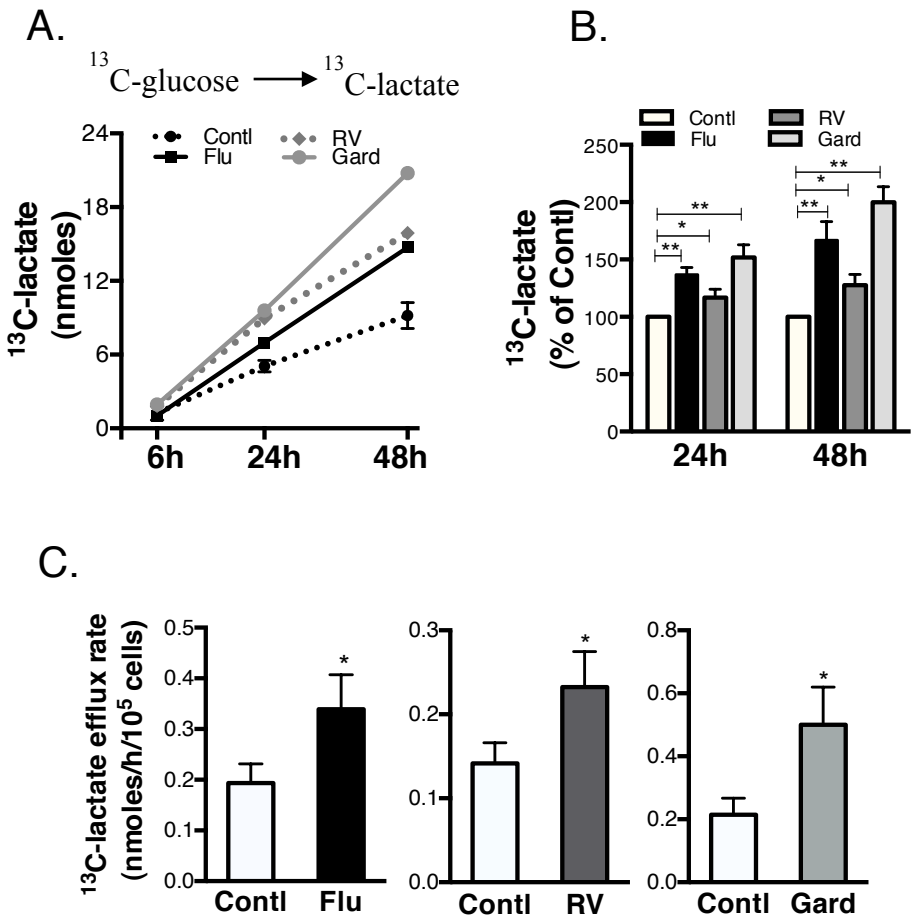

Figure 10. Exposure to viruses and TLR7 agonist results in increased lactate efflux in pDCs. *A*, pDCs (10^5) were cultured in complete RPMI medium (containing 10% dialyzed serum and 10 mM 1,6 ^{13}C-glucose) with or without influenza, RV or gardiquimod for 6 h, 24 h and 48 h and total concentration of glucose-derived ^{13}C-lactate was measured in the supernatants using mass spectrometry. *B*, The bar graph displays mean ± SEM values of ^{13}C-lactate normalized to control in influenza-, RV- or gardiquimod-treated pDC at 24 and 48 h time points. *C*, Rate of lactate efflux calculated in nmoles/h/10^5 cells in pDCs treated with or without influenza, RV or gardiquimod is shown. Data are from one experiment representative of 3 independent experiments (*A*), or mean values ± SEM from 4-8 independent experiments (*B*-*C*) (*$p \leq 0.05$, **$p \leq 0.01$; paired t-test).

In Vivo Exposure to LAIV Results in Increased Lactate Efflux in Human pDCs

To begin to assess the *in vivo* impact of viral infection on glycolysis in human pDCs, we utilized administration of the live attenuated influenza vaccine (LAIV) in our study model. We purified blood pDCs from 10 healthy human donors (Table 1) before, 1 day and/or 3 days after LAIV administration, and compared lactate efflux by pDCs isolated before and after vaccination (Fig. 11*A*). Plasmacytoid DCs isolated after vaccination had greater *ex vivo* lactate accumulation and higher rates of lactate efflux compared to pDCs isolated before vaccination (Fig. 11*B* and 11*C*). We also found that pDC expression of surface HLA-DR was higher after vaccination compared to pDCs isolated before vaccination in most cases, providing evidence that pDCs were activated *in vivo* by the influenza vaccine (Fig. 11*D*). To further investigate if the pDC activation state (assessed by HLA-DR expression) was related to glycolytic flux, we measured the correlation between pDC HLA-DR expression and pDC lactate efflux. A significant positive correlation was observed between pDC HLA-DR expression and *ex vivo* lactate accumulation (Fig. 11*E*). Results from our *in vivo* vaccination model thus complement the *ex vivo* findings of viral-mediated induction of glycolysis, and highlight a potential role of glycolysis in human pDC in response to viral infections.

Table 1. *Participant information*

Age, mean (range)	32 (23-42)
Gender	
Male	5
Female	5
Ethnicity	
Non-Hispanic	10
Hispanic	0
Race	
Black	1
Asian	4
Caucasian	5
Influenza/LAIV during past 4 months	None
pDC count ($x10^5$), mean (range)	
Pre vaccination	5.4 (1.7 – 10.0)
Post day 1 vaccination	3.3 (1.9 - 5.7)
Post day 3 vaccination	5.7 (2.0 – 14.0)

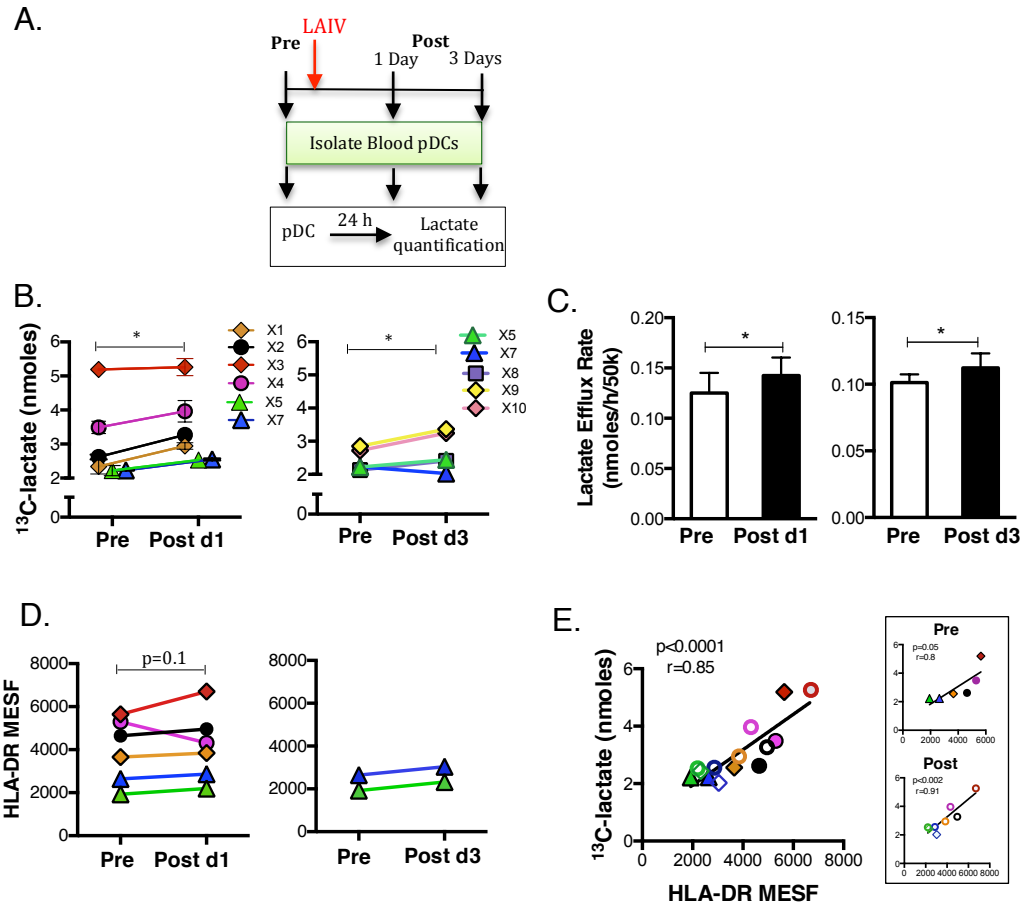

Figure 11. Influenza vaccination increases glycolysis in human pDCs. *A*, Schematic demonstrating the timing of influenza vaccination (live attenuated influenza vaccine; "LAIV") in relation to experimental measurements. *B*, Blood pDCs were purified from healthy donors before, 1 and/or 3 days after vaccination, cultured *ex vivo* for 24 h, and 13C-lactate was measured in pDC supernatants. *C*, Rate of lactate production in pDC supernatants before and 1 day and/or 3 days after vaccination. *D*, PBMCs were isolated from blood before, 1 day and/or 3 days after vaccination and stained with following fluorochrome-conjugated Abs: Lineage FITC, HLA-DR-APC-Cy7, CD123-PE and CD11c-APC. HLA-DR MESF was measured on pDCs gated from PBMCs based on Lin^- $HLA\text{-}DR^+CD123^+$ using flow cytometry. *E*, Correlation between HLA-DR MESF on blood pDCs (gated from PBMCs) and amount of lactate accumulated in purified pDC 24 h *ex vivo* cultures are shown. Inset- correlations between HLA-DR and lactate efflux pre and post vaccination. In *B* and *D*, lines connect data from individual donors In *C*, data represent mean ± SEM of 4-6 individual experiments. *$p \leq 0.05$, paired t-test.

Inhibition of Glycolysis Impairs pDC Antiviral Responses

As influenza and RV exposure induced secretion of IFN-α, phenotypic maturation (Fig. 4 and 5) and induced glycolysis in pDCs, we wanted to investigate whether glycolysis plays a role in pDC antiviral responses. To achieve this, we assessed the effect of 2-DG, a known glycolytic inhibitor. Two-deoxyglucose is a glucose analog and a competitive inhibitor of hexokinase, the first enzyme of the glycolytic pathway (126). Plasmacytoid DCs were exposed to influenza with different doses (1- 20 mM) of 2-DG and ^{13}C-lactate and IFN-α was assayed in the supernatants. Addition of 2-DG significantly reduced ^{13}C-lactate efflux in influenza-exposed pDCs and concomitantly impaired IFN-α secretion in a dose-dependent manner (Fig. 12*A*), without impacting pDC viability (Fig. 12*B*). In experiments using fixed-concentration (10 mM) 2-DG, we found a significant reduction in the influenza-, RV- and gardiquimod-induced IFN-α (64%, 54% and 79% respectively, Fig. 13*A*-13*C*) in pDCs. These results indicate that inhibition of glycolysis impairs viral-induced IFN-α production, a crucial pDC function.

In addition to secreting IFN-α on viral exposure, pDCs play a major role in developing immune responses by presenting antigen to naïve T lymphocytes. The initial process of priming T lymphocyte is mediated by the expression of MHC molecules and other costimulatory molecules on pDCs. We next investigated the role of glycolysis in maturation of pDC, in terms of increased expression of viral-induced surface markers. To assess this, surface expression of HLA-DR, CD80, CD86 and CD83 (another marker that

augments pDC and T cell interaction) was measured in the presence of 2-DG in viral- and gardiquimod-exposed pDCs. Addition of 2-DG interrupted influenza-, RV- and gardiquimod-induced HLA-DR, CD80, CD86 and CD83 expression in pDCs (Fig. 14*A*-14*D*). Collectively, our data highlight a critical role for glycolysis in pDC responses to viruses including both IFN-α production and up regulation of co-stimulatory molecules required for pDC maturation.

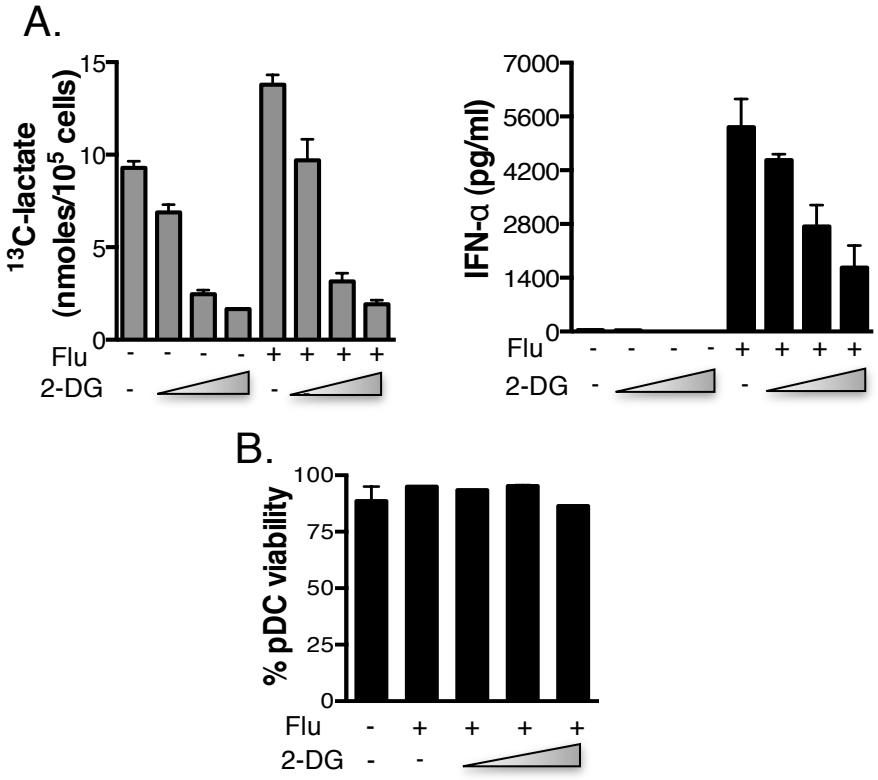

Figure 12. 2-DG, a potent inhibitor of glycolysis, interrupts influenza-induced IFN-α production in pDCs. *A*, The bar graph depicts simultaneous quantification of ^{13}C-lactate and IFN-α in supernatants of pDCs exposed to influenza with or without 1 mM, 10 mM and 20 mM 2-DG. Results are from one experiment representative of 3 individual experiments. *B*, Percent viability of pDCs treated with or without influenza in the presence of 1, 10 and 20 mM 2-DG assessed by trypan blue staining.

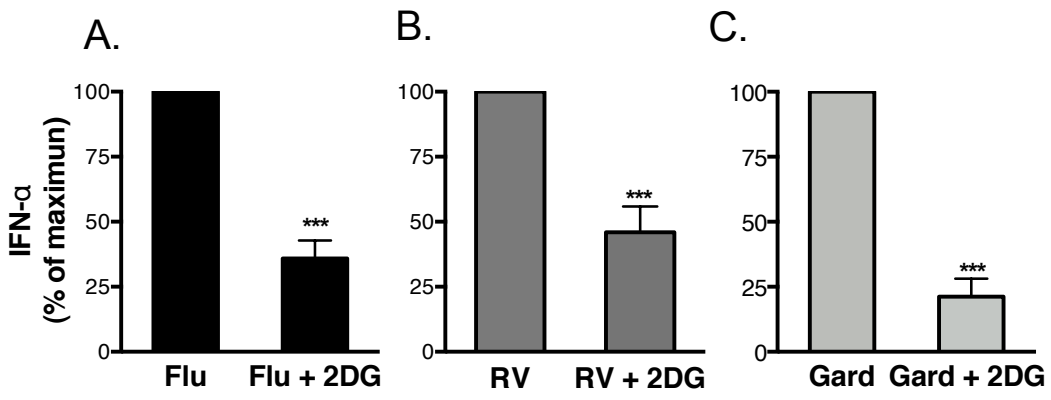

Figure 13. Inhibition of glycolysis results in impairment of viral-induced-IFN-α production in human pDCs. IFN-α concentration in supernatants of pDCs exposed to (*A*) influenza, (*B*) RV or (*C*) gardiquimod in the presence or absence of 10 mM 2-DG for 24 h assessed by ELISA. Data were normalized to maximum IFN-α induction and represent mean values ± SEM from 5 independent experiments, *** denotes $p \leq 0.001$, paired t-test.

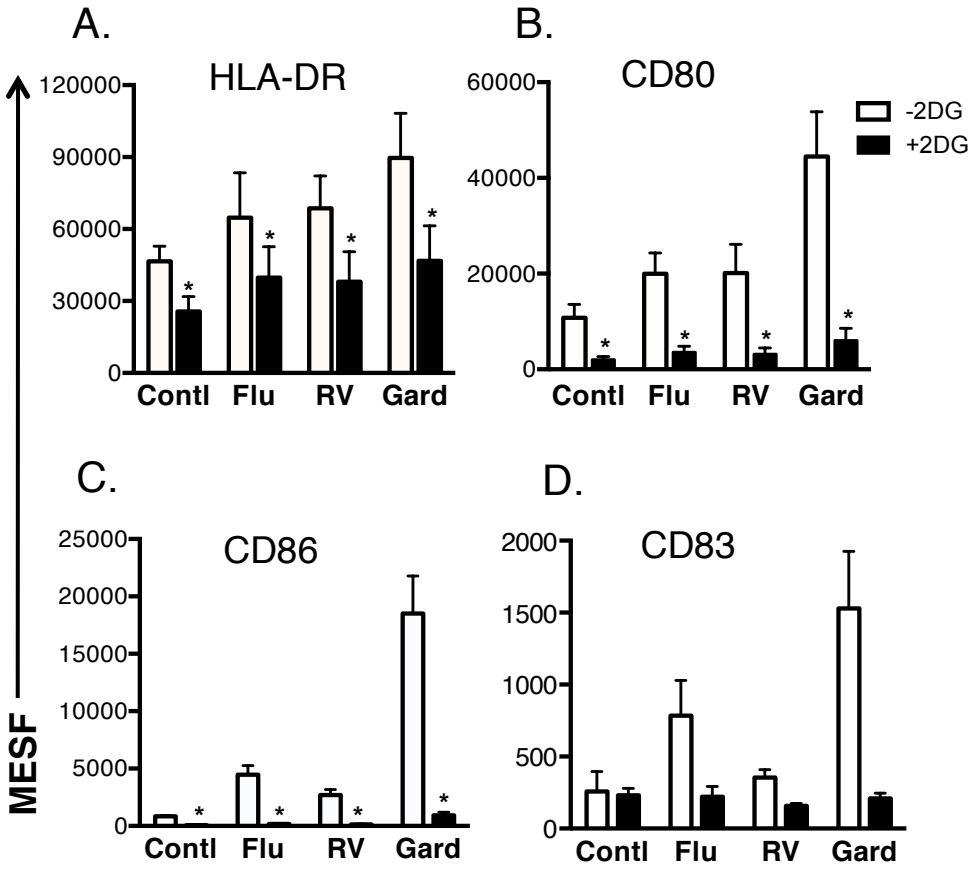

Figure 14. Inhibition of glycolysis interrupts viral-induced pDC maturation. Purified pDCs were cultured with IL-3 alone (control), influenza, RV or gardiquimod in the presence (the white bar) or absence (the black bar) of 10 mM 2-DG for 48 h. Cells were harvested, stained with HLA-DR APC-Cy7, CD80-FITC, CD86 PE-Cy5 and CD83 PE Abs, and surface expression (MESF) of (*A*) HLA-DR, (*B*) CD80, (*C*) CD86 and (*D*) CD83 was measured by flow cytometry. Results represent mean ± SEM from 3-4 independent experiments in *A*-*C* and N=2 in *D*, * $p \leq 0.05$.

Viral-Induced Glycolysis is Independent of Type I IFN Signaling in Human pDCs

To determine a potential role for type I IFN, the major chemokine group secreted by viral-activated pDCs, we measured ^{13}C-lactate in supernatants of pDCs exposed to IFN-α itself. Interestingly, there was no increase in the lactate efflux in pDCs in response to IFN-α compared to control (Fig. 15*A*). Additionally, blocking the receptor for IFN-α/β with the IFNRA blocking antibody had no impact on influenza-induced increases in lactate efflux (Fig. 15*A*), suggesting that type I IFN signaling is not required for influenza-induced glycolysis in pDCs. To investigate the role of TLR7 activation in viral-induced glycolysis, we exposed pDCs to chloroquine, an agent that interrupts TLR7 signaling by disrupting endosomal acidification. Chloroquine exposure significantly reduced both influenza and gardiquimod-induced increases in ^{13}C-lactate and IFN-α, providing evidence that TLR7 signaling likely plays a role in viral-induced up-regulation of glycolytic pathways in pDCs (Fig. 15*B*, 15*C*). However, heat inactivation of influenza virus had no impact on influenza-induced IFN-α or lactate production (Fig. 16*A*, 16*B*) confirming that replication of influenza is not essential for induction of IFN-α and glycolysis.

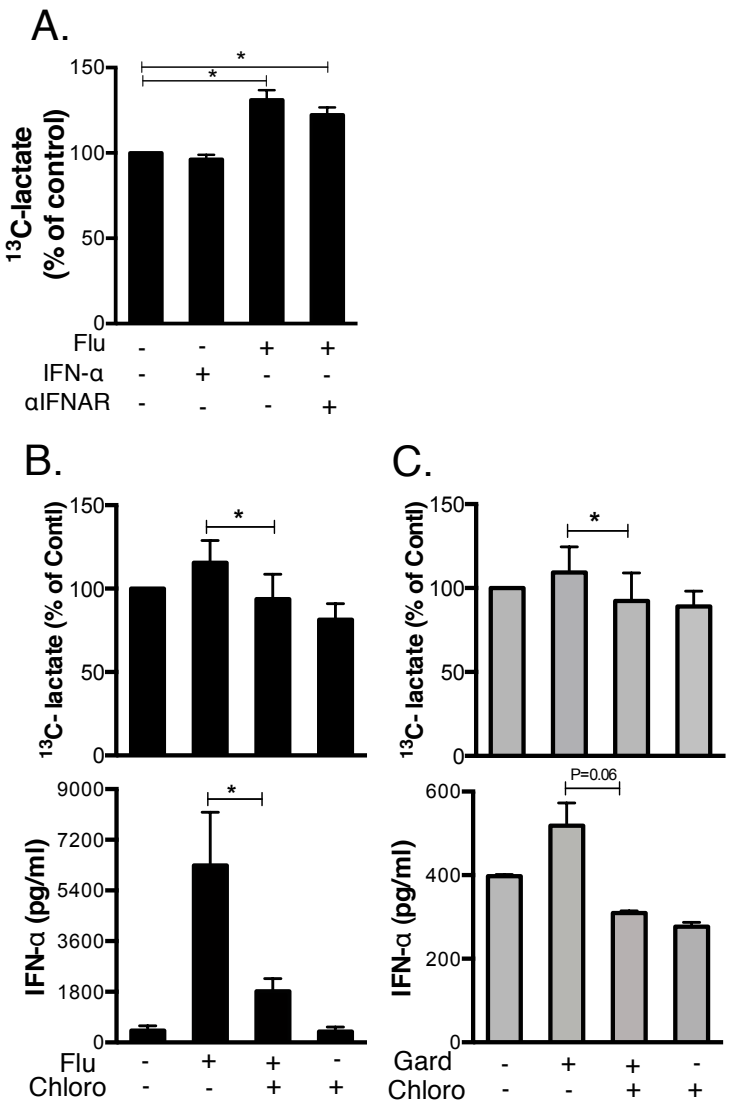

Figure 15. Viral-induced glycolysis is independent of type I IFN signaling in human pDCs. *A*, pDCs were treated with or without IFN-α (1000 U/ml) or influenza in the presence or absence of anti-human IFNAR monoclonal Ab (5 μg/ml) for 24 h and ^{13}C-lactate was quantified in the supernatants using mass spectrometry. ^{13}C-lactate amount and IFN-α in pDCs treated with (*B*) influenza or (*C*) gardiquimod with or without chloroquine (2 μg/ml) for 24 h. Lactate amounts are normalized to the control condition. Data represent mean ± SEM of 3-4 individual experiments. *$p \leq 0.05$, Two-way ANOVA followed by Tukey's test.

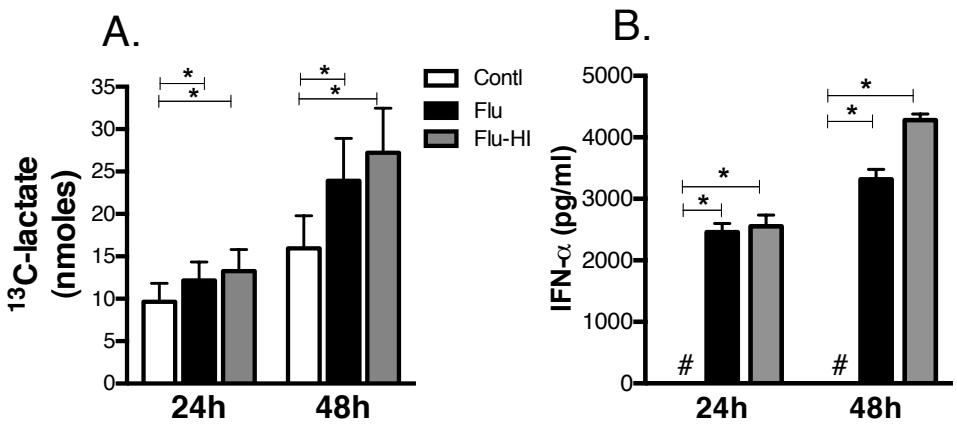

Figure 16. Heat-inactivation of influenza virus does not prevent influenza-induced lactate and IFN-α production. (*A*) Total ^{13}C-lactate quantification and (*B*) IFN-α in the supernatants of pDCs treated with or without influenza, or equal amount of heat-inactivated (HI) influenza at 24 h and 48 h time points. Data represent mean ± SEM of 3 independent experiments. *p ≤ 0.05, Two-way ANOVA followed by Tukey's test, # represents not detected.

Influenza Exposure Promotes Increased Expression and Nuclear Translocation of HIF-1α in pDCs

In mammals, HIF-1α is a principal transcription factor that controls the metabolic state of the cell (127), and most of the genes encoding glycolytic enzymes are regulated by HIF-1α (128). We next investigated the impact of influenza on pDC expression of HIF-1α. Our DNA microarray analysis revealed a significant increase in HIF-1α gene expression in influenza-exposed pDCs (Fig. 17*A*). We confirmed these findings by qRT-PCR, and observed ~2.5 fold increase in HIF-1α mRNA in influenza-exposed pDCs compared to control pDCs (Fig. 17*B*). We next evaluated the effect of influenza exposure on pDC HIF-1α protein expression and nuclear translocation. To this end, pDCs were exposed to influenza for 24 h, stained for intracellular HIF-1α Ab and HIF-1α fluorescence intensities and nuclear translocation were subsequently assessed using the ImageStream MKII quantitative imaging flow cytometer. We found a significant increase in total HIF-1α expression in pDCs exposed to influenza (Fig. 18*A*). Additionally, we observed increased HIF-1α in the nuclear compartments of influenza-treated pDCs, suggesting that influenza promotes nuclear translocation of HIF-1α, a critical step in activation of the HIF-1α pathway (Fig 18*B*, 18*C*). Collectively, these data suggest that influenza promotes HIF-1α transcription, protein expression and nuclear translocation in pDCs and provide evidence that viral-induced glycolysis in pDCs may be regulated, at least in part, by HIF-1α signaling.

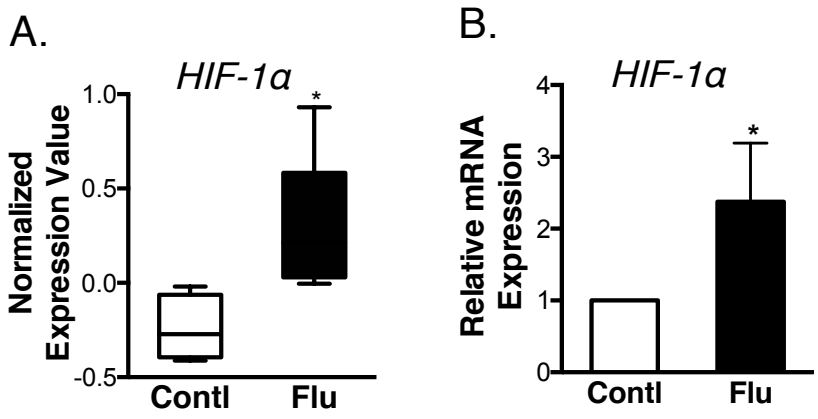

Figure 17. Influenza exposure induces *HIF-1α* gene expression in human pDCs. Purified pDCs were cultured with or without influenza for 8 h, total RNA was isolated from the cell pellets, and (*A*) DNA microarray or (*B*) qRT-PCR analysis was performed. *A*, Normalized expression values of the *HIF-1α* gene in pDCs exposed to influenza versus control conditions is shown. *B*, Induction of *HIF-1α expression* in influenza-treated versus control pDCs was confirmed by RT-PCR, using *HPRT* as an endogenous control. Data represent mean values ±SEM from 3-5 independent experiments (*A-B*), * depicts $p \leq 0.05$.

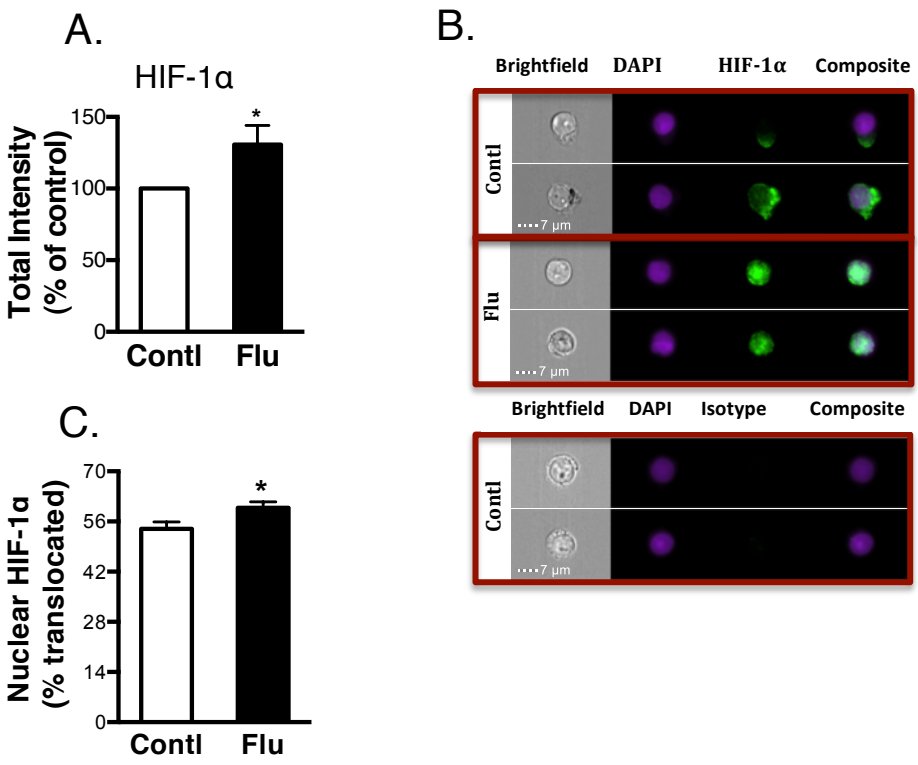

Figure 18. Influenza exposure induces expression and promotes nuclear translocation of HIF-1α in pDCs. Purified pDCs were treated with influenza for 24 h, cells were harvested and stained intracellularly with HIF-1α Dyl-488 or rabbit IgG isotype control-488 Ab and DAPI. Samples were acquired and analyzed using the ImageStream Mark II Quantitative Imaging Flow Cytometer. *A*, Total HIF-1α intensities in pDCs exposed to influenza versus control conditions at 24 h are shown as percent of control. *B*, Images comparing nuclear HIF-1α Ab or isotype control Ab staining in pDCs cultured with or without Flu are displayed. *C*, A quantitative summary of influenza-induced HIF-1α nuclear translocation in pDCs is shown (N=8-9). Data represent mean ± SEM from 8-10 independent experiments in *A*-*C*, * denotes $p \leq 0.05$.

CHAPTER IV

DISCUSSION

In this study, we determined the viral-induced metabolic changes in pDCs, a unique and rare cell type highly important for directing antiviral immune responses. Recent immunometabolism studies indicate that cellular metabolism regulates function and activation of many immune cells, but no information about metabolism in pDCs in mouse or humans is available. Our *ex vivo* results demonstrate that exposure to two common respiratory viruses, influenza and RV, in human pDCs leads to the secretion of significant amounts of IFN-α and upregulate expression of HLA-DR, CD80 and CD83 molecules. Exposure to influenza and RV, as well as TLR7 agonist gardiquimod, *ex vivo* induces glycolysis in human pDCs. A proposed model is shown in Fig. 19. Increased ATP content, up-regulation of glycolytic genes including *HK2*, *LDHA* and *PFKP*, elevated ECAR and higher lactate efflux characterized this induction. Results from our *in vivo* influenza immunization model demonstrate that vaccination with live attenuated influenza virus increases glycolytic flux in circulating pDCs, confirming our *ex vivo* results and emphasizing the biological relevance of glycolysis during human respiratory viral infections. Increased trends in the surface expression of HLA-DR molecules on pDCs after vaccination in the majority of donors verifies that pDCs are activated after *in vivo* live viral infection. In addition, we found a significant positive correlation between

HLA-DR expression and glycolytic flux in pDCs providing evidence of a link between metabolic state of pDCs and their activation during *in vivo* viral infection.

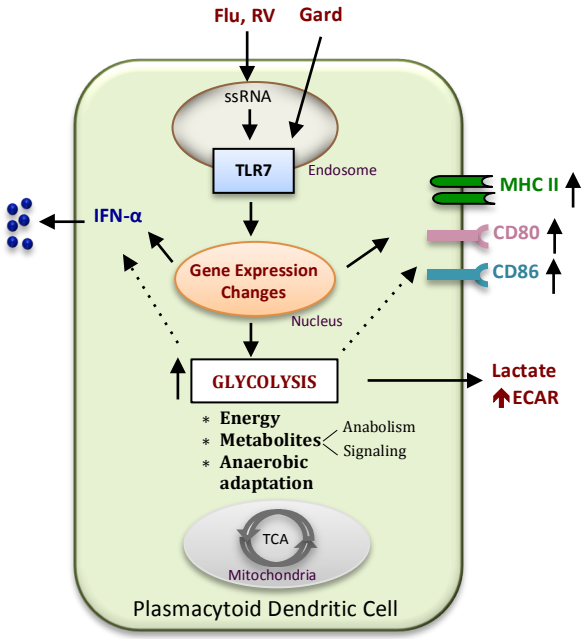

Figure. 19. Overview of viral-induced metabolic changes in human pDCs. Exposure to ssRNA respiratory viruses such as influenza and RV or TLR7 agonist, gardiquimod induces glycolysis in pDCs. This induction of glycolysis plays an important role in key pDC antiviral functions; viral-induced IFN-α secretion and up-regulation of co-stimulatory molecules such as MHC II, CD80 and CD86, necessary to prime T cells. Induction of glycolysis potentially provides rapid energy and metabolites (could be utilized for anabolism of macromolecules or for signaling) for pDC antiviral responses.

Our data reveals that *ex vivo* inhibition of glycolysis by 2-DG significantly impairs two key pDC functions: viral-induced IFN-α secretion and viral-induced activation as measured by surface expression of HLA-DR, CD80, CD86 and CD83 molecules, suggesting a vital role of glucose metabolism in pDC antiviral responses. Our type I IFN blocking experiments demonstrate that this influenza-induced glycolysis is not mediated by IFN-α, a major cytokine secreted by pDCs, and is independent of type I IFN signaling. However, chloroquine, a drug that disrupts TLR7 signaling, reduced viral-induced lactate efflux and IFN-α indicating that endosomal acidification, a requirement for TLR7 signaling is essential for directing metabolic changes in these cells. In addition, heat-inactivation of influenza did not inhibit influenza-induced pDC lactate and IFN-α production, indicating that influenza replication is not a prerequisite for enhancing glycolysis. Influenza exposure in pDCs induces expression and promotes nuclear translocation of HIF-1α, providing evidence that viral-induced glycolysis is likely mediated in part by activation of the HIF-1 pathway.

Viral-Induced pDC Activation and Glycolysis is Independent of Viral Replication

In this study, we show that heat inactivation of influenza did not interrupt influenza-induced IFN-α and influenza-induced glycolysis suggesting that both pDC activation and induction of glycolysis upon influenza exposure is independent of viral replication. Our data thus demonstrate that viral-induced IFN-α responses and metabolic changes are mediated by replication-independent mechanism of influenza in human pDCs. Previous studies have also indicated that pDCs do not support the productive

replication cycle of influenza virus and the viral-mediated immune responses are independent of influenza viral replication (72, 129-131). Dipucchio and group have demonstrated that influenza exposure results in rapid cross-presentation of internalized exogenous viral antigens via MHC I to $CD8^+$ cells. This processing of exogenous antigen occurred within the pDC endocytic vesicles and was independent of the proteasomal pathway. In addition, the endocytic vesicles stored MHC I molecules and were the sites of peptide loading onto MHC I (107). Therefore, pDCs can present antigen to $CD8^+$ T cell without the requirement of viral replication. Kumagai et al. have demonstrated that antiviral responses to Newcastle disease virus (NDV) are independent of viral replication in murine pDCs (130). This study shows that TLR7-mediated NDV recognition and positive feedback of IFN-α signaling circumvents the requirement of viral replication for IFN-α responses (130). However, the molecular mechanism explaining replication-independent recognition of viral infection and antiviral responses in pDCs is unclear.

The exact mechanism why influenza cannot replicate in pDCs is not clearly understood, and a few potential explanations are discussed here. In an epithelial cell, binding of influenza HA glycoproteins to the sialic acid residues (N-acetyl neuraminic acid) on the host cell leads to receptor-mediated endocytosis, and the virus enters the endosome. The acidic pH (5-6) of the endocytic vesicle causes cleavage of HA monomers by trypsin-like enzymes, resulting in a conformational change of HA, which then activates fusion of the viral envelope with the endosomal membrane. In addition, the low pH leads to opening of viral M2 protein ion channels, resulting in acidification of the

viral core and release of viral ribonucleoprotein (vRNP) into the cytoplasm. The viral proteins – nucleoprotein (NP), polymerase acidic (PA), polymerase basic 1 (PB1) and PB2 have nuclear localization signals that direct the associated viral RNA (vRNA, - strand) into the nucleus, where RNA-dependent RNA polymerase (RdRp) initiates synthesis of complementary mRNA (+ strand) and vRNA. The viral mRNA is exported to the cytoplasm and is translated, followed by interaction of viral proteins with vRNA to form vRNP. Influenza virus uses the host plasma membrane to form its envelope and buds off from the host cell (132). Therefore, influenza replication in pDCs could potentially be obstructed at any of the following steps beyond the uptake of virus into the endocytic vesicles: i) M2 ion channel, preventing the release of vRNP into the cytoplasm; ii) transport of vRNA into the nucleus; iii) transcription of viral mRNA by RdRp; iv) replication of viral RNA; v) transport of vRNA or viral mRNA into the cytoplasm; vi) translation of viral proteins; vii) assembly of viral proteins with vRNA; or viii) budding off the viral particles from pDCs. Plasmacytoid DCs secrete vast amounts of IFNs upon TLR7 activation and constitutively express transcription factor IRF7, a master regulator of type I IFN production. The inability of influenza to replicate in pDCs could potentially be mediated by components of the TLR7 pathway such as MyD88 or IRF7. Kumagai et al. have shown that TLR signaling and IFNR feedback suppresses NDV replication in murine pDCs (130). A study by Smed-Sorensen and group has shown that constitutive expression of myxovirus resistant protein 1 (MxA) in unstimulated pDCs is higher compared to the other cell types such as mDCs (129). MxA protein is an antiviral protein

that oligomerizes and targets the nucleocapsid of virus and prevents viral transcription and replication (133, 134). The higher expression of MxA even without the viral exposure could hinder that transcription and replication of influenza genome obstructing viral replication in pDCs.

However, not much is known about the replication of RV in pDCs. Experiments conducted in our laboratory in collaboration with Dr. James Gern, University of Wisconsin, reveal that RV replicate poorly in pDCs (unpublished data).

Manipulation of Glucose Metabolism by Viruses

Many oncogenic and non-oncogenic viruses are known to modulate glucose metabolism (115, 117, 135-137). For example, HSV-1 activates glycolysis in Vero cells by inducing 6-phosphofructo-1-kinase (PFK-1), a glycolytic enzyme (117). HCMV up-regulates expression of glucose transporter 4 to increase the uptake of glucose and elevates glycolytic metabolism by calmodulin-KK-dependent activation of PFK-1 in human fibroblasts (135, 136). KSHV infection induces aerobic glycolysis and decreases oxygen consumption in endothelial cells (115). A gene product of adenovirus, E4ORF1, promotes increased anabolic glucose metabolism in epithelial cells, leading to increased glucose consumption, higher lactate production and increased transcription of glycolytic genes including *HK2* and *PFKM* (137). Most of these studies have been done in non-immune cells, however, how these and other viruses impact glucose metabolism in immune cells is unknown. Why these viruses regulate metabolism is also unclear. Some studies suggest that the switch from OXPHOS to glycolysis might be advantageous for

viruses, resulting in increased production of biomass, including fatty acids, nucleotides and lipids, necessary to promote viral replication and budding of enveloped viruses (115, 135). In our study, the similar induction of glycolysis in pDCs exposed to heat-inactivated influenza indicate that viral replication is not required for this metabolic transition in these cells, raising the possibility that this influenza-induced glycolytic adaptation is potentially also beneficial to the pDC itself. However with low m.o.i. of influenza used in our experiments, viral-induced metabolic changes could also possibly be mediated by influenza virion proteins such as HA or NA and need to be investigated further.

Potential Benefits of Glycolysis in pDC Antiviral Responses

Increasing glycolytic flux upon viral encounter could be advantageous to pDCs for several reasons. First, secretion of high concentrations of IFN-α rapidly upon viral encounter represents an energy-demanding process, and higher glycolytic flux could allow a pDC to generate ATP more quickly although less efficiently than the OXPHOS pathway (112, 113). Second, increased glycolysis could support the anabolic demands of viral-exposed pDCs by providing intermediate precursors for the biosynthesis of nucleotides (via the pentose phosphate pathway, PPP), non-essential amino acids and lipids. In support of this concept, a study of murine moDCs demonstrated that TLR agonist-induced anabolic demands were met by early induction of glycolysis in these cells. This facilitated the *de novo* synthesis of fatty acids, required for the expansion of endoplasmic reticulum and Golgi apparatus, both integral for protein synthesis and

secretion during moDC activation (119). Elevation of glycolysis could thus serve as a mechanism employed by pDCs to similarly meet increasing metabolic demands encountered during viral infections. In our experiments, viral exposure did not increase mitochondrial respiration in pDCs, providing further evidence that these cells preferentially utilize glycolysis and not OXPHOS as a main pathway to fulfill viral-induced elevated energy requirements.

Many enzymes involved in glycolysis, the Krebs cycle and other metabolic pathways have been shown to have dual functions; besides driving metabolic pathways, these enzymes can regulate gene expression by binding to specific target mRNA (138). For example, glyceraldehyde-3-phospahtase (GAPDH), a glycolytic enzyme, has been reported to regulate posttranscriptional production of IFN-γ in T cells (139). From this perspective, a third potential benefit of increased glycolysis in viral-exposed pDCs could be a link between glycolysis and the IFN-α pathway itself. Glycolytic enzyme(s) could potentially be involved in posttranscriptional regulation of pDC IFN-α. Furthermore, metabolites generated within the cell can also act as signaling molecules; the availability of these intermediates can thus influence both cellular metabolism and signaling cascades (140). For example, succinate, a Krebs cycle intermediate, can also act as an inflammatory signal by inducing IL-1β in mouse macrophages (141). We speculate that the positive oxidative redox potential (high NAD^+/NADH ratio) generated in pDCs due to elevated glycolysis or even lactate produced could result in activation of transcription factors involved in IFN-α synthesis. Therefore, a viral-induced alteration in cell

metabolism could be advantageous to pDCs by providing additional mechanisms to regulate the production of innate cytokines (IFN-α) and expression of key surface co-stimulatory molecules (MHC II, CD80, CD86 and CD83).

Metabolic Regulation of Immune Responses

Metabolic reprogramming, involving a switch from one metabolic state to the other, for example, a switch from OXPHOS to glycolysis or vice versa, has been intricately linked to myeloid DC and T cell activation, yet nothing was known about its contribution to the activation of pDCs. In mouse mDCs, TLR4 agonists induce transition from OXPHOS to glycolysis, which is essential for DC maturation and function (118). Early induction of glycolysis is required to fulfill the anabolic demands of TLR agonist-activated murine moDCs (119). Recently, it has been demonstrated by Malinarich and group that human tolerogenic myeloid DCs show metabolic phenotype of high OXPHOS and glycolytic capacity (120).

In T cells, the effector functions are dependent on metabolic reprogramming and adopting a correct metabolism is important for a cell to perform specific effector function (109). T cell receptor (TCR) signaling directs metabolic changes in T cells, leading to a shift to aerobic glycolysis, higher nutrient uptake and reduced mitochondrial OXPHOS, essential for effector T cell proliferation and function (108, 109, 121). Our data provide evidence of induction of glycolysis upon viral exposure in pDCs and demonstrate that inhibition of glycolysis significantly impairs influenza and RV-induced IFN-α secretion and maturation responses in these cells. MHC II, CD80 and CD86 molecules on pDCs

are crucial for priming Th cells (interacts with CD4 and CD28 receptor on Th cells). Inhibition of glycolysis suppresses the ability of viral-exposed pDCs to upregulate expression of these maturation markers and would potentially impact adaptive immune responses to viruses. Therefore, changes in glucose metabolism play an important role in modulating pDC antiviral responses and could be potentially beneficial to pDCs during viral infections.

Regulation of Viral-Induced Metabolic Changes in pDCs

Role of type I IFN signaling

Type I IFNs represent a critical component of pDC-mediated functions; a potential role for these innate cytokines in viral-induced glycolysis in pDCs thus seemed plausible. In support of this possibility is the recent demonstration that type I IFN is required for the metabolic shift from OXPHOS to glycolysis after poly I:C stimulation in mouse mDCs (142). Another study in mouse embryonic fibroblasts indicates that IFN-β modulates glucose metabolism, which is important for rapid antiviral responses against coxsackievirus B3 infection (143). In contrast, our data reveal that type I IFN signaling is not required for viral-induced glycolysis in pDCs. Moreover, blockade of IFN-α signaling itself did not interrupt influenza-induced glycolysis in pDCs, confirming that IFN signaling does not regulate the viral-induced glycolysis in these cells. This observed difference in regulation of DC metabolism could be attributable to species variations (human versus mouse), DC subset differences (mDCs versus pDCs) or even in the ways different TLRs are activated in these cells.

In our experiments, chloroquine, a drug that disrupts TLR7 signaling, inhibited viral-induced lactate efflux and IFN-α secretion in pDCs. Chloroquine inhibits endosomal acidification, a prerequisite for the activation of endosomal TLRs, and interferes with binding of viral nucleic acid to TLRs (144, 145). Our data thus provide additional evidence that endosomal acidification, a requirement for the interaction of ssRNA or gardiquimod with TLR7, is an essential component of these metabolic changes in pDCs. However, additional studies evaluating inhibitors with greater specificity are needed to define the specific components of the TLR7 pathway required for induction of glycolysis in pDCs.

Role of HIF-1α signaling

We further investigated mechanism(s) by which human pDCs modulate metabolism upon viral encounter by studying the effects of influenza on the hypoxia pathway. A central role for HIF-1α in regulating glycolysis induction is well established in cancerous cells and has also been observed during inflammation and viral infections (111, 146-148). HIF-1α is a principal transcriptional regulator of cellular metabolism, induced under hypoxic (low O_2) conditions and mediates up-regulation of enzymes of the glycolytic pathway (128). HIF is composed of an oxygen labile α-subunit, which exists in three forms, -1α, -2α and -3α, and a constitutive active β–subunit. Under normoxic conditions, HIF-1α is hydroxylated at the conserved prolyl residues by prolyl hydroxylases (PHD) and is degraded via ubiquitin-mediated proteasomal destruction (149). However, under hypoxic conditions, degradation of HIF-1α is prevented, leading

to its stabilization and translocation into the nucleus. HIF-1α along with HIF-1β and other transcription factors binds hypoxia response elements (HRE) and initiates transcription of various genes including many involved in glycolysis (150). In this study, we demonstrate that influenza induces HIF-1α transcription, nuclear translocation and increased expression of HIF-responsive genes (*HK2* and *LDHA*) in pDCs. Our findings add to the growing body of knowledge about viral induction of the hypoxia pathway in pDCs.

HIF-1α is induced by a variety of DNA viruses such as vaccinia virus, Epstein-Barr virus (EBV), human papillomaviruses (HPV), KSHV, varicella zoster virus (VZV) and RNA viruses such as hepatitis C virus (HCV), human immunodeficiency virus (HIV) and respiratory syncytial virus (RSV). For example, the C16 protein of vaccina virus promotes stabilization and nuclear translocation of HIF-1α in HeLa cells and leads to the activation of HIF-responsive genes including *GLUT1* and *VEGF* (151). Vaccinia C16 mediates HIF-1α stabilization by binding to the oxygen-sensing domain of prolyl-hydroxylase and inhibits hydroxylation of HIF-1α. EBV transformed human B-cells stabilize HIF-1α at the protein level leading to elevated production of lactate, lactate dehydrogenase and pyruvate (152). Similar stabilization of HIF-1α is seen with HPV as well; a keratinocyte-derived cell line expressing HPV31 genome shows elevated levels of HIF-1α protein stability and increased expression of HIF-1α target genes including *GLUT1* and *VEGF* (153). KSHV infection activates HIF-1α (154) and induces aerobic glycolysis (115). HCV, a ssRNA virus, impairs mitochondrial OXPHOS, which consequently promotes stabilization of HIF-1α and up-regulates glycolytic gene

expression in a human hepatoma cell line (155). The Vpr protein of HIV induces expression and accumulation of HIF-1α in neural cells (156). RSV promotes stabilization of HIF-1α in bronchial epithelial cells via inducing nitric oxide (NO) (157). HIF-1α activation has also been detected in biopsies of human skin infected with KSHV and VZV (147), and in liver biopsy samples form HCV-infected patients (155).

HIF-α has also been shown to play a role in immune responses. Innate immune response is another stress response (158) that has been linked to the hypoxic pathway. In mice, HIF-1α has been reported to be essential for the regulation of glycolysis in macrophages and neutrophils. HIF-1α null macrophages exhibited reduced cellular ATP pools, resulting in a metabolic defect and profound impairment in cell migration and their ability to kill intracellular bacteria (148). In one study, *in vivo* infection of macaques with a highly pathogenic H5N1 influenza strain induced HIF-1α expression in peripheral blood leukocytes, lung macrophages and liver macrophages (159). Our data corroborate and extend these findings in human pDCs and indicate a potential role for HIF-1α in pDCs immune response to viral infections. Plasmacytoid DCs are recruited to the site of inflammation or infection (54, 160), where the oxygen and nutrient levels are lower than the blood (161). The induction of HIF-1 could thus promote antiviral functions of pDCs in such anaerobic microenvironments by maintaining energy homeostasis and regulating glycolysis.

The molecular mechanism leading to the induction of HIF-1α in immune cells is not well understood. A study by Rius and group has demonstrated that NF-κβ, a

transcription factor of the innate immune response, is a critical activator of HIF-1α in mouse macrophages (162). NF-κβ activation is regulated by IκB kinases (IKK) including IKK-β. This report showed that IKK regulates HIF-1α and HIF-responsive genes in mouse macrophages. In pDCs, the precise mechanism by which influenza induces and stabilizes HIF-1α is unclear. However, there are several possible mechanisms by which influenza could mediate induction of HIF-1α in human pDCs. Induction of HIF-1α could be facilitated by i) influenza virion proteins such as HA or NA, because the virus does not replicate in pDCs; ii) the interaction of viral genome with TLR7 in the endocytic vesicle; iii) activation of components of the TLR7 pathway such as MyD88, IRF7 or by NF-κβ as in LPS-stimulated mouse macrophages (162); or iv) by a mechanism independent of TLR7 signaling, such as by binding of viral ribonucleoprotein to the PHD containing protein, inhibiting the hydroxylation of HIF and promoting its stabilization as induced by VV (151) or by binding of viral protein to HIF-1α itself leading to its stabilization. These proposed mechanisms of influenza-induced HIF-1α could be characterized by i) investigating the induction of HIF-1α in pDCs in the presence of viral proteins such as HA, NA or ribonucleoprotein; or ii) by using specific inhibitors or small interfering RNA (siRNA) molecules against TLR7, MyD88, IRF7 and NF-κβ. Further studies are therefore needed to better understand how influenza regulates HIF-1α induction and to confirm the role of HIF-1α in antiviral immune responses in human pDCs.

Significance and Future Directions

The significance of our findings is underscored by the results from our *in vivo* LAIV immunization experiments, demonstrating induction of glycolysis in pDC after live virus infection and confirming our *ex vivo* data. We observed increased trends in surface expression of HLA-DR on pDCs in majority of the donors after vaccination suggesting that pDCs were activated upon *in vivo* influenza exposure. A significant positive correlation between HLA-DR expression on pDCs and their glycolytic flux provides further evidence that function/maturation responses and metabolic activity are linked in these immune cells. These data provide the first indication that pDCs potentially utilize glycolysis *in vivo* to perform key antiviral functions such as up-regulation of co-stimulatory molecules necessary for priming T cells and initiating optimal adaptive immune responses. These findings could have potential implication in vaccine therapy. Plasmacytoid DCs have been shown to play a pivotal role in response to various preventive vaccines including yellow fever virus and influenza (163-166). For example, depletion of pDCs in mice before and during whole-inactivated influenza vaccination leads to reduction in influenza-specific cytotoxic T lymphocytes (CTL) suggesting their role in promoting a robust CTL response to influenza vaccine (166). One *in vitro* study has indicated that pDCs are important for generation of plasma cells and influenza-specific IgG production, suggesting a key role of pDCs in generating humoral response to influenza (102). In mice, type I IFN acts as a powerful adjuvant for vaccination against influenza by promoting significant increases in influenza-specific antibody titers and

induction of long-lasting immune responses (101, 167). Targeted manipulation of glycolysis in pDCs by HIF inducers like cobalt sulfate heptahydrate, known to induce HIF-1α activity in human cell lines (168), or TLR7 agonists such as gardiquimod or imiqumod, could potentially improve viral vaccine responses. Such studies are complex, and would require either infusion of pDCs activated *ex vivo*, or specific *in vivo* targeting of pDCs. Autologous pDCs could be manipulated *ex vivo* and could be re-infused in the patients. These infusion techniques have been safely utilized in several human studies, such as in DC vaccine trials in leukemia patients (169) and in $CD4^+$ T–cell transfer therapy in HIV patients (170). Alternatively, to specifically target pDCs, the therapeutic molecules could be encapsulated inside liposomes linked to fragments of the antibody against BDCA-4 antigen, expressed particularly by pDCs. This technique has already been explored in targeted drug delivery in cancer patients (171). In addition, glucose infusion therapy during vaccination (mucosal or intranasal) represents another potential strategy since this would fuel the glycolysis pathway. This strategy does not represent a pDC-specific targeting because it would fuel glycolysis in all cells, but from DC standpoint, it could potentially be beneficial for vaccine improvement.

Another significance of our work linking pDC metabolism to its immune function could also provide potential strategies for modulating antiviral responses to viruses. The TLR7 agonists resiquimod and imiquimod are currently used to treat human cutaneous viral infections (172); it is postulated that their antiviral effects are mediated by secretion of IFN-α and other innate cytokines (173). Our data suggest that modulation of the

antiviral activity induced by these TLR7 agonists is perhaps regulated by glucose metabolism, and reveal additional (metabolic) pathways that could be potentially targeted to impact antiviral responses *in vivo*. In clinical conditions such as the WHIM (warts, hypogammaglobulinemia, infections, myelokathexis) syndrome (174) and the dedicator of cytokinesis 8 (DOCK8) deficiency syndrome (175), increased viral infections are associated with defects in pDC numbers. Patients with allergic asthma are more susceptible to viral-associated-asthma exacerbations (176), in part due to deficient viral-induced pDC IFN-α responses (177). Theoretically, targeting pDC metabolism in these clinical scenarios via TLR7 agonists and/or HIF inducers could be investigated as a potential strategy to augment antiviral responses. However, higher glycolytic flux results in increased lactate levels and lower blood pH, which could be disadvantageous to the host cell in certain disease conditions when lactic acid is not cleared.

On the other hand, strategies such as inhibition of glycolysis with 2-DG, approved for its safe use in patients with solid tumors (178), could be investigated to potentially control excessive inflammation, such as in patients with systemic lupus erythematosus (SLE), where excessive pDC IFN-α secretion has been hypothesized to contribute to disease pathogenesis (179), or with chronic viral infections with dysregulated type I IFN production (86). Further investigating the effects of TLR7 and TLR9 activation on DC (pDCs and mDCs) metabolism and function will be intriguing, as it would provide novel therapies for other RNA and DNA viruses, bacterial infections such as *Staphylococcus*

aureus and *Escherichia coli* (sensed by TLR9) (180, 181) and strengthen the role of metabolism in human disease.

Limitations

Demonstration of metabolic pathway induction in rare primary human immune cells signifies a major strength of our study. However, there were several limitations in this study. pDCs represent a minuscule fraction (about 0.1-0.4%) of circulating blood cells (182); the limited number available from human donors poses a huge challenge in working with these cells, restricting both the number of assessable targets and the scope of achievable experiments. For example, although we hypothesize that viral-induced glycolysis in pDCs may support pDC functions by fueling other anabolic pathways such as the PPP or fatty acid synthesis, we were unable to demonstrate this concept in our study model. This could have been achieved by quantifying intermediate metabolic precursors of these anabolic pathways which require at least 1-2 million pDCs for analysis. We were unable to measure any of the metabolite precursor macromolecules such as acetyl CoA (utilized for fatty acid synthesis), oxaloacetate or citrate in our system.

Although our data clearly demonstrate that TLR7 stimulation itself induces glycolysis in human pDCs, the specific component(s) of TLR7 signaling that potentially contributes to viral-induced pDC up-regulation of glycolysis needs to be further investigated. Our data from the chloroquine experiments suggest that the interaction between ssRNA and the endosomal TLR represents one essential component of this

effect on pDC metabolism. It is also possible, however, that the chloroquine effects we observed were not specific for the endosome/TLR7 and that it impacted other processes in the cells such as inhibition of DNA and RNA polymerases (183). Inhibition of RNA polymerase by chloroquine could also possibly hinder transcription of genes encoding IFN-α or glycolytic pathway. Utilization of techniques such as post-transcriptional gene silencing with siRNA targeting the TLR7 represents one potential method to address such questions (184). Such studies to address these limitations were impractical in our system due to the limited availability of the starting material.

Lastly, while our data reveal induction and activation of the HIF-1α pathway in influenza-exposed pDCs, the precise regulatory role(s) of HIF-1α in glycolysis and antiviral responses in pDCs needs further investigation. These questions could have been answered by knocking down HIF-1α in human pDCs using siRNA and analyzing influenza-induced glycolysis (including quantification of *HK2*, *LDHA* and lactate) and antiviral responses (such as IFN-α production and upregulation of costimulatory molecules). Our data support that influenza-induced glycolysis is independent of IFN-α signaling pathway. However, determining the role of IFN-α in HIF-1α induction and stabilization could provide additional insight on crosstalk between HIF-1 and type I IFN signaling pathways. Plasmacytoid DC cell lines such as GEN2.2 (185) could serve as a valuable resource in future immunometabolism experiments, but were beyond the scope of our current study.

Despite these limitations, to our knowledge this study is the first to demonstrate a role for alterations in the glucose metabolism in human pDC antiviral immune responses. Manipulation of metabolism in these immune cells represents a potential target for modulation of immune responses to both vaccines and infections with viral pathogens such as influenza and RV. In addition, understanding and modulating pDC metabolism could also be potentially exploited in other areas of human disease, such as in the development of therapies for bacterial infections, allergic asthma and autoimmune diseases such as SLE, and in the development of cancer vaccines.

BIBLIOGRAPHY

1. Mizgerd, J. P. 2006. Lung infection--a public health priority. *PLoS medicine* 3: e76.
2. Murray, C. J., and A. D. Lopez. 2013. Measuring the global burden of disease. *The New England journal of medicine* 369: 448-457.
3. World Lung Foundation. 2010. The Acute Respiratory Infections Atlas. *BMJ (Clinical research ed.)*.
4. Vissers, M., R. de Groot, and G. Ferwerda. 2014. Severe viral respiratory infections: are bugs bugging? *Mucosal Immunol* 7: 227-238.
5. Anaïs Legand, S. B., Nikki Shindo, W Abdullah Brooks, Menno D de Jong, Jeremy Farrar, Ximena Aguilera, and Frederick G Hayden. 2013. *Addressing the public health burden of respiratory viruses: the Battle against Respiratory Viruses (BRaVe) Initiative*, Future Virology.
6. Jacobs, S. E., D. M. Lamson, K. St George, and T. J. Walsh. 2013. Human rhinoviruses. *Clinical microbiology reviews* 26: 135-162.
7. Rambaut, A., O. G. Pybus, M. I. Nelson, C. Viboud, J. K. Taubenberger, and E. C. Holmes. 2008. The genomic and epidemiological dynamics of human influenza A virus. *Nature* 453: 615-619.
8. Cox, N. J., and K. Subbarao. 2000. Global epidemiology of influenza: past and present. *Annual review of medicine* 51: 407-421.

9. Lin, T. Y., and A. L. Brass. 2013. Host genetic determinants of influenza pathogenicity. *Current opinion in virology* 3: 531-536.

10. Dawood, F. S., A. D. Iuliano, C. Reed, M. I. Meltzer, D. K. Shay, P. Y. Cheng, D. Bandaranayake, R. F. Breiman, W. A. Brooks, P. Buchy, D. R. Feikin, K. B. Fowler, A. Gordon, N. T. Hien, P. Horby, Q. S. Huang, M. A. Katz, A. Krishnan, R. Lal, J. M. Montgomery, K. Molbak, R. Pebody, A. M. Presanis, H. Razuri, A. Steens, Y. O. Tinoco, J. Wallinga, H. Yu, S. Vong, J. Bresee, and M. A. Widdowson. 2012. Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study. *The Lancet infectious diseases* 12: 687-695.

11. Prchal, M., A. Pilz, O. Simma, K. Lingnau, A. von Gabain, B. Strobl, M. Muller, and T. Decker. 2009. Type I interferons as mediators of immune adjuvants for T- and B cell-dependent acquired immunity. *Vaccine* 27 Suppl 6: G17-20.

12. Bracci, L., I. Canini, S. Puzelli, P. Sestili, M. Venditti, M. Spada, I. Donatelli, F. Belardelli, and E. Proietti. 2005. Type I IFN is a powerful mucosal adjuvant for a selective intranasal vaccination against influenza virus in mice and affects antigen capture at mucosal level. *Vaccine* 23: 2994-3004.

13. McCullers, J. A., T. Saito, and A. R. Iverson. 2004. Multiple genotypes of influenza B virus circulated between 1979 and 2003. *Journal of virology* 78: 12817-12828.

14. Watts, G. 2009. A/H1N1 influenza virus: the basics. *BMJ (Clinical research ed.)* 339: b3046.

15. Hers, J. F. 1966. Disturbances of the ciliated epithelium due to influenza virus. *The American review of respiratory disease* 93: Suppl:162-177.

16. Uiprasertkul, M., P. Puthavathana, K. Sangsiriwut, P. Pooruk, K. Srisook, M. Peiris, J. M. Nicholls, K. Chokephaibulkit, N. Vanprapar, and P. Auewarakul. 2005. Influenza A H5N1 replication sites in humans. *Emerging infectious diseases* 11: 1036-1041.

17. Matrosovich, M. N., T. Y. Matrosovich, T. Gray, N. A. Roberts, and H. D. Klenk. 2004. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proc Natl Acad Sci U S A* 101: 4620-4624.

18. Couch, R. B. 2008. Seasonal inactivated influenza virus vaccines. *Vaccine* 26 Suppl 4: D5-9.

19. Fiore, A. E., C. B. Bridges, and N. J. Cox. 2009. Seasonal influenza vaccines. *Current topics in microbiology and immunology* 333: 43-82.

20. Couch, R. B., R. L. Atmar, T. R. Cate, J. M. Quarles, W. A. Keitel, N. H. Arden, J. Wells, D. Nino, and P. R. Wyde. 2009. Contrasting effects of type I interferon as a mucosal adjuvant for influenza vaccine in mice and humans. *Vaccine* 27: 5344-5348.

21. Gwaltney, J. M., Jr. 1985. Virology and immunology of the common cold. *Rhinology* 23: 265-271.

22. Winther, B. 2011. Rhinovirus infections in the upper airway. *Proceedings of the American Thoracic Society* 8: 79-89.

23. Greenberg, S. B. 2003. Respiratory consequences of rhinovirus infection. *Archives of internal medicine* 163: 278-284.

24. Gern, J. E. 2010. The ABCs of rhinoviruses, wheezing, and asthma. *Journal of virology* 84: 7418-7426.

25. Gern, J. E. 2015. How rhinovirus infections cause exacerbations of asthma. *Clin Exp Allergy* 45: 32-42.

26. Durrani, S. R., D. J. Montville, A. S. Pratt, S. Sahu, M. K. DeVries, V. Rajamanickam, R. E. Gangnon, M. A. Gill, J. E. Gern, R. F. Lemanske, Jr., and D. J. Jackson. 2012. Innate immune responses to rhinovirus are reduced by the high-affinity IgE receptor in allergic asthmatic children. *J Allergy Clin Immunol* 130: 489-495.

27. Ruuskanen, O., E. Lahti, L. C. Jennings, and D. R. Murdoch. 2011. Viral pneumonia. *Lancet* 377: 1264-1275.

28. Greve, J. M., G. Davis, A. M. Meyer, C. P. Forte, S. C. Yost, C. W. Marlor, M. E. Kamarck, and A. McClelland. 1989. The major human rhinovirus receptor is ICAM-1. *Cell* 56: 839-847.

29. Hofer, F., M. Gruenberger, H. Kowalski, H. Machat, M. Huettinger, E. Kuechler, and D. Blaas. 1994. Members of the low density lipoprotein receptor family

mediate cell entry of a minor-group common cold virus. *Proc Natl Acad Sci U S A* 91: 1839-1842.

30. Marlovits, T. C., C. Abrahamsberg, and D. Blaas. 1998. Very-low-density lipoprotein receptor fragment shed from HeLa cells inhibits human rhinovirus infection. *Journal of virology* 72: 10246-10250.

31. Khan, A. G., J. Pichler, A. Rosemann, and D. Blaas. 2007. Human rhinovirus type 54 infection via heparan sulfate is less efficient and strictly dependent on low endosomal pH. *Journal of virology* 81: 4625-4632.

32. Khan, A. G., A. Pickl-Herk, L. Gajdzik, T. C. Marlovits, R. Fuchs, and D. Blaas. 2011. Entry of a heparan sulphate-binding HRV8 variant strictly depends on dynamin but not on clathrin, caveolin, and flotillin. *Virology* 412: 55-67.

33. Bochkov, Y. A., A. C. Palmenberg, W. M. Lee, J. A. Rathe, S. P. Amineva, X. Sun, T. R. Pasic, N. N. Jarjour, S. B. Liggett, and J. E. Gern. 2011. Molecular modeling, organ culture and reverse genetics for a newly identified human rhinovirus C. *Nature medicine* 17: 627-632.

34. Koyama, S., K. J. Ishii, C. Coban, and S. Akira. 2008. Innate immune response to viral infection. *Cytokine* 43: 336-341.

35. Kohlmeier, J. E., and D. L. Woodland. 2009. Immunity to respiratory viruses. *Annu Rev Immunol* 27: 61-82.

36. Pulendran, B., K. Palucka, and J. Banchereau. 2001. Sensing pathogens and tuning immune responses. *Science* 293: 253-256.

37. Steinman, R. M., M. Pack, and K. Inaba. 1997. Dendritic cells in the T-cell areas of lymphoid organs. *Immunological reviews* 156: 25-37.

38. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu Rev Immunol* 18: 767-811.

39. Belz, G. T., and S. L. Nutt. 2012. Transcriptional programming of the dendritic cell network. *Nat Rev Immunol* 12: 101-113.

40. Steinman, R. M., and H. Hemmi. 2006. Dendritic cells: translating innate to adaptive immunity. *Current topics in microbiology and immunology* 311: 17-58.

41. Liu, Y. J. 2001. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* 106: 259-262.

42. van de Laar, L., P. J. Coffer, and A. M. Woltman. 2012. Regulation of dendritic cell development by GM-CSF: molecular control and implications for immune homeostasis and therapy. *Blood* 119: 3383-3393.

43. Liu, Y. J. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* 23: 275-306.

44. Blom, B., S. Ho, S. Antonenko, and Y. J. Liu. 2000. Generation of interferon alpha-producing predendritic cell (Pre-DC)2 from human CD34(+) hematopoietic stem cells. *J Exp Med* 192: 1785-1796.

45. Gilliet, M., A. Boonstra, C. Paturel, S. Antonenko, X. L. Xu, G. Trinchieri, A. O'Garra, and Y. J. Liu. 2002. The development of murine plasmacytoid dendritic

cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *J Exp Med* 195: 953-958.

46. Ishikawa, F., H. Niiro, T. Iino, S. Yoshida, N. Saito, S. Onohara, T. Miyamoto, H. Minagawa, S. Fujii, L. D. Shultz, M. Harada, and K. Akashi. 2007. The developmental program of human dendritic cells is operated independently of conventional myeloid and lymphoid pathways. *Blood* 110: 3591-3660.

47. Reizis, B. 2010. Regulation of plasmacytoid dendritic cell development. *Current opinion in immunology* 22: 206-211.

48. Cisse, B., M. L. Caton, M. Lehner, T. Maeda, S. Scheu, R. Locksley, D. Holmberg, C. Zweier, N. S. den Hollander, S. G. Kant, W. Holter, A. Rauch, Y. Zhuang, and B. Reizis. 2008. Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. *Cell* 135: 37-48.

49. Pulendran, B., J. Banchereau, S. Burkeholder, E. Kraus, E. Guinet, C. Chalouni, D. Caron, C. Maliszewski, J. Davoust, J. Fay, and K. Palucka. 2000. Flt3-ligand and granulocyte colony-stimulating factor mobilize distinct human dendritic cell subsets in vivo. *J Immunol* 165: 566-572.

50. Chen, W., S. Antonenko, J. M. Sederstrom, X. Liang, A. S. Chan, H. Kanzler, B. Blom, B. R. Blazar, and Y. J. Liu. 2004. Thrombopoietin cooperates with FLT3-ligand in the generation of plasmacytoid dendritic cell precursors from human hematopoietic progenitors. *Blood* 103: 2547-2553.

51. Demedts, I. K., G. G. Brusselle, K. Y. Vermaelen, and R. A. Pauwels. 2005. Identification and characterization of human pulmonary dendritic cells. *American journal of respiratory cell and molecular biology* 32: 177-184.

52. O'Doherty, U., M. Peng, S. Gezelter, W. J. Swiggard, M. Betjes, N. Bhardwaj, and R. M. Steinman. 1994. Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology* 82: 487-493.

53. Swiecki, M., and M. Colonna. 2010. Accumulation of plasmacytoid DC: roles in disease pathogenesis and targets for immunotherapy. *Eur J Immunol* 40: 2094-2098.

54. Gill, M. A., K. Long, T. Kwon, L. Muniz, A. Mejias, J. Connolly, L. Roy, J. Banchereau, and O. Ramilo. 2008. Differential recruitment of dendritic cells and monocytes to respiratory mucosal sites in children with influenza virus or respiratory syncytial virus infection. *The Journal of infectious diseases* 198: 1667-1676.

55. Bratke, K., M. Lommatzsch, P. Julius, M. Kuepper, H. D. Kleine, W. Luttmann, and J. Christian Virchow. 2007. Dendritic cell subsets in human bronchoalveolar lavage fluid after segmental allergen challenge. *Thorax* 62: 168-175.

56. Jahnsen, F. L., F. Lund-Johansen, J. F. Dunne, L. Farkas, R. Haye, and P. Brandtzaeg. 2000. Experimentally induced recruitment of plasmacytoid (CD123high) dendritic cells in human nasal allergy. *J Immunol* 165: 4062-4068.

57. Nestle, F. O., C. Conrad, A. Tun-Kyi, B. Homey, M. Gombert, O. Boyman, G. Burg, Y. J. Liu, and M. Gilliet. 2005. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. *J Exp Med* 202: 135-143.

58. Lande, R., V. Gafa, B. Serafini, E. Giacomini, A. Visconti, M. E. Remoli, M. Severa, M. Parmentier, G. Ristori, M. Salvetti, F. Aloisi, and E. M. Coccia. 2008. Plasmacytoid dendritic cells in multiple sclerosis: intracerebral recruitment and impaired maturation in response to interferon-beta. *Journal of neuropathology and experimental neurology* 67: 388-401.

59. Lande, R., E. Giacomini, B. Serafini, B. Rosicarelli, G. D. Sebastiani, G. Minisola, U. Tarantino, V. Riccieri, G. Valesini, and E. M. Coccia. 2004. Characterization and recruitment of plasmacytoid dendritic cells in synovial fluid and tissue of patients with chronic inflammatory arthritis. *J Immunol* 173: 2815-2824.

60. Gottenberg, J. E., N. Cagnard, C. Lucchesi, F. Letourneur, S. Mistou, T. Lazure, S. Jacques, N. Ba, M. Ittah, C. Lepajolec, M. Labetoulle, M. Ardizzone, J. Sibilia, C. Fournier, G. Chiocchia, and X. Mariette. 2006. Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjogren's syndrome. *Proc Natl Acad Sci U S A* 103: 2770-2775.

61. Lund, J. M., M. M. Linehan, N. Iijima, and A. Iwasaki. 2006. Cutting edge: Plasmacytoid dendritic cells provide innate immune protection against mucosal viral infection in situ. *J Immunol* 177: 7510-7514.

62. Smit, J. J., B. D. Rudd, and N. W. Lukacs. 2006. Plasmacytoid dendritic cells inhibit pulmonary immunopathology and promote clearance of respiratory syncytial virus. *J Exp Med* 203: 1153-1159.

63. Cella, M., D. Jarrossay, F. Facchetti, O. Alebardi, H. Nakajima, A. Lanzavecchia, and M. Colonna. 1999. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nature medicine* 5: 919-923.

64. O'Brien, M., O. Manches, and N. Bhardwaj. 2013. Plasmacytoid dendritic cells in HIV infection. *Advances in experimental medicine and biology* 762: 71-107.

65. Wendland, M., N. Czeloth, N. Mach, B. Malissen, E. Kremmer, O. Pabst, and R. Forster. 2007. CCR9 is a homing receptor for plasmacytoid dendritic cells to the small intestine. *Proc Natl Acad Sci U S A* 104: 6347-6352.

66. Swiecki, M., and M. Colonna. 2011. Disparate antiviral responses in Molluscum contagiosum virus-induced skin lesions. *J Invest Dermatol* 131: 288-290.

67. Noah, T. L., and S. Becker. 2000. Chemokines in nasal secretions of normal adults experimentally infected with respiratory syncytial virus. *Clinical immunology (Orlando, Fla.)* 97: 43-49.

68. Saraya, T., D. Kurai, H. Ishii, A. Ito, Y. Sasaki, S. Niwa, N. Kiyota, H. Tsukagoshi, K. Kozawa, H. Goto, and H. Takizawa. 2014. Epidemiology of virus-induced asthma exacerbations: with special reference to the role of human rhinovirus. *Frontiers in microbiology* 5: 226.

69. Diacovo, T. G., A. L. Blasius, T. W. Mak, M. Cella, and M. Colonna. 2005. Adhesive mechanisms governing interferon-producing cell recruitment into lymph nodes. *J Exp Med* 202: 687-696.

70. Yoneyama, H., K. Matsuno, Y. Zhang, T. Nishiwaki, M. Kitabatake, S. Ueha, S. Narumi, S. Morikawa, T. Ezaki, B. Lu, C. Gerard, S. Ishikawa, and K. Matsushima. 2004. Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules. *International immunology* 16: 915-928.

71. Coccia, E. M., M. Severa, E. Giacomini, D. Monneron, M. E. Remoli, I. Julkunen, M. Cella, R. Lande, and G. Uze. 2004. Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. *Eur J Immunol* 34: 796-805.

72. Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303: 1529-1531.

73. Pritchard, A. L., M. L. Carroll, J. G. Burel, O. J. White, S. Phipps, and J. W. Upham. 2012. Innate IFNs and plasmacytoid dendritic cells constrain Th2 cytokine responses to rhinovirus: a regulatory mechanism with relevance to asthma. *J Immunol* 188: 5898-5905.

74. Isaacs, A., J. Lindenmann, and R. C. Valentine. 1957. Virus interference. II. Some properties of interferon. *Proceedings of the Royal Society of London. Series B, Biological sciences* 147: 268-273.

75. Sadler, A. J., and B. R. Williams. 2008. Interferon-inducible antiviral effectors. *Nat Rev Immunol* 8: 559-568.

76. Young, H. A. 1996. Regulation of interferon-gamma gene expression. *J Interferon Cytokine Res* 16: 563-568.

77. Frucht, D. M., T. Fukao, C. Bogdan, H. Schindler, J. J. O'Shea, and S. Koyasu. 2001. IFN-gamma production by antigen-presenting cells: mechanisms emerge. *Trends in immunology* 22: 556-560.

78. Flaishon, L., R. Hershkoviz, F. Lantner, O. Lider, R. Alon, Y. Levo, R. A. Flavell, and I. Shachar. 2000. Autocrine secretion of interferon gamma negatively regulates homing of immature B cells. *J Exp Med* 192: 1381-1388.

79. Colonna, M., G. Trinchieri, and Y. J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat Immunol* 5: 1219-1226.

80. Kumar, H., T. Kawai, and S. Akira. 2009. Toll-like receptors and innate immunity. *Biochemical and biophysical research communications* 388: 621-625.

81. Lester, S. N., and K. Li. 2014. Toll-like receptors in antiviral innate immunity. *Journal of molecular biology* 426: 1246-1264.

82. Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 5: 987-995.

83. Lund, J., A. Sato, S. Akira, R. Medzhitov, and A. Iwasaki. 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *J Exp Med* 198: 513-520.

84. Kawai, T., and S. Akira. 2007. TLR signaling. *Semin Immunol* 19: 24-32.

85. Der, S. D., A. Zhou, B. R. Williams, and R. H. Silverman. 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc Natl Acad Sci U S A* 95: 15623-15628.

86. Ivashkiv, L. B., and L. T. Donlin. 2014. Regulation of type I interferon responses. *Nat Rev Immunol* 14: 36-49.

87. Kadowaki, N., and Y. J. Liu. 2002. Natural type I interferon-producing cells as a link between innate and adaptive immunity. *Hum Immunol* 63: 1126-1132.

88. Marrack, P., J. Kappler, and T. Mitchell. 1999. Type I interferons keep activated T cells alive. *J Exp Med* 189: 521-530.

89. Zhang, X., S. Sun, I. Hwang, D. F. Tough, and J. Sprent. 1998. Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. *Immunity* 8: 591-599.

90. Braun, D., I. Caramalho, and J. Demengeot. 2002. IFN-alpha/beta enhances BCR-dependent B cell responses. *International immunology* 14: 411-419.

91. Kaminski, M. M., A. Ohnemus, M. Cornitescu, and P. Staeheli. 2012. Plasmacytoid dendritic cells and Toll-like receptor 7-dependent signalling

promote efficient protection of mice against highly virulent influenza A virus. *The J Gen Virol* 93: 555-559.

92. Yoneyama, H., K. Matsuno, E. Toda, T. Nishiwaki, N. Matsuo, A. Nakano, S. Narumi, B. Lu, C. Gerard, S. Ishikawa, and K. Matsushima. 2005. Plasmacytoid DCs help lymph node DCs to induce anti-HSV CTLs. *J Exp Med* 202: 425-435.

93. Boogaard, I., M. van Oosten, L. S. van Rijt, F. Muskens, T. G. Kimman, B. N. Lambrecht, and A. M. Buisman. 2007. Respiratory syncytial virus differentially activates murine myeloid and plasmacytoid dendritic cells. *Immunology* 122: 65-72.

94. Davidson, S., G. Kaiko, Z. Loh, A. Lalwani, V. Zhang, K. Spann, S. Y. Foo, N. Hansbro, S. Uematsu, S. Akira, K. I. Matthaei, H. F. Rosenberg, P. S. Foster, and S. Phipps. 2011. Plasmacytoid dendritic cells promote host defense against acute pneumovirus infection via the TLR7-MyD88-dependent signaling pathway. *J Immunol* 186: 5938-5948.

95. Moschen, A. R., S. Geiger, I. Krehan, A. Kaser, and H. Tilg. 2008. Interferon-alpha controls IL-17 expression in vitro and in vivo. *Immunobiology* 213: 779-787.

96. Huber, J. P., and J. D. Farrar. 2011. Regulation of effector and memory T-cell functions by type I interferon. *Immunology* 132: 466-474.

97. Huber, J. P., H. J. Ramos, M. A. Gill, and J. D. Farrar. 2010. Cutting edge: type I IFN reverses human Th2 commitment and stability by suppressing GATA3. *J Immunol* 185: 813-817.

98. Romagnani, S. 2000. T-cell subsets (Th1 versus Th2). *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* 85: 9-18; quiz 18, 21.

99. Licona-Limon, P., L. K. Kim, N. W. Palm, and R. A. Flavell. 2013. TH2, allergy and group 2 innate lymphoid cells. *Nat Immunol* 14: 536-542.

100. Koyama, S., T. Aoshi, T. Tanimoto, Y. Kumagai, K. Kobiyama, T. Tougan, K. Sakurai, C. Coban, T. Horii, S. Akira, and K. J. Ishii. 2010. Plasmacytoid dendritic cells delineate immunogenicity of influenza vaccine subtypes. *Science translational medicine* 2: 25ra24.

101. Proietti, E., L. Bracci, S. Puzelli, T. Di Pucchio, P. Sestili, E. De Vincenzi, M. Venditti, I. Capone, I. Seif, E. De Maeyer, D. Tough, I. Donatelli, and F. Belardelli. 2002. Type I IFN as a natural adjuvant for a protective immune response: lessons from the influenza vaccine model. *J Immunol* 169: 375-383.

102. Jego, G., A. K. Palucka, J. P. Blanck, C. Chalouni, V. Pascual, and J. Banchereau. 2003. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. *Immunity* 19: 225-234.

103. Tovey, M. G., C. Lallemand, J. F. Meritet, and C. Maury. 2006. Adjuvant activity of interferon alpha: mechanism(s) of action. *Vaccine* 24 Suppl 2: S2-46-47.

104. Tel, J., E. H. Aarntzen, T. Baba, G. Schreibelt, B. M. Schulte, D. Benitez-Ribas, O. C. Boerman, S. Croockewit, W. J. Oyen, M. van Rossum, G. Winkels, P. G. Coulie, C. J. Punt, C. G. Figdor, and I. J. de Vries. 2013. Natural human plasmacytoid dendritic cells induce antigen-specific T-cell responses in melanoma patients. *Cancer research* 73: 1063-1075.

105. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245-252.

106. Vermaelen, K. Y., I. Carro-Muino, B. N. Lambrecht, and R. A. Pauwels. 2001. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J Exp Med* 193: 51-60.

107. Di Pucchio, T., B. Chatterjee, A. Smed-Sorensen, S. Clayton, A. Palazzo, M. Montes, Y. Xue, I. Mellman, J. Banchereau, and J. E. Connolly. 2008. Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I. *Nat Immunol* 9: 551-557.

108. Fox, C. J., P. S. Hammerman, and C. B. Thompson. 2005. Fuel feeds function: energy metabolism and the T-cell response. *Nat Rev Immunol* 5: 844-852.

109. Pearce, E. L., M. C. Poffenberger, C. H. Chang, and R. G. Jones. 2013. Fueling immunity: insights into metabolism and lymphocyte function. *Science* 342: 1242454.

110. Vander Heiden, M. G., L. C. Cantley, and C. B. Thompson. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324: 1029-1033.

111. Warburg, O. 1956. On the origin of cancer cells. *Science* 123: 309-314.

112. Guppy, M., E. Greiner, and K. Brand. 1993. The role of the Crabtree effect and an endogenous fuel in the energy metabolism of resting and proliferating thymocytes. *European journal of biochemistry / FEBS* 212: 95-99.

113. Pfeiffer, T., S. Schuster, and S. Bonhoeffer. 2001. Cooperation and competition in the evolution of ATP-producing pathways. *Science* 292: 504-507.

114. Semenza, G. L. 2008. Tumor metabolism: cancer cells give and take lactate. *J Clin Invest* 118: 3835-3837.

115. Delgado, T., P. A. Carroll, A. S. Punjabi, D. Margineantu, D. M. Hockenbery, and M. Lagunoff. 2010. Induction of the Warburg effect by Kaposi's sarcoma herpesvirus is required for the maintenance of latently infected endothelial cells. *Proc Natl Acad Sci U S A* 107: 10696-10701.

116. Yu, Y., A. J. Clippinger, and J. C. Alwine. 2011. Viral effects on metabolism: changes in glucose and glutamine utilization during human cytomegalovirus infection. *Trends in microbiology* 19: 360-367.

117. Abrantes, J. L., C. M. Alves, J. Costa, F. C. Almeida, M. Sola-Penna, C. F. Fontes, and T. M. Souza. 2012. Herpes simplex type 1 activates glycolysis

through engagement of the enzyme 6-phosphofructo-1-kinase (PFK-1). *Biochimica et biophysica acta* 1822: 1198-1206.

118. Krawczyk, C. M., T. Holowka, J. Sun, J. Blagih, E. Amiel, R. J. DeBerardinis, J. R. Cross, E. Jung, C. B. Thompson, R. G. Jones, and E. J. Pearce. 2010. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood* 115: 4742-4749.

119. Everts, B., E. Amiel, S. C. Huang, A. M. Smith, C. H. Chang, W. Y. Lam, V. Redmann, T. C. Freitas, J. Blagih, G. J. van der Windt, M. N. Artyomov, R. G. Jones, E. L. Pearce, and E. J. Pearce. 2014. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. *Nat Immunol* 15: 323-332.

120. Malinarich, F., K. Duan, R. A. Hamid, A. Bijin, W. X. Lin, M. Poidinger, A. M. Fairhurst, and J. E. Connolly. 2015. High mitochondrial respiration and glycolytic capacity represent a metabolic phenotype of human tolerogenic dendritic cells. *J Immunol* 194: 5174-5186.

121. MacIver, N. J., R. D. Michalek, and J. C. Rathmell. 2013. Metabolic regulation of T lymphocytes. *Annu Rev Immunol* 31: 259-283.

122. George, T. C., S. L. Fanning, P. Fitzgerald-Bocarsly, R. B. Medeiros, S. Highfill, Y. Shimizu, B. E. Hall, K. Frost, D. Basiji, W. E. Ortyn, P. J. Morrissey, and D. H. Lynch. 2006. Quantitative measurement of nuclear translocation events using

similarity analysis of multispectral cellular images obtained in flow. *Journal of immunological methods* 311: 117-129.

123. Huang da, W., B. T. Sherman, and R. A. Lempicki. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols* 4: 44-57.

124. Kaisho, T. 2010. Molecular mechanisms for plasmacytoid dendritic cell function and development. *Vaccine* 28: 8046-8047.

125. Board, M., S. Humm, and E. A. Newsholme. 1990. Maximum activities of key enzymes of glycolysis, glutaminolysis, pentose phosphate pathway and tricarboxylic acid cycle in normal, neoplastic and suppressed cells. *The Biochemical journal* 265: 503-509.

126. Pelicano, H., D. S. Martin, R. H. Xu, and P. Huang. 2006. Glycolysis inhibition for anticancer treatment. *Oncogene* 25: 4633-4646.

127. Maxwell, P. H. 2005. Hypoxia-inducible factor as a physiological regulator. *Experimental physiology* 90: 791-797.

128. Semenza, G. L., P. H. Roth, H. M. Fang, and G. L. Wang. 1994. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem* 269: 23757-23763.

129. Smed-Sorensen, A., C. Chalouni, B. Chatterjee, L. Cohn, P. Blattmann, N. Nakamura, L. Delamarre, and I. Mellman. 2012. Influenza A virus infection of

human primary dendritic cells impairs their ability to cross-present antigen to CD8 T cells. *PLoS pathogens* 8: e1002572.

130. Kumagai, Y., H. Kumar, S. Koyama, T. Kawai, O. Takeuchi, and S. Akira. 2009. Cutting edge: TLR-Dependent viral recognition along with type I IFN positive feedback signaling masks the requirement of viral replication for IFN-{alpha} production in plasmacytoid dendritic cells. *J Immunol* 182: 3960-3964.

131. Marcus, P. I., J. M. Rojek, and M. J. Sekellick. 2005. Interferon induction and/or production and its suppression by influenza A viruses. *Journal of virology* 79: 2880-2890.

132. Fields BN, K. D., Howley PM. 2007. *Fields virology*. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins.

133. Haller, O., and G. Kochs. 2011. Human MxA protein: an interferon-induced dynamin-like GTPase with broad antiviral activity. *J Interferon Cytokine Res* 31: 79-87.

134. Matzinger, S. R., T. D. Carroll, J. C. Dutra, Z. M. Ma, and C. J. Miller. 2013. Myxovirus resistance gene A (MxA) expression suppresses influenza A virus replication in alpha interferon-treated primate cells. *Journal of virology* 87: 1150-1158.

135. Munger, J., S. U. Bajad, H. A. Coller, T. Shenk, and J. D. Rabinowitz. 2006. Dynamics of the cellular metabolome during human cytomegalovirus infection. *PLoS pathogens* 2: e132.

136. McArdle, J., X. L. Schafer, and J. Munger. 2011. Inhibition of calmodulin-dependent kinase kinase blocks human cytomegalovirus-induced glycolytic activation and severely attenuates production of viral progeny. *Journal of virology* 85: 705-714.

137. Thai, M., N. A. Graham, D. Braas, M. Nehil, E. Komisopoulou, S. K. Kurdistani, F. McCormick, T. G. Graeber, and H. R. Christofk. 2014. Adenovirus E4ORF1-induced MYC activation promotes host cell anabolic glucose metabolism and virus replication. *Cell metabolism* 19: 694-701.

138. Castello, A., B. Fischer, K. Eichelbaum, R. Horos, B. M. Beckmann, C. Strein, N. E. Davey, D. T. Humphreys, T. Preiss, L. M. Steinmetz, J. Krijgsveld, and M. W. Hentze. 2012. Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell* 149: 1393-1406.

139. Chang, C. H., J. D. Curtis, L. B. Maggi, Jr., B. Faubert, A. V. Villarino, D. O'Sullivan, S. C. Huang, G. J. van der Windt, J. Blagih, J. Qiu, J. D. Weber, E. J. Pearce, R. G. Jones, and E. L. Pearce. 2013. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* 153: 1239-1251.

140. Locasale, J. W., and L. C. Cantley. 2011. Metabolic flux and the regulation of mammalian cell growth. *Cell metabolism* 14: 443-451.

141. Tannahill, G. M., A. M. Curtis, J. Adamik, E. M. Palsson-McDermott, A. F. McGettrick, G. Goel, C. Frezza, N. J. Bernard, B. Kelly, N. H. Foley, L. Zheng, A. Gardet, Z. Tong, S. S. Jany, S. C. Corr, M. Haneklaus, B. E. Caffrey, K.

Pierce, S. Walmsley, F. C. Beasley, E. Cummins, V. Nizet, M. Whyte, C. T. Taylor, H. Lin, S. L. Masters, E. Gottlieb, V. P. Kelly, C. Clish, P. E. Auron, R. J. Xavier, and L. A. O'Neill. 2013. Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. *Nature* 496: 238-242.

142. Pantel, A., A. Teixeira, E. Haddad, E. G. Wood, R. M. Steinman, and M. P. Longhi. 2014. Direct type I IFN but not MDA5/TLR3 activation of dendritic cells is required for maturation and metabolic shift to glycolysis after poly IC stimulation. *PLoS biology* 12: e1001759.

143. Burke, J. D., L. C. Platanias, and E. N. Fish. 2014. Beta interferon regulation of glucose metabolism is PI3K/Akt dependent and important for antiviral activity against coxsackievirus B3. *Journal of virology* 88: 3485-3495.

144. Sepulveda, F. E., S. Maschalidi, R. Colisson, L. Heslop, C. Ghirelli, E. Sakka, A. M. Lennon-Dumenil, S. Amigorena, L. Cabanie, and B. Manoury. 2009. Critical role for asparagine endopeptidase in endocytic Toll-like receptor signaling in dendritic cells. *Immunity* 31: 737-748.

145. Kuznik, A., M. Bencina, U. Svajger, M. Jeras, B. Rozman, and R. Jerala. 2011. Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. *J Immunol* 186: 4794-4804.

146. Semenza, G. L. 2003. Targeting HIF-1 for cancer therapy. *Nature reviews. Cancer* 3: 721-732.

147. Werth, N., C. Beerlage, C. Rosenberger, A. S. Yazdi, M. Edelmann, A. Amr, W. Bernhardt, C. von Eiff, K. Becker, A. Schafer, A. Peschel, and V. A. Kempf. 2010. Activation of hypoxia inducible factor 1 is a general phenomenon in infections with human pathogens. *PLoS One* 5: e11576.

148. Cramer, T., Y. Yamanishi, B. E. Clausen, I. Forster, R. Pawlinski, N. Mackman, V. H. Haase, R. Jaenisch, M. Corr, V. Nizet, G. S. Firestein, H. P. Gerber, N. Ferrara, and R. S. Johnson. 2003. HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell* 112: 645-657.

149. Maxwell, P. H., M. S. Wiesener, G. W. Chang, S. C. Clifford, E. C. Vaux, M. E. Cockman, C. C. Wykoff, C. W. Pugh, E. R. Maher, and P. J. Ratcliffe. 1999. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 399: 271-275.

150. Brahimi-Horn, M. C., J. Chiche, and J. Pouyssegur. 2007. Hypoxia signalling controls metabolic demand. *Curr Opin Cell Biol* 19: 223-229.

151. Mazzon, M., N. E. Peters, C. Loenarz, E. M. Krysztofinska, S. W. Ember, B. J. Ferguson, and G. L. Smith. 2013. A mechanism for induction of a hypoxic response by vaccinia virus. *Proc Natl Acad Sci U S A* 110: 12444-12449.

152. Darekar, S., K. Georgiou, M. Yurchenko, S. P. Yenamandra, G. Chachami, G. Simos, G. Klein, and E. Kashuba. 2012. Epstein-Barr virus immortalization of human B-cells leads to stabilization of hypoxia-induced factor 1 alpha, congruent with the Warburg effect. *PLoS One* 7: e42072.

153. Nakamura, M., J. M. Bodily, M. Beglin, S. Kyo, M. Inoue, and L. A. Laimins. 2009. Hypoxia-specific stabilization of HIF-1alpha by human papillomaviruses. *Virology* 387: 442-448.

154. Carroll, P. A., H. L. Kenerson, R. S. Yeung, and M. Lagunoff. 2006. Latent Kaposi's sarcoma-associated herpesvirus infection of endothelial cells activates hypoxia-induced factors. *Journal of virology* 80: 10802-10812.

155. Ripoli, M., A. D'Aprile, G. Quarato, M. Sarasin-Filipowicz, J. Gouttenoire, R. Scrima, O. Cela, D. Boffoli, M. H. Heim, D. Moradpour, N. Capitanio, and C. Piccoli. 2010. Hepatitis C virus-linked mitochondrial dysfunction promotes hypoxia-inducible factor 1 alpha-mediated glycolytic adaptation. *Journal of virology* 84: 647-660.

156. Deshmane, S. L., R. Mukerjee, S. Fan, L. Del Valle, C. Michiels, T. Sweet, I. Rom, K. Khalili, J. Rappaport, S. Amini, and B. E. Sawaya. 2009. Activation of the oxidative stress pathway by HIV-1 Vpr leads to induction of hypoxia-inducible factor 1alpha expression. *J Biol Chem* 284: 11364-11373.

157. Kilani, M. M., K. A. Mohammed, N. Nasreen, R. S. Tepper, and V. B. Antony. 2004. RSV causes HIF-1alpha stabilization via NO release in primary bronchial epithelial cells. *Inflammation* 28: 245-251.

158. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124: 783-801.

159. Tolnay, A. E., C. R. Baskin, T. M. Tumpey, P. J. Sabourin, C. L. Sabourin, J. P. Long, J. A. Pyles, R. A. Albrecht, A. Garcia-Sastre, M. G. Katze, and H. Bielefeldt-Ohmann. 2010. Extrapulmonary tissue responses in cynomolgus macaques (Macaca fascicularis) infected with highly pathogenic avian influenza A (H5N1) virus. *Archives of virology* 155: 905-914.

160. Hashizume, H., T. Horibe, H. Yagi, N. Seo, and M. Takigawa. 2005. Compartmental imbalance and aberrant immune function of blood CD123+ (plasmacytoid) and CD11c+ (myeloid) dendritic cells in atopic dermatitis. *J Immunol* 174: 2396-2403.

161. Saadi, S., L. E. Wrenshall, and J. L. Platt. 2002. Regional manifestations and control of the immune system. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology* 16: 849-856.

162. Rius, J., M. Guma, C. Schachtrup, K. Akassoglou, A. S. Zinkernagel, V. Nizet, R. S. Johnson, G. G. Haddad, and M. Karin. 2008. NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. *Nature* 453: 807-811.

163. Querec, T., S. Bennouna, S. Alkan, Y. Laouar, K. Gorden, R. Flavell, S. Akira, R. Ahmed, and B. Pulendran. 2006. Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *J Exp Med* 203: 413-424.

164. de Vries, I. J., J. Tel, D. Benitez-Ribas, R. Torensma, and C. G. Figdor. 2011. Prophylactic vaccines mimic synthetic CpG oligonucleotides in their ability to modulate immune responses. *Mol Immunol* 48: 810-817.

165. Tel, J., E. L. Smits, S. Anguille, R. N. Joshi, C. G. Figdor, and I. J. de Vries. 2012. Human plasmacytoid dendritic cells are equipped with antigen-presenting and tumoricidal capacities. *Blood* 120: 3936-3944.

166. Budimir, N., A. de Haan, T. Meijerhof, S. Waijer, L. Boon, E. Gostick, D. A. Price, J. Wilschut, and A. Huckriede. 2013. Critical role of TLR7 signaling in the priming of cross-protective cytotoxic T lymphocyte responses by a whole inactivated influenza virus vaccine. *PLoS One* 8: e63163.

167. Bracci, L., I. Canini, M. Venditti, M. Spada, S. Puzelli, I. Donatelli, F. Belardelli, and E. Proietti. 2006. Type I IFN as a vaccine adjuvant for both systemic and mucosal vaccination against influenza virus. *Vaccine* 24 Suppl 2: S2-56-57.

168. Xia, M., R. Huang, Y. Sun, G. L. Semenza, S. F. Aldred, K. L. Witt, J. Inglese, R. R. Tice, and C. P. Austin. 2009. Identification of chemical compounds that induce HIF-1alpha activity. *Toxicological sciences: an official journal of the Society of Toxicology* 112: 153-163.

169. Van Driessche, A., A. L. Van de Velde, G. Nijs, T. Braeckman, B. Stein, J. M. De Vries, Z. N. Berneman, and V. F. Van Tendeloo. 2009. Clinical-grade manufacturing of autologous mature mRNA-electroporated dendritic cells and

safety testing in acute myeloid leukemia patients in a phase I dose-escalation clinical trial. *Cytotherapy* 11: 653-668.

170. Levine, B. L., W. B. Bernstein, N. E. Aronson, K. Schlienger, J. Cotte, S. Perfetto, M. J. Humphries, S. Ratto-Kim, D. L. Birx, C. Steffens, A. Landay, R. G. Carroll, and C. H. June. 2002. Adoptive transfer of costimulated CD4+ T cells induces expansion of peripheral T cells and decreased CCR5 expression in HIV infection. *Nature medicine* 8: 47-53.

171. Mamot, C., D. C. Drummond, C. O. Noble, V. Kallab, Z. Guo, K. Hong, D. B. Kirpotin, and J. W. Park. 2005. Epidermal growth factor receptor-targeted immunoliposomes significantly enhance the efficacy of multiple anticancer drugs in vivo. *Cancer research* 65: 11631-11638.

172. Meyer, T., C. Surber, L. E. French, and E. Stockfleth. 2013. Resiquimod, a topical drug for viral skin lesions and skin cancer. *Expert opinion on investigational drugs* 22: 149-159.

173. Dockrell, D. H., and G. R. Kinghorn. 2001. Imiquimod and resiquimod as novel immunomodulators. *The Journal of antimicrobial chemotherapy* 48: 751-755.

174. Tassone, L., D. Moratto, W. Vermi, M. De Francesco, L. D. Notarangelo, F. Porta, V. Lougaris, F. Facchetti, A. Plebani, and R. Badolato. 2010. Defect of plasmacytoid dendritic cells in warts, hypogammaglobulinemia, infections, myelokathexis (WHIM) syndrome patients. *Blood* 116: 4870-4873.

175. Keles, S., H. H. Jabara, I. Reisli, D. R. McDonald, I. Barlan, R. Hanna-Wakim, G. Dbaibo, G. Lefranc, W. Al-Herz, R. S. Geha, and T. A. Chatila. 2014. Plasmacytoid dendritic cell depletion in DOCK8 deficiency: rescue of severe herpetic infections with IFN-alpha 2b therapy. *J Allergy Clin Immunol* 133: 1753-1755.e1753.

176. Busse, W. W., R. F. Lemanske, Jr., and J. E. Gern. 2010. Role of viral respiratory infections in asthma and asthma exacerbations. *Lancet* 376: 826-834.

177. Gill, M. A., G. Bajwa, T. A. George, C. C. Dong, Dougherty, II, N. Jiang, V. N. Gan, and R. S. Gruchalla. 2010. Counterregulation between the FcepsilonRI pathway and antiviral responses in human plasmacytoid dendritic cells. *J Immunol* 184: 5999-6006.

178. Raez, L. E., K. Papadopoulos, A. D. Ricart, E. G. Chiorean, R. S. Dipaola, M. N. Stein, C. M. Rocha Lima, J. J. Schlesselman, K. Tolba, V. K. Langmuir, S. Kroll, D. T. Jung, M. Kurtoglu, J. Rosenblatt, and T. J. Lampidis. 2013. A phase I dose-escalation trial of 2-deoxy-D-glucose alone or combined with docetaxel in patients with advanced solid tumors. *Cancer chemotherapy and pharmacology* 71: 523-530.

179. Banchereau, J., and V. Pascual. 2006. Type I interferon in systemic lupus erythematosus and other autoimmune diseases. *Immunity* 25: 383-392.

180. Dalpke, A., J. Frank, M. Peter, and K. Heeg. 2006. Activation of toll-like receptor 9 by DNA from different bacterial species. *Infection and immunity* 74: 940-946.

181. Parcina, M., C. Wendt, F. Goetz, R. Zawatzky, U. Zahringer, K. Heeg, and I. Bekeredjian-Ding. 2008. Staphylococcus aureus-induced plasmacytoid dendritic cell activation is based on an IgG-mediated memory response. *J Immunol* 181: 3823-3833.

182. Siegal, F. P., N. Kadowaki, M. Shodell, P. A. Fitzgerald-Bocarsly, K. Shah, S. Ho, S. Antonenko, and Y. J. Liu. 1999. The nature of the principal type 1 interferon-producing cells in human blood. *Science* 284: 1835-1837.

183. Cohen, S. N., and K. L. Yielding. 1965. Inhibition of DNA and RNA polymerase reactions by chloroquine. *Proc Natl Acad Sci U S A* 54: 521-527.

184. Ozcan, G., B. Ozpolat, R. L. Coleman, A. K. Sood, and G. Lopez-Berestein. 2015. Preclinical and clinical development of siRNA-based therapeutics. *Advanced drug delivery reviews*.

185. Angelot, F., E. Seilles, S. Biichle, Y. Berda, B. Gaugler, J. Plumas, L. Chaperot, F. Dignat-George, P. Tiberghien, P. Saas, and F. Garnache-Ottou. 2009. Endothelial cell-derived microparticles induce plasmacytoid dendritic cell maturation: potential implications in inflammatory diseases. *Haematologica* 94: 1502-1512.