LOOKING BEYOND PATTERN RECOGNITION: PERTURBATIONS IN CELLULAR HOMEOSTASIS AND METABOLISM AS EMERGING REGULATORS OF DENDRITIC CELL FUNCTION

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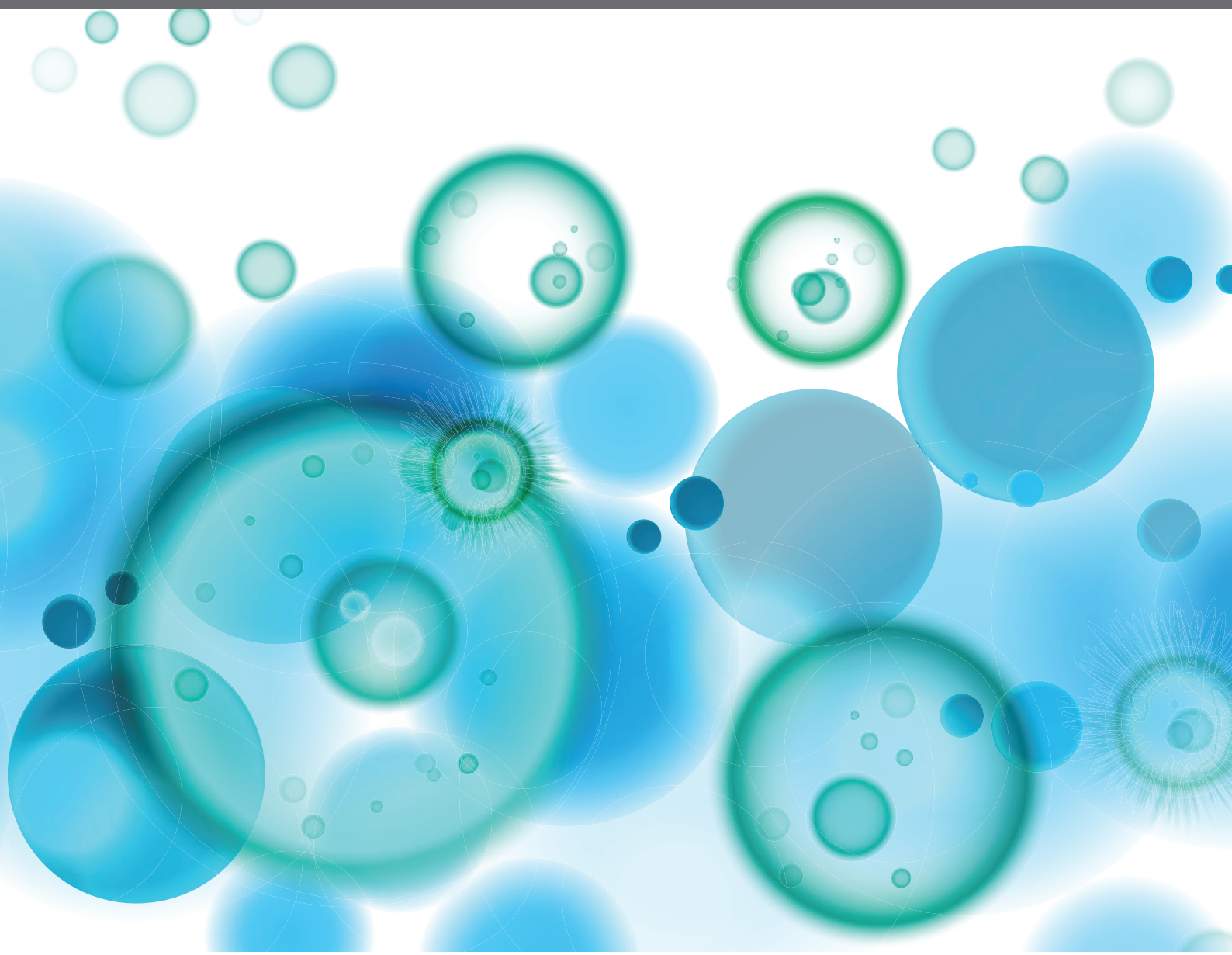

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LOOKING BEYOND PATTERN RECOGNITION: PERTURBATIONS IN CELLULAR HOMEOSTASIS AND METABOLISM AS EMERGING REGULATORS OF DENDRITIC CELL FUNCTION

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Table of Contents

CHAPTER 3

NON-CANONICAL ENVIRONMENTAL REGULATORS OF DC FUNCTION

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EDITORIAL
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Editorial: Looking Beyond Pattern Recognition: Perturbations in Cellular Homeostasis and Metabolism as Emerging Regulators of Dendritic Cell Function

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Editorial on the Research Topic

Looking Beyond Pattern Recognition: Perturbations in Cellular Homeostasis and Metabolism as Emerging Regulators of Dendritic Cell Function

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As professional antigen presenting cells, Dendritic cells (DCs), undergo a well-defined activation process that render them competent to activate adaptive immune responses and also to control tolerance. As a result, DCs are considered key regulators of the immune system. DC activation via pathogen recognition or by tissue injury occurs by virtue of expression of pattern recognition receptors, which has been extensively documented in previous years. However, there is a growing body of evidence demonstrating that DC activation and function can be also finely adjusted by perturbations in cellular mechanisms that are normally associated with homeostasis, and that include processes such as cell polarity, changes in the secretory demand, endoplasmic reticulum (ER) stress, epigenetics, autophagy, and metabolism among others. Moreover, evidence is now emerging that environmental cues such as nutrient availability, antibody complexes, and sodium levels are also important regulators of DC biology. In this Research Topic, we have brought together a collection of 10 primary research and review papers from experts in their respective fields to home in novel and emerging regulators of DC function that go beyond canonical pattern recognition.

Several cellular "household" processes that normally function to maintain intracellular homeostasis can also serve as regulators of DC function and when changed or perturbed, may act as instigators of DC activation. In our Research Topic, five of these emerging processes are highlighted in a total of five reviews and three primary research articles. First, in a review by Münz, the most recent studies and evidence for a previously unappreciated role of macro-autophagy in regulating antigen processing and presentation are discussed. Second, Barbier et al. explore the role of myosin in actin remodeling, which in the case of DCs is important for migration. They performed a convincing set of experiments to show a key role for myosin II in maintaining fast cell speed specifically in confined microenvironments. Third, despite the relatively short lifespan of DCs, there is a growing body of evidence that suggests that epigenetic changes and chromatin remodeling are pivotal in determining DC fate and activation state. Boukhaled et al. delve into this topic and discuss in their review what signals control epigenetic changes in DCs and what the functional consequences are of such changes. Fourth, Medel et al. have investigated the relevance of ER stress in sensing tumor cell lysates by DCs. They showed that bone marrow derived DCs activate the unfolded protein response sensor IRE1 and the transcription factor XBP1s upon recognition of tumor cell lysates, which boosts proinflammatory cytokine production and cross-presentation

of tumor cell-associated antigens to CD8+ T cells. Finally, currently one of the most rapidly developing immunological research areas is the field of immunemetabolism, which focuses on the role of cellular metabolism in shaping immune cell function. This concept has also permeated the DC field and there have been major recent advances in our understanding of the importance of central metabolic pathways and metabolic reprogramming in all aspects in DC biology. Four different contributions highlight the current status of, and provide exciting new insights in this field. Wculek et al. provide an insightful overview of how glucose, lipid, and amino acid metabolism shape DC differentiation and activation. In addition, they discuss the distinct metabolic programs in DCs that underlie induction of tolerance vs. immunity, as well as what the current evidence is for differences in metabolism between different DC subsets. In a study by Basit et al., this latter aspect is experimentally tested in different DC subsets from human blood. Their work reveals that CD1c+ and plasmacytoid DCs rely on glycolysis and oxidative phosphorylation for their activation, respectively, indicating that different DC subsets have very distinct metabolic requirements for their activation. In a timely review by Snyder and Amiel the most recent developments on the role of nutrient sensor mTOR in DC biology are discussed. From this article, it becomes clear that mTOR acts a central signaling hub that orchestrates the integration of nutrient and danger signals with DC metabolism, activation, and differentiation. Finally, given the growing appreciation that metabolic reprogramming plays a key role in DC differentiation, He et al. have dedicated a review on this specific topic. From their review, it becomes clear that the type metabolic programs involved in DC differentiation largely depend on the DC subset involved and whether it is studied *in vitro* or *in vivo*, or is still simply not known, illustrating that this field is still maturing.

One of the implications of the articles on metabolism and myosin, as highlighted in the previous section, is that nutrient availability in the micro-environment or the degree of spatial confinement that DCs reside in are important determinants of their function. However, there are other factors in the microenvironment, apart from canonical danger signals, that can have an equally significant impact on DCs. One such example is elegantly provided by Hoepel et al., who show that DCs when exposed to IgG immune complexes, in an FcγR and IRF5 dependent manner, boost pro-inflammatory cytokine production through cross-talk with Toll-like receptors. Likewise, it has become apparent that extracellular sodium can have a significant impact on immune cell function, including DCs. Neubert et al. review the recent literature on this topic and provide compelling examples of how local Na^+ shapes DC function, but also how immune cells impact Na^+ homeostasis, revealing that there is reciprocal regulation of DC biology and fluid homeostasis. This clearly illustrates that there is a wide variety of extrinsic cues DCs receive in a given microenvironment that go well beyond classical pattern recognition and that profoundly impact their function.

We thank all authors for their time and effort they put into their excellent contributions. Without their commitment this collection would not have been possible. We hope that you will enjoy reading the articles within this Frontiers collection, give you new insights into the factors that regulate DC function and spark new ideas. We believe that these studies and reviews are a testimony of the complex set of cell intrinsic and extrinsic cues that govern DC biology, but also demonstrate that we still have a long way to go before we have a complete understanding of the multilayered control of DC function.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Non-canonical Functions of Macroautophagy Proteins During Endocytosis by Myeloid Antigen Presenting Cells

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Endocytosis by myeloid antigen presenting cells such as dendritic cells and macrophages regulates both antigen processing and major histocompatibility complex (MHC) molecule trafficking during antigen presentation. The molecular machinery of macroautophagy, a catabolic pathway that delivers cytoplasmic constituents to lysosomal degradation, has recently been found to modulate both MHC class I internalization and phagocytosis of antigens for efficient MHC class II presentation. In this review, I will discuss the respective studies and how these alternative pathways of macroautophagy protein usage differ from their canonical functions. A better understanding of these additional functions of the macroautophagy machinery should allow us to interpret biological effects of macroautophagy protein deficiencies more comprehensively and to therapeutically target the different pathways which utilize the molecular machinery of macroautophagy.

Keywords: LC3-associated phagocytosis (LAP), MHC class I, MHC (HLA) class II proteins, Phagocytosis, autophagy (macroautophagy)

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INTRODUCTION

Adaptive immune responses are coordinated and in part executed by T cells. Their activation requires the presentation of non-self peptides on major histocompatibility complex (MHC) molecules (1, 2). These peptides originate from the main proteolytic machineries in cells with the proteasome mainly responsible to produce MHC class I ligands to stimulate $CD8^+$ T cells and lysosomal proteases, like cathepsins, predominantly generating MHC class II ligands. These proteolytic machineries do not discriminate between self and non-self, including pathogen derived proteins, but central (e.g., clonal T cell deletion) and peripheral (e.g., regulatory T cells) tolerance mechanisms prevent most T cell activation by complexes of self-peptides plus MHC molecules. Proteasome products reach MHC class I molecules mainly after import into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP), where they are loaded onto co-translationally inserted MHC class I molecules in the MHC class I loading complex containing chaperones and protein disulfide isomerases (1). MHC class I molecules with their octa- to nonameric peptide ligands get then transported to the cell surface for interaction with the T cell receptor (TCR) and CD8 co-receptor of $CD8^+$ T cells. The longer MHC class II ligands, often around 15 amino acid long peptides, are primarily generated by lysosomal proteolysis and loaded in late endosomal compartments, that often present as multivesicular bodies (MVBs), called MIICs, onto MHC class II molecules (2). These reach MIICs under the guidance of the invariant chain (Ii) chaperone, which is then degraded by lysosomal proteases and the final Ii

peptide remnant is exchanged for high affinity peptide ligands with the help of the HLA-DM chaperone. The resulting complexes of MHC class II molecules and their peptide ligands then gets transported to the cell membrane for interaction with the TCR and CD4 co-receptor of $CD4^+$ T cells. According to these cell biological requirements for MHC class I and II ligand generation, $CD8^+$ T cells recognize mainly intracellular antigens, and $CD4^+$ T cells extracellular antigens after their endocytosis into late endosomes. However, alternative pathways exist for access to proteasomes and lysosomal proteases in MIICs, namely mechanistically poorly defined escape from endosomes during MHC class I cross-presentation, recently coined type 1 cross-presentation, and cytoplasmic constituent delivery to MIICs via autophagy for MHC class II presentation, recently coined type 2 cross-presentation (3).

Autophagy consists of at least three pathways, micro-autophagy, chaperone-mediated autophagy and macroautophagy (4, 5). So far, only chaperone-mediated and macroautophagy have been implicated in antigen processing for MHC class II presentation (6–9). However, microautophagy also delivers cytoplasmic material to MVBs (10–13) and, thereby, might also contribute to MHC class II presentation of intracellular antigens. While chaperone-mediated autophagy utilizes LAMP2A and cytosolic as well as lysosomal chaperones to transport proteins with a KFERQ-like signal peptide across lysosomal and possibly MVB membranes, macroautophagy employs more than 30 autophagy-related gene (atg) products to build double-membrane surrounded autophagosomes and regulate their fusion with lysosomes and late endosomes, including MVBs (5, 14). The macroautophagy machinery mainly consists of five complexes. The ULK1/Atg1 complex integrates metabolic cues. It is relieved of its inhibition by mTOR and activated via phosphorylation by the AMP-activated protein kinase (AMPK) during starvation. The ULK1/Atg1 complex itself then phosphorylates the VPS34 PI3 kinase complex containing Atg14 on its Beclin-1/Atg6 component, which in turn phosphorylates membranes at which autophagosome formation is initiated, the so called phagophore. Via PI3P the LC3/Atg8 lipidation complex is recruited which consists of Atg5, 12, and 16L1 and conjugates the ubiquitin-like Atg8 proteins, including LC3B/Atg8 and GABARAP/Atg8, to phosphatidylethanolamine (PE) at the phagophore. LC3/Atg8 lipidation then allows for membrane elongation and substrate recruitment, either by directly binding proteins that contain LC3-interacting regions (LIRs) or adaptors that include LIRs and ubiquitin-binding domains to deliver ubiquitinated aggregates, damaged organelles or pathogens into autophagosomes. A fourth complex, that also facilitates endosome maturation, containing VPS34, Beclin-1/Atg6, and UVRAG then regulates fusion with lysosomes, which is executed by the HOPS complex, Rab7 and syntaxin 17 (STX17). While the Atg4 protease recycles LC3/Atg8 proteins from the outer autophagosomal membrane upon vesicle completion, LC3/Atg8 on the inner autophagosomal membrane and its cargo including ubiquitin-binding LIR adaptors like sequestosome/p62 are then degraded by lysosomes. This highly sophisticated machinery of membrane tagging by phosphorylation and then LC3/Atg8 protein conjugation that is used to generate autophagosomes and regulate their fusion with other membrane compartments, is, however, also used for alternative functions including the regulation of endocytosis. This alternative use of the Atg machinery will be discussed in this review.

RECEPTOR INTERNALIZATION VIA LC3/ATG8 BINDING PROTEINS

The first protein for which an involvement of the macroautophagy machinery for its internalization was discovered is the amyloid precursor protein (APP) (15–17). APP proteolysis gives rise to Aβ peptide, the main component of extracellular proteaneous plaques in the central nervous system of patients with Alzheimer's disease (18). It was noted that stimulation of the macroautophagy machinery stimulates APP degradation in a fashion that neurodegenerative Aβ peptides are not produced (15). This protective APP degradation depends on the membrane conjugation machinery of LC3/Atg8 (16) as well as Beclin-1/Atg6 (17). However, lipidated LC3/Atg8 does not recruit APP itself, but rather components of the clathrin dependent internalization machinery, primarily the adaptor protein complex 2 (AP2) (16) (**Figure 1**). Its AP2A1 subunit contains a LIR motif, which was found to be required for its binding to LC3. Boosting the macroautophagy machinery via starvation or mTOR inhibition increased APP internalization and degradation, and this process was inhibited by RNA silencing of Atg5 (16). In addition to AP2A1, another component of clathrin mediated endocytosis, namely clathrin itself, also contains another LIR motif (19). Furthermore, APP binds directly to Beclin-1/Atg6 via APP's evolutionary conserved domain (ECD) (17). This binding facilitates internalization via the recruitment of the VPS34, Beclin-1/Atg6 and UVRAG containing complex that enhances endosome maturation. Thus, both binding of the clathrin dependent internalization machinery to LC3/Atg8 that is coupled to the cell membrane and Beclin-1/Atg6 binding to APP itself facilitates internalization and degradation of the Aβ precursor and thereby inhibits amyloid generation.

This enhanced internalization with the support of the macroautophagy machinery is also hijacked by viruses (**Figure 1**). The single stranded RNA virus ECHO (enteric cytopathic human orphan) virus 7 of the picornaviridae family was shown to require core components of macroautophagy for its clathrin dependent internalization (20). RNA silencing of Beclin-1/Atg6, Atg12, Atg14, Atg16, or LC3/Atg8 inhibited echovirus 7 entry prior to uncoating. Of these Atg16L1 was required for echovirus internalization from the cell membrane of intestinal epithelial cells, while attachment was unchanged. Furthermore, the double stranded DNA virus white spot syndrome virus (WSSV) of the Nimaviridae family requires GABARAP/Atg8 for clathrin mediated entry into crayfish cells (21). During entry WSSV colocalized with GABARAP/Atg8 at the cell membrane. Thus, echovirus 7 and WSSV seem to use clathrin mediated endocytosis for surface internalization and this process is facilitated by LC3/Atg8 and GABARAP/Atg8 conjugation to the

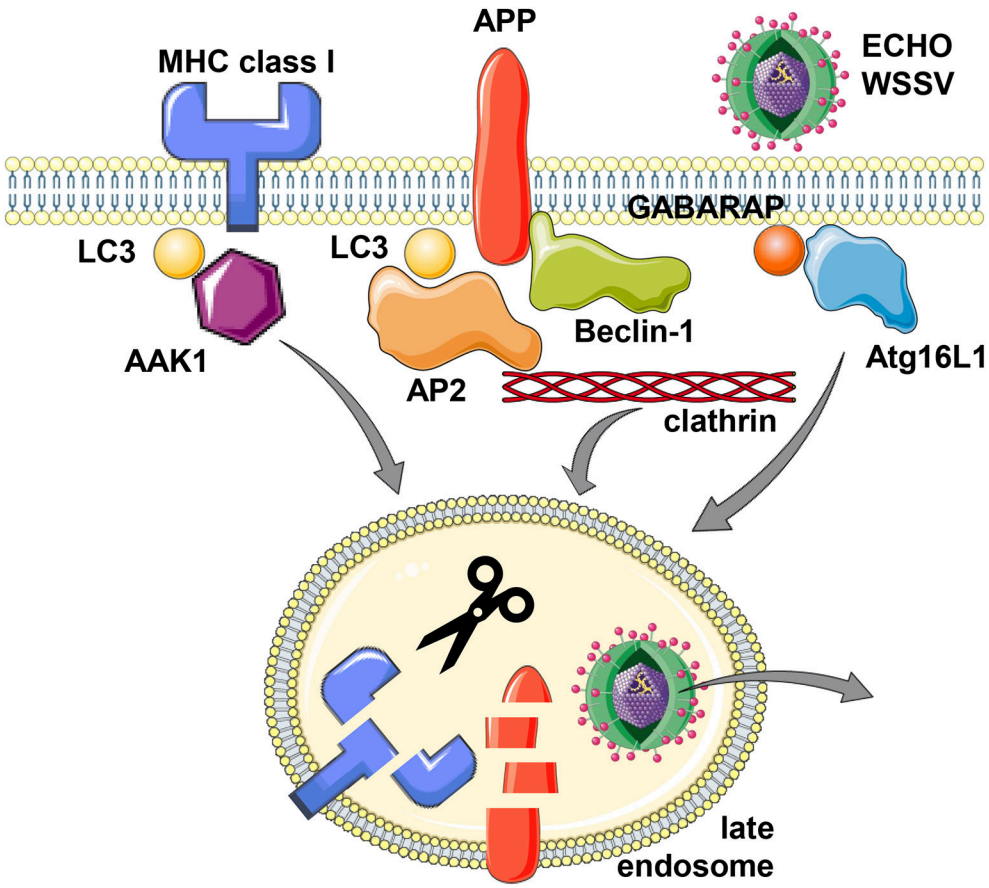

FIGURE 1 | Support of the macroautophagy machinery for receptor and virus internalization. MHC class I molecules, Alzheimer precursor protein (APP) and the two viruses Echovirus 7 (ECHO) and white spot syndrome virus (WSSV) seem to utilize LC3/Atg8 or GABARAP/Atg8 lipidation of the cell membrane for more efficient clathrin dependent internalization. For MHC class I molecules, LC3/Atg8 (LC3) mediated recruitment of the adaptor associated kinase 1 (AAK1), for APP, LC3/Atg8 binding of adaptor protein complex 2 (AP2) and direct binding to Beclin-1/Atg6, and for Echovirus 7 and WSSV, Atg16L1 and GABARAP/Atg8, respectively, have been implicated in their internalization from the cell membrane. While this internalization leads to lysosomal degradation for MHC class I molecules and APP, the two viruses escape to the cytosol from the respective endosomes. This figure was created in part with modified Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License: https://smart.servier.com.

cell membrane, which might support recruitment of the clathrin mediated endocytosis machinery.

THE ROLE OF ATG ASSISTED ENDOCYTOSIS FOR MHC CLASS I RESTRICTED ANTIGEN PRESENTATION

The internalization mechanism that benefits from Atg8 ortholog mediated recruitment of components of the clathrin mediated endocytosis machinery seems to also influence both classical and non-classical MHC class I molecules (22, 23). In mice that are deficient in the LC3/Atg8 lipidation complex components Atg5 or Atg7 in their dendritic cells and some macrophage populations, these myeloid cells have increased classical MHC class Ia (H2-K^b and H2-D^b) and non-classical MHC class Ib (CD1d) surface expression levels. While these molecules are transported to the cell surface at similar rate in Atg5 or Atg7 deficient cells, their internalization is significantly attenuated. Other surface molecules like MHC class II, CD86 and CD40, as well as MHC class I on B and T cells were not affected by Atg5 or Atg7 deficiency in dendritic cells and some macrophages (22–24). In immunoprecipitation experiments it was found that the adaptor associated kinase 1 (AAK1) interacts with MHC class Ia molecules less efficiently in the absence of Atg5 or Atg7 (22). This kinase phosphorylates the μ subunit of the AP2 complex (AP2M1) for more efficient clathrin mediated endocytosis (25, 26). AAK1 also associates with LC3/Atg8 and contains predicted LIR motifs, suggesting that LC3/Atg8 lipidation at the plasma membrane localizes AAK1 in proximity to MHC class I molecules for their more efficient internalization (**Figure 1**). Accordingly, RNA silencing of AAK1 stabilizes MHC class Ia molecules on the surface of mouse dendritic cells (22). This MHC class I stabilization is of functional relevance because influenza and lymphocytic choriomeningitis virus (LCMV) specific $CD8^+$ T cell responses are more efficiently primed and/or expanded in mice with Atg5 deficiency in their dendritic cells and some macrophage compartments, including

alveolar macrophages (22). Influenza infected Atg5 deficient dendritic cells also stimulate virus specific $CD8^+$ T cells more efficiently *in vitro* (22). The increased $CD8^+$ T cell expansion in mice with Atg5 or Atg7 deficiency in dendritic cells and some macrophage populations also correlates with improved control of viral titers and pathology in the influenza infected mice (22). Similarly, Atg5 or Atg7 deficiency in macrophages rescues mice from influenza induced pathology only after priming of adaptive immune responses (10 days post-infection), while components of the ULK1/Atg1 complex and the VPS34 PI3 kinase complex, Fip200 and Atg14, are also required for the influenza induced pathology during innate immunity at earlier timepoints (27). In addition to this regulation of $CD8^+$ T cell responses by altered classical MHC class Ia internalization, NKT cell responses that are restricted by the non-classical MHC class Ib molecule CD1d are also altered in mice with Atg5 deficiency in dendritic cells and some macrophage populations (23). Invariant NKT cells recognize phospholipids on CD1d molecules (28). CD1d accumulation on the surface of Atg5 deficient dendritic cells leads to increased α-galactosylceramide (αGalCer) presentation to NKT cells *in vitro*, and αGalCer injection leads to elevated cytokine production in mice with Atg5 deficiency in dendritic cells and some macrophage populations (23). Furthermore, the pathogen *Sphingomonas paucimobilis*, which is exquisitely sensitive to NKT cell mediated immune control during early infection, reached lower bacterial loads associated with higher cytokine production in the absence of Atg5 in dendritic cells and some macrophages (23). These findings suggest that also NKT cell responses are elevated in the absence of efficient CD1d internalization that is supported by components of the macroautophay machinery.

While endogenous antigen presentation by MHC class I molecules seems to be increased in the absence of LC3/Atg8 lipidation, cross-presentation of exogenous antigens might be compromised (29, 30). Indeed, the pool of MHC class I molecules that is internalized into early endosomes of dendritic cells has been suggested to be required for efficient cross-presentation (31). Dendritic cells with VPS34 deficiency displayed increased LCMV derived antigen presentation on MHC class I molecules to $CD8^+$ T cells, but failed to cross-present cell associated ovalbumin efficiently (29, 30). Accordingly, mice with VPS34 deficiency in their dendritic cells and some macrophage populations were more susceptible to challenge with B16 melanoma cells (30). This might suggest that loss of Atg supported MHC class I internalization improves endogenous antigen presentation, but inhibits cross-presentation to $CD8^+$ T cells.

LC3 ASSOCIATED PHAGOCYTOSIS (LAP)

In addition to LC3/Atg8 lipidation events at the cell membrane that might support receptor as well as virus internalization, such modifications have also been found to take place at endosomal membranes (32, 33). The respective process was coined LC3 associated phagocytosis or LAP (32). It depends on the VPS34 and LC3/Atg8 lipidation complexes, but does not require the ULK1/Atg1 complex (34). This machinery gets engaged when extracellular material is phagocytosed that binds to distinct receptors, including the pathogen associated molecular pattern receptor toll-like receptor 2 (TLR2), antibody Fc receptors, the C-type lectin Dectin-1 and the apoptotic body receptor TIM4 (32, 33, 35, 36) (**Figure 2**). VPS34 then introduces PI3P marks on the phagosomal membrane, which allow the recruitment of NADPH oxidase 2 (NOX2), whose reactive oxygen species (ROS) generation is required for LAP (33, 34, 37). So far it is unclear how ROS production by NOX2 regulates LAP, but macrophages of chronic granulomatous disease (CGD) patients with defined NOX2 mutations are not able to form LAP phagosomes after TLR2 ligand internalization (33). The LC3/Atg8 lipidation complex can be recruited to phagosomal membranes via the WD40 domain of Atg16L1, which is not present in yeast Atg16 and might be an adaptation in higher eukaryotes to allow for LAP (38). How this WD40 domain, however, recognizes phagosomes that are in need of LC3/Atg8 modification remains unclear. Nevertheless, Atg16L1 mediated recruitment of Atg5 and Atg12 then allows LC3/Atg8 conjugation to the cytosolic side of phagosomes and these membrane tags are only removed prior to phagosome fusion with lysosomes and MIICs (32, 33). LC3/Atg8 conjugation to phagosomes influences their fate differently depending on the cellular background. While it accelerates fusion with lysosomes, possibly by improving endosome transport along microtubules and recruitment of the fusion machinery, in mouse macrophages (39–41), LC3/Atg8 attenuates phagosome maturation and fusion with lysosomes in human macrophages and monocyte-derived dendritic cells (33). In plasmacytoid dendritic cells LC3/Atg8 seems to divert phagosomes to TLR containing endosomes for efficient type I interferon production after phagocytosed pathogen sensing (42). Therefore, LAP seems to regulate endocytosis to adapt the fate of the internalized cargo to the functional needs of the respective phagocyte.

THE ROLE OF LAP DURING MHC CLASS II RESTRICTED ANTIGEN PRESENTATION

Irrespective of the different kinetics of LC3 associated phagosome fusion with lysosomes in human and mouse macrophages, in both species LAP seems to enhance MHC class II restricted antigen presentation (33, 35) (**Figure 2**). This was shown for *Candida albicans* antigens in human macrophages and for ovalbumin expressed in *Saccharomyces cerevisiae* in mouse macrophages. Accordingly, exogenous antigen presentation on MHC class II molecules to $CD4^+$ T cells is also compromised in mice with Atg5 deficiency in dendritic cells and some macrophage populations (24). This also extends to autoantigens, because experimental autoimmune encephalomyelitis (EAE) upon myelin oligodendrocyte glycoprotein (MOG) specific $CD4^+$ T cell transfer is severely attenuated in mice with Atg5 deficiency in dendritic cells and some macrophage populations (43). The respective dendritic cells are less efficient in processing apoptotic MOG expressing oligodendrocytes for MHC class II presentation to $CD4^+$ T cells also *in vitro*. Commensal specific

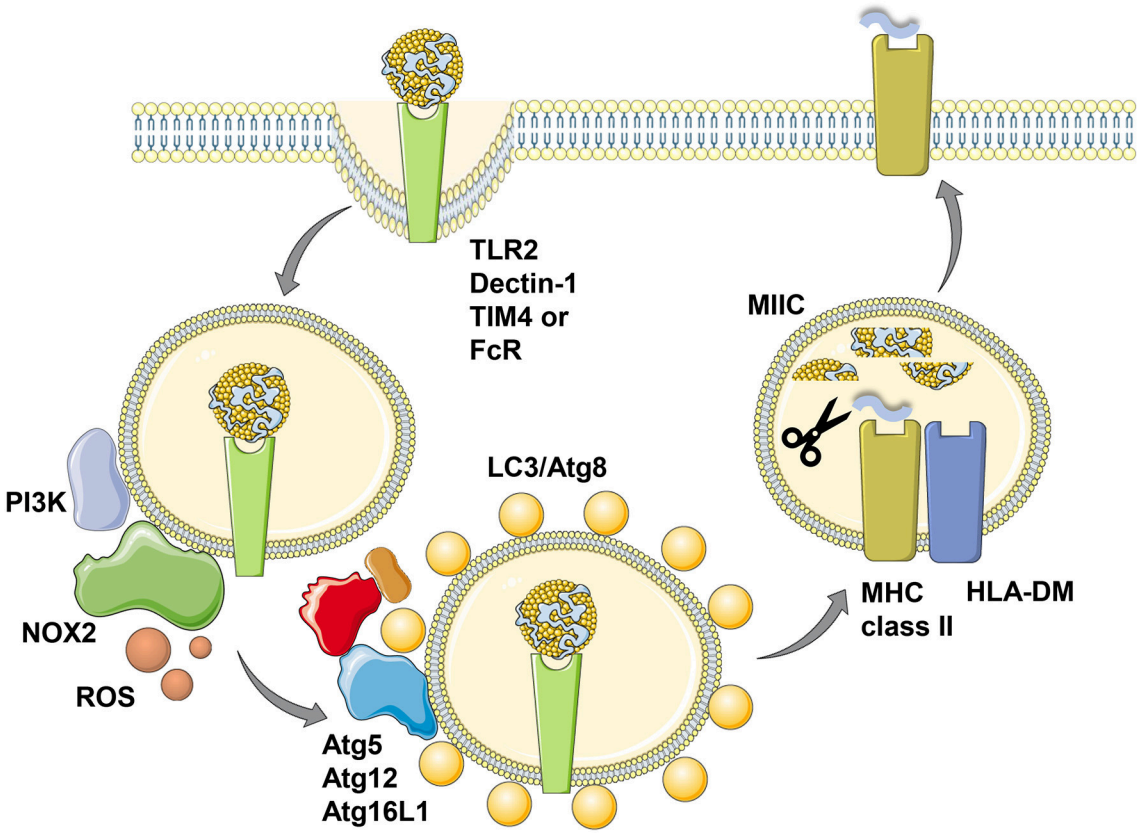

FIGURE 2 | LC3-associated phagocytosis (LAP) of antigens for improved MHC class II presentation. Phagocytosis of ligands for toll-like receptor 2 (TLR2), Dectin-1, T-cell immunoglobulin and mucin domain-containing molecule 4 (TIM4) or antibody Fc receptors (FcR) leads to LC3/Atg8 conjugation to the cytosolic side of the phagosomal membrane in a process called LC3-associated phagocytosis (LAP). Presumably prior to LC3 conjugation this membrane is modified by the PI3 kinase (PI3K) to recruit NADPH oxidase 2 (NOX2), whose reactive oxygen species (ROS) production is required for LAP. The cargo of LC3/Atg8-associated phagosomes is then more efficiently processed for prolonged antigen presentation on MHC class II molecules (MHC class II) which are loaded with lysosomal degradation products in MHC class II containing compartments (MIICs) with the help of the chaperone HLA-DM. This figure was created in part with modified Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License: https://smart.servier.com.

regulatory $CD4^+$ T cells are also less well-induced in mice with Atg16L1 deficiency in dendritic cells and some macrophage compartments (44). Atg16L1 mutations are associated with inflammatory bowel disease (IBD) in humans and patients with such mutations have a decreased frequency of regulatory $CD4^+$ T cells. Accordingly, mice with Atg16L1 deficient dendritic cells and some macrophage populations develop inflammatory bowel disease. In this study, the missing regulatory $CD4^+$ T cell induction was proposed to be mediated by outer membrane vesicles (OMVs) of commensals. Similarly, mice that lack LAP components in their macrophages were less able to clear apoptotic cells, which led to the induction of autoantibodies resulting in a lupus erythematosus like systemic autoimmunity (45). Interestingly, this phenotype was observed in macrophage deficiencies in Atg5, Atg7, Beclin-1/Atg6 or NOX2, but not affected by loss of the ULK1/Atg1 complex components ULK1 or Fip200. In this experimental system inefficient regulatory $CD4^+$ T cell induction could have also contributed to the observed autoimmune phenotype. Therefore, LAP supports anti-fungal, autoimmune and regulatory $CD4^+$ T cell responses *in vitro* and *in vivo* by regulating endocytosed antigen processing for MHC class II presentation.

CONCLUSIONS

The above summarized studies suggest that the macroautophagy machinery fulfills important functions for the optimization of endocytosis. So far two stages of endocytosis have been found to be affected by deficiencies in the LC3/Atg8 lipidation machinery, namely early internalization from the membrane, presumably by a more efficient recruitment of the clathrin dependent internalization machinery, and governing phagosome fate by LC3/Atg8 conjugation to the cytosolic side of these vesicles during LAP (46). The ability of the macroautophagy machinery to conjugate LC3/Atg8 to other membranes than autophagosomes has already been realized by Yoshinori Ohsumi, who received for the discovery of the atg genes the Nobel prize for physiology and medicine in 2016. He observed in yeast that was deficient in the Atg4 protease that cleaves LC3/Atg8

from the outer membrane of completed autophagosomes, and that was transgenic for C-terminally truncated Atg8 which is ready for conjugation to PE that LC3/Atg8 could be found on lysosomal, endosomal and ER membranes (47). These findings suggested that LC3/Atg8 deconjugation by Atg4 restricts this membrane tag to autophagosomes in yeast and that any regulatory mechanism of Atg4 mediated deconjugation would allow LC3/Atg8 to be used for other membrane trafficking functions. The identification of such regulatory mechanisms that allows LC3/Atg8 lipidation to be retained at endosome membranes and then used for phagocytosis should clarify in the future how the macroautophagy machinery can fulfill its different tasks during intracellular and extracellular cargo degradation in lysosomes and MIICs.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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ORIGINAL RESEARCH
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Myosin II Activity Is Selectively Needed for Migration in Highly Confined Microenvironments in Mature Dendritic Cells

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Upon infection, mature dendritic cells (mDCs) migrate from peripheral tissue to lymph nodes (LNs) to activate T lymphocytes and initiate the adaptive immune response. This fast and tightly regulated process is tuned by different microenvironmental factors, such as the physical properties of the tissue. Mechanistically, mDCs migration mostly relies on acto-myosin flow and contractility that depend on non-muscular Myosin IIA (MyoII) activity. However, the specific contribution of this molecular motor for mDCs navigation in complex microenvironments has yet to be fully established. Here, we identified a specific role of MyoII activity in the regulation of mDCs migration in highly confined microenvironments. Using microfluidic systems, we observed that during mDCs chemotaxis in 3D collagen gels under defined CCL21 gradients, MyoII activity was required to sustain their fast speed but not to orientate them toward the chemokine. Indeed, despite the fact that mDCs speed declined, these cells still migrated through the 3D gels, indicating that this molecular motor has a discrete function during their motility in this irregular microenvironment. Consistently, using microchannels of different sizes, we found that MyoII activity was essential to maintain fast cell speed specifically under strong confinement. Analysis of cell motility through micrometric holes further demonstrated that cell contractility facilitated mDCs passage only over very small gaps. Altogether, this work highlights that high contractility acts as an adaptation mechanism exhibited by mDCs to optimize their motility in restricted landscapes. Hence, MyoII activity ultimately facilitates their navigation in highly confined areas of structurally irregular tissues, contributing to the fine-tuning of their homing to LNs to initiate adaptive immune responses.

Keywords: confinement, contractility, chemotaxis, microfabrication, microchannel, collagen

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INTRODUCTION

Antigen delivery from peripheral tissues to LNs by mDCs is critical to initiate the adaptive immune response (1). To ensure its adequacy, this antigen transport needs to occur within a few hours. Consequently, DCs migration to LNs is boosted by signals that trigger their activation, such as pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs, respectively)

(2–5). In this context, we have recently shown that DCs activation leads to a fast and persistent mode of migration, which is linked to the concentration of the acto-myosin cytoskeleton at the cell rear (4–6). MyoII activity generates the force required for mDCs migration in 3D confined microenvironments (7) and is needed for fast and persistent motility during chemotaxis in a dense extracellular matrix (4). Accordingly, failure in inducing MyoII activity is sufficient to delay mDCs homing to draining LNs, with important consequences in the development of immune responses (8).

Importantly, during navigation from the infected tissue to the draining LN, mDCs need to adapt their morphology to the evolving geometrical properties of their microenvironment (9). Recently, several articles have evidenced that distinct cell types increase their MyoII-dependent contractility to migrate in confined microenvironments (10–13). In mesenchymal cells, we have shown that combination of high confinement and low adhesion result in MyoII-dependent fast cell motility *in vitro* (13). In analogy to this observation, fully mature DCs are intrinsically non-adhesive *in vitro* and do not require specific adhesions to migrate in dense 3D microenvironments *in vivo* (7). However, how MyoII activity regulates mDCs motility in response to the degree of confinement remains unexplored.

Here, we combined the use of *ex vivo* imaging and precise *in vitro* microfabricated tools to demonstrate that MyoII activity is important to sustain efficient mDCs navigation exclusively in highly confined microenvironments. Since migratory mDCs possess a high basal level of MyoII activity (6), we propose that this property allows them to adapt their motility to irregular microenvironments found in different tissue compartments. This property might be key to bypass natural physical obstacles in order to reach efficiently the draining LN, ensuring the prompt initiation of the adaptive immune response.

INHIBITION OF CELL CONTRACTILITY REDUCES mDCs MIGRATION SPEED IN A DENSE EXTRACELLULAR MATRIX

To assess the contribution of MyoII to cell migration in a complex microenvironment, we first used an *ex vivo* model tissue. For that, we evaluated the capacity of exogenous mDCs to reach the LVs in mouse ear explants (4, 14). Briefly, *in vitro* differentiated bone marrow-derived DCs were activated with bacterial lipopolysaccharide (LPS), labeled and seeded in the dermal side of open ear explants either in the absence or presence of the MyoII inhibitor Blebbistatin (**Figure 1A**). After 1 h of migration, the tissue was fixed and imaged to quantify the number of mDCs that reached the LVs (**Figure 1B**). Control cells were mostly observed near the lymphatic system or overlapping it, reflecting their strong capacity to migrate toward the LVs. Conversely, in the presence of Blebbistatin, the localization of mDCs was mainly restricted to the area surrounding the LVs (**Figure 1B**). Accordingly, the ratio of mDCs overlapping the LVs over those remaining in the interstitial space decreased upon MyoII inhibition (**Figure 1C**). Importantly, these differences were not due to changes in the expression of CCR7, chemokine receptor responsible for driving mDCs migration toward the lymphatic system (**Figure 1D**). Altogether, these data indicate that MyoII activity is required for the migration of mDCs from the interstitial space toward the LVs in the confined microenvironment of this model tissue.

One limit of this experimental setup is that migration of mDCs in both, the absence or presence of Blebbistatin, cannot be evaluated in the exact same tissue. In addition, despite the short duration of the experiment, the drug might have adverse effects on the tissue itself. Thus, we decided to confirm this result by evaluating the capacity of MyoII conditional knock-out mice mDCs to reach the LVs *ex vivo* (15). For that, exogenous mDCs derived from MyoII-flox/flox/CD11c-Cre- (WT) and MyoII-flox/flox/CD11c-Cre+ (KO) mice were differentially labeled, mixed 50–50% and seeded in the dermal side of the open ear explants (**Supplementary Figures 1A,B**). In our *in vitro* cultures, we observed a reduction by half in the total amount of MyoII, as measured by western blot (**Supplementary Figure 1C**). However, this drop was sufficient to reduce significantly the number of mDCs reaching the LVs (**Supplementary Figure 1D**).

Altogether, these results indicate that MyoII activity is needed for the proper migration of mDCs in ear explants, and a partial decrease in the protein abundance is enough to impair their arrival at LVs, highlighting the relevance of this molecule for mDCs migration in tissues.

Based on these observations, we hypothesized that the decreased mDCs arrival at the lymphatic system might be due to (a) reduced directional migration toward CCL21, the chemokine that guides mDCs toward the lymphatic vessels (14) and/or to (b) a diminished efficiency of mDCs to migrate in the dense extracellular matrix. To test these hypothesis, we used an *in vitro* chemotactic assay in which we assessed the capacity of mDCs to follow a gradient of CCL21 in a dense 3D collagen gel (7, 16). Since MyoII depletion was incomplete in the KO mice, we decided to restrict our experiments to different small inhibitors of this molecular motor. In the control condition, mDCs migrated directionally toward CCL21 (**Figure 1E**), while in areas of the same gel that were not exposed to the chemokine, their motility remained random (**Figure 1E**). In Blebbistatin-treated mDCs, directionality toward the chemokine was not affected (**Figure 1E**, **Supplementary Figure 1E** and **Supplementary Movie 1**) while cell speed was markedly reduced (**Figure 1F**). Slower cell migration was also observed during random motility, indicating that MyoII activity is required for fast cell migration in the 3D extracellular matrix, but is dispensable for sensing or orientation of mDCs toward CCL21 (**Supplementary Figure 1F**). Similar results were obtained from inhibiting the rho-activated kinase (ROCK) using Y27632 (Y27), which also leads to decreased MyoII activity (**Figures 1E,F**, **Supplementary Figure 1F**). These results indicate that MyoII inhibition causes a strong decrease in mDCs migration in 3D microenvironments.

MYOSIN II IS REQUIRED FOR mDCs MIGRATION IN HIGHLY CONFINED MICROENVIRONMENTS

A striking property of 3D collagen gels is the geometrical irregularity imposed to cells, which forces them to transit

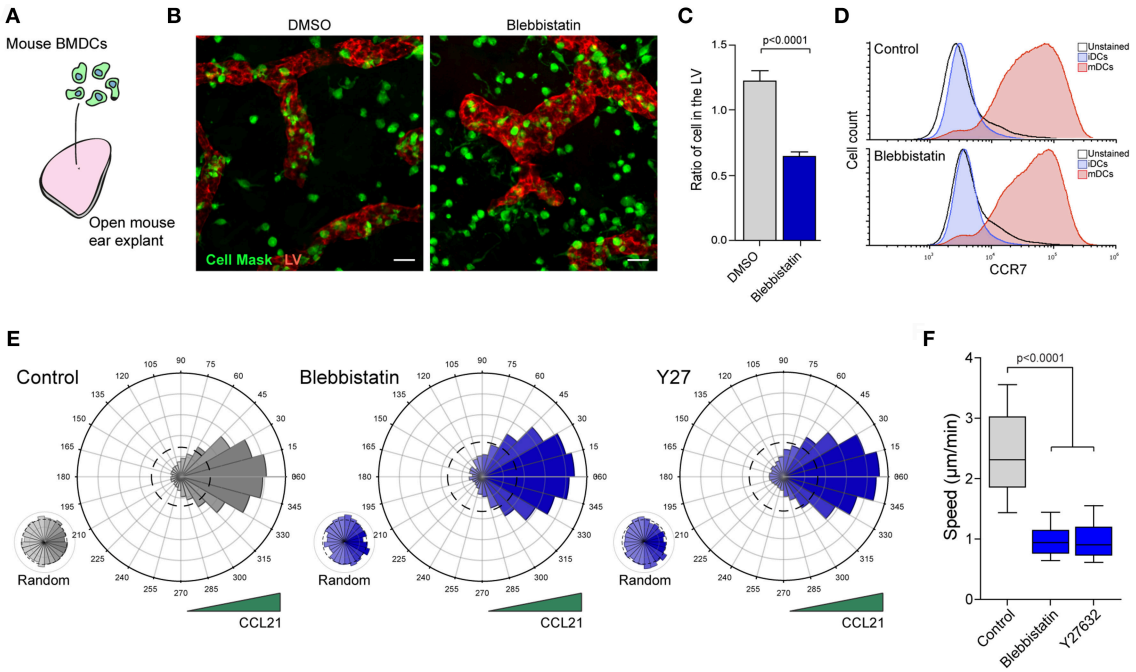

FIGURE 1 | MyoII activity regulates mDCs migration in dense extracellular matrices. **(A–C)** Analysis of mDCs migration in mouse ear explants. **(A)** Schematic representation of the experimental set-up in which *in vitro* differentiated and labeled mDCs were seeded on the dermal side of mouse ear explants. **(B)** Sum z-projection of a representative field from a skin ear explant imaged at 20X on a spinning disk. mDCs are shown in green, LVs stained with anti Lyve-1 in red. Scale bar = 25 µm. **(C)** Quantification of the ratio of mDCs overlapping with the LVs vs. those in the interstitial space. Data from 2 independent experiments, 2 ears explant per experiment and 4 fields of view per explant. Mean and SEM are showed. Unpaired *t*-test with Welch's correction was applied as statistical test. **(D)** Flow cytometry analysis of CCR7 surface expression in mDCs treated or not with Blebbistatin. **(E,F)** Analysis of mDCs trajectories in 3D collagen gels along a CCL21 gradient. **(E)** Polar plots show cell directionality during chemotaxis of control, blebbistatin or Y27632 treated mDCs. Random motility was analyzed in the same gels, but in areas with no access to CCL21. The dashed line in the polar plots indicate the theoretical random motility. One representative experiment out of three is shown (n = 323 cells in control, 219 in blebbistatin and 199 in Y27632) **(F)** Mean speed of control, blebbistatin or Y27632 treated mDCs migrating in the directional zone of the gel. Data correspond to the same trajectories as shown in **E**. In the boxplot, the bar and the box include 90 and 75% of the points, respectively. The line inside the box corresponds to the median. The Mann-Whitney test was used as statistical test.

through zones of variable confinement. Interestingly, inhibition of MyoII activity in mDCs reduces their migration speed in the 3D gel, but without stopping completely their movement (**Supplementary Movie 1**). Based on this observation, we hypothesized that MyoII activity could influence specific steps of mDCs motility depending on the degree of confinement encountered in irregular 3D landscapes.

To test this idea, we took advantage of the precise and diverse geometries that can be generated by using microfabrication (17, 18). With this technology, we designed microchannels of different sizes (8, 5, or 3 µm width by 4 µm height) to challenge cells to migrate in microenvironments with increasing degrees of confinement (**Figure 2A**). First, we observed that mDCs were able to migrate spontaneously independently of the confinement level. Strikingly, despite a strong effect on the cell shape due to the confinement in small microchannels (i.e., 3 µm) (**Figure 2B**), mDCs speed was only slightly reduced in these tubes (**Figure 2C**, **Supplementary Figure 2A**). This suggests that mDCs activate a specific cellular machinery to maintain efficient migration in very confined geometries. Surprisingly, mDCs treatment with Blebbistatin did not significantly affect their speeds in larger channels (i.e., 8 µm) (**Figure 2C**, **Supplementary Figure 2A**, and **Supplementary Movie 2**), but decreased them only under higher confinement (30 and 50% speed reduction in 5 and 3 µm channels, respectively) (**Figure 2C**, **Supplementary Figure 2A**, and **Supplementary Movie 2**). Similar results were obtained from using the ROCK inhibitor Y27, indicative of the phenotype robustness (**Supplementary Figure 2B**). For us, the simplest interpretation of this result is that large channels impose little resistance to migration, and thus the force provided by MyoII is not needed. In contrast, MyoII becomes critical to maintain cell speed in small channels, which impose more resistance to their motility. These results suggest that mDCs regulate MyoII activity depending on the degree of confinement.

MYOII ACTIVITY IS REQUIRED FOR mDCs PASSAGE THROUGH SMALL GAPS

The constant confinement provided by straight microchannels does not recapitulates the complex geometry of dense extracellular matrices, which display multiple irregularities (19). Thus, we decided to study mDCs migration in microchannels coupled to micrometric constrictions, to evaluate the precise

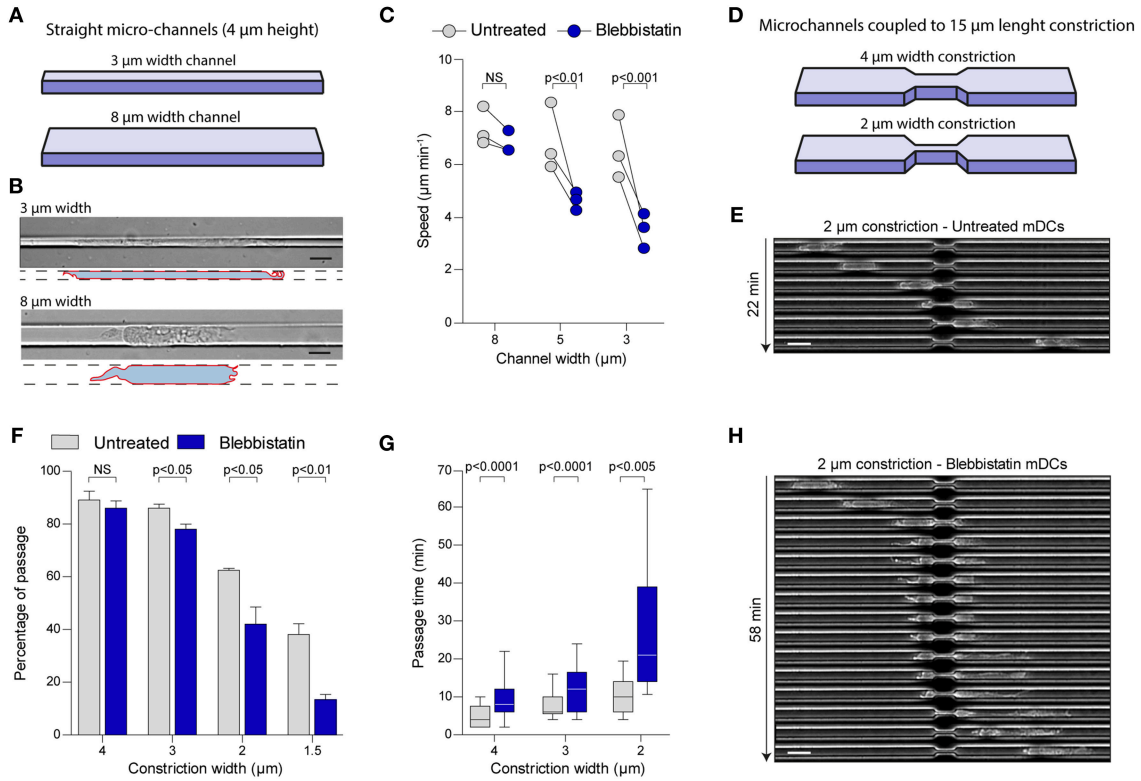

FIGURE 2 | MyoII motor activity is specifically required for mDCs migration in very confined microenvironments. **(A–C)** Analysis of mDCs migration in straight microchannel of different width. **(A)** Schematic representation of the microchannel used, where the height of the microchannel is fixed at 4 µm, and the width of the channel vary from 3 to 8 µm. **(B)** Representative mDCs in 3 and 8 µm width microchannel imaged at 40X with DIC. The drawing highlights the contour of the cells. Scale bar = 10 µm. **(C)** Mean instantaneous speed of untreated or blebbistatin treated mDCs in microchannel of 3, 5, and 8 µm width obtained in three independent experiments. Each dot represents the median of one experiment ($n > 30$ cells for each condition in each experiment). Anova with Tukey's Multiple Comparison Test was applied as statistical test. **(D–H)** Analysis of mDCs passage through constrictions of different sizes. **(D)** Schematic representation of the constrictions added in the microchannel, the height and the length of the constriction are fixed to 4 and 15 µm, respectively, the width of the constriction varies from 1.5 to 4 µm. **(E)** Sequential image of a mDCs passing through a 2 µm width constriction acquired with phase contrast and a 10X objective. Scale bar = 20 µm. **(F)** Percentage of the untreated and treated mDCs passing through the first constriction of the chamber amongst all cells touching it. Bars represent mean and SEM from three independent experiments ($n > 95$ cells for each condition in each experiment). Unpaired *t*-test was applied as statistical test. **(G)** Time spent in the constriction by mDCs passing the constriction. $N = 72$, 142, and 156 untreated mDCs in 2, 3, and 4 µm width constrictions; $n = 42$, 70, and 142 for blebbistatin treated mDCs in 2, 3, and 4 µm width constrictions. The bar and the box include, respectively, 90 and 75% of the points, the center corresponds to the median. One representative experiment out of three is shown. Unpaired *t*-test with Welch's correction was applied for statistical test. **(H)** Sequential image of a blebbistatin treated mDCs passing through a 2 µm width constriction acquired with phase contrast and a 10X objective. Scale bar = 20 µm.

contribution of MyoII activity to migration through an irregular landscape (**Figure 2D**) (9, 16). For that, we used 8 µm width microchannels and added constrictions of different sizes, ranging from 4 to 1.5 µm width, over 15 µm length (**Figure 2D**) (9). Since in these channels MyoII inhibition does not affect cell speed (**Figure 2B** and **Supplementary Figure 2A**), this system allowed us to evaluate the specific role of cell contractility in the passage of mDCs through small pores (**Figure 2E**). First, we evaluated the percentage of cells effectively passing through the different constrictions (**Figure 2F**). Our experiments showed that in control cells, more than 80% of the mDCs passed through 4 and 3 µm width constrictions, while 60 and 40% of them were able to overcome 2 and 1.5 µm pores, respectively (**Figure 2F**). No significant effect was observed on the fraction of cells migrating through 4 µm width constrictions upon MyoII inhibition with Blebbistatin (**Figure 2F**). However, the drug impact intensified progressively with the constriction narrowing, ending up with a 75% inhibition of the passage through the smallest pores (1.5 µm width) (**Figure 2F**). A similar effect was induced by the ROCK inhibitor Y27 (**Supplementary Figure 2C**). Altogether, these data indicate that MyoII activity in mDCs is needed exclusively to pass through narrow gaps smaller than 3 µm in width.

Next, as an indicator of the cell efficiency in deforming and passing through the small gaps, we calculated the time spent by each cell inside a constriction, only if they succeeded to migrate through it. In the analysis, we observed that this time also increased with the pore narrowing, starting at 5 min in average for the 4 µm constriction, and ending with 10 min when moving through the 2 µm gaps (**Figure 2G**). These observations indicate that unlike straight microchannels, the

pore size of an irregular microenvironment can be determinant to restrict mDCs migration. In this setup, MyoII inhibition using blebbistatin systematically increased the time spent by mDCs in the constriction as compared to the control condition, independently of gap size (**Figures 2G,E,H**). Similarly, ROCK inhibition also doubled the passage time of cells through the constrictions for all pore sizes (**Supplementary Figure 2D**). Of note, due to the low proportion of mDCs able to pass through the 1.5 μm constrictions upon MyoII inhibition, the passage time was not considered for this specific condition.

Collectively, these data indicate a dual role of MyoII activity in the migration of mDCs through small gaps: (i) it is required for cell passage through very small holes and (ii) to maintain cell speed while squeezing and deforming in an irregular landscape. Combined with the data obtained from collagen gels and straight microchannels, our experiments demonstrate that MyoII activity in mDCs helps them to adapt their migration to irregular and restrictive microenvironments. This suggests a specific adaptation of mDCs that ensures fast homing from infected tissues to LNs, a situation that imposes a series of physical constrains as cells translocate between distant locations in the organism.

DISCUSSION

Migration from peripheral tissues to lymph nodes is a challenging function for mDCs, which must constantly move through different body compartments adapting to the changing properties of the tissues. Here, we found that MyoII activity in mDCs is required specifically to maintain their speed and squeeze through highly confined microenvironments. In ear explants, MyoII inhibition impaired mDCs arrival at the lymphatic system, which was not due to a defect in the detection of CCL21 gradient but rather to a defect in their intrinsic motile capacity, as measured in collagen gels. This is in agreement with data showing that cell speed can be dissociated from directionality during chemotaxis, having as consequence a decrease in the quality of cell migration *in vitro* and *ex vivo* (4, 20, 21). Interestingly, partial depletion of MyoII was enough to decrease cell migration of mDCs in ear explants. Recently, similar observations were obtained in neutrophils, in which partial depletion of MyoII diminished migration in confinement (22). Together with our data, these evidences indicate that motility of mDCs and neutrophils in confinement is highly sensitive to the levels or activity of MyoII.

Interestingly, inhibition of MyoII reduces the speed, but does not fully stops cells as they migrate in a collagen gel. Cell speed during random motility and chemotaxis decreases in both cases, but cells are still able to move. Since the microenvironment in a 3D gel is irregular, this can be explained by a specific role of MyoII during migration in the more restricted areas of the gel. In agreement with this idea, recent articles have shown MyoII activation under compression (10–13).

Surprisingly, in our experiments, migration speed in large channels was not affected by MyoII inhibition. One possibility to explain this result is that MyoII is simply not required for their movement in such a microenvironment. However, in previous studies obtained by our group, we have seen that MyoII inhibition triggers a global reorganization of the actin cytoskeleton in mDCs when migrating in large channels (6). This suggests the existence of distinct modes of motility that can operate in these cells, resulting in both cases in fast migration *in vitro* when confinement is not strong. Interestingly, mDCs have been previously shown to adapt their motility to the adhesive properties of their microenvironment, alternating distinct modes of migration that sustain fast speed (23). Altogether, these observations indicate the existence of different types of migratory machineries in mDCs that depending on the properties of their microenvironment can compensate to ensure their migratory function.

Migration under strong confinement (small channels and constrictions) required MyoII activity. This indicates that despite the existence of different modes of motility in mDCs, extreme confinement needs a specific migratory mechanism that relies on cell contractility. This mechanism also applies for mDCs migration in dense collagen gels, showing that this need is maintained during migration in more complex landscapes (7). This adaptation is not universal, since some tumor cells and human mesenchymal stem cells have been shown to use a contractility-independent mode of motility under confinement (24, 25). Interestingly, the MyoII requirement to migrate in small holes seems specific to mDCs, since we have shown that passage of immature DCs through constrictions was independent of cell contractility and required Arp2/3-mediated actin nucleation (9). These differences might result in an additional level of control for the migration of mDCs, in which the regulation of MyoII contractility by inflammatory or environmental factors might participate in the fine tuning of their migration to LNs. This MyoII-dependency might be a global requirement for leukocyte migration between tissues, since neutrophils and T lymphocytes also require contractility to squeeze through confined landscapes (13, 26).

Our study also showed that the geometry of the microenvironment has a strong impact on mDCs migration, especially in irregular spaces where the size of the pores limits mDCs passage. Thus, modification in the density of the extracellular matrix may also modulate mDCs arrival at LNs. This property can be particularly relevant during mDCs migration through distinct organs, which display intrinsic differences in their stiffness (27) or during inflammation, known to alter the physical properties of the tissue (28). The same principle can apply during cancer progression, that often alter the properties of the extracellular matrices surrounding the core of the tumor (29, 30) and might prevent immune cells infiltration (31). Thus, modulation of contractility could be used as a general approach to optimize mDCs motility in pathological conditions.

Importantly, the behavior of mDCs relative to the different degrees of confinement suggests a mechano-response in these cells. However, the mechanism(s) that sense the geometry of the tissue and adapt MyoII activity remain unknown. A local control of mDCs contractility has been already reported to promote their transmigration, where MyoII activity was modulated by chemical signals from lymphatic endothelial cells (32). Also, a possible

specific regulatory mechanism might come from lysosomal signaling, that we have recently shown to regulate MyoII-induced contractility at the back of mDCs (6). Understanding how MyoII activity is modulated in response to confinement might provide molecular tools to modulate cell migration through specific tissues. In particular, in inflammatory diseases such as auto-immune encephalopathies or allergic contact dermatitis, down regulation of MyoII activity prevents mDCs migration to LNs and limits inflammation (8), while in other situations, such as tumors, increasing their migration might be beneficial (33). A better understanding of the mechanisms regulating mDCs contractility under confinement can create new routes to the development of molecules to tune the adaptive immune response with therapeutic purposes.

METHODS

Cells

Bone marrow derived dendritic cells (BMDCs) were obtain by differentiation of bone morrow precursors for 10 days in DCs medium (IMDM-Glutamax, FCS 10%, pen-strep 100 U ml^{-1}, and 2-ME 50 μM) supplemented with granulocyte-macrophage colony stimulating factor (GM-CSF)-containing supernatant (50 ng ml^{-1}) obtained from transfected J558 cell line, as previously described (34). Briefly, at day 10 of differentiation, semi-adherent DCs were treated with LPS (100 ng ml^{-1}) for 30 min, then washed 3 times with DCs medium and cultured overnight (ON) to reach full DC maturation. Migration of mDCs was recorded between 24 and 34 h post LPS treatment.

Mice

BMDCs were obtained from wild-type C57BL/B6 mice (Charles River). In the case of MyoII KO, BMDCs were differentiated from MyoIIA-flox/flox-CD11c-Cre+ mice, as previously described (15, 35, 36). Littermate MyoIIA-flox/flox CD11c-Cre- were used as a control. In general, 6 to 10 weeks old mice were used as source for bone marrows; 4 to 6 weeks old mice were used as ear explant donors. For animal care, we strictly followed the European and French National Regulation for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Directive 2010/63; French Decree 2013-118). The present experiments, which used mouse strains displaying non-harmful phenotypes, did not require a project authorization and benefited from guidance of the Animal Welfare Body, Research Centre, Institut Curie.

Antibodies and Reagents

For drug treatment, Blebbistatin (50 μM, Sigma) and the equivalent amount of DMSO (Sigma-Aldrich), or Y-27632 (10 μM, Tocris Bioscience) and the equivalent amount of distillated water were used. For labeling of lymphatic vessels in mouse era explants, Alexa Fluor 655-coupled anti-Lyve-1 antibody was used (R&D System, 1/50). To label BMDCs for migration in ear explants we used Hoechst 33342 (200 ng ml^{-1}, Life Technologies) and CellMask CFSE or CMTMR (2.5 μM) (Life Technologies). For western blot, Non-muscle Myosin Heavy Chain II-A Antibody (Biolegend, clone Ply19098, 1/200) and GAPDH Antibody (Cell Signaling, clone 14c10, 1/5,000) were used. For flow cytometry analysis: Mouse CCL19-Fc Fusion Recombinant Protein (1/400, eBioscience) and Alexa-Fluor 488-coupled anti-human Fc (1/400, eBioscience).

Migration in Ear Explant

Migration of DCs was performed as previously described (14) but modified to work with fixed samples. Briefly, ears were excised from C57BL/6 mice and the ventral part of the skin was peeled off to expose their dermal side. 100,000 colored LPS-activated BMDCs were added on the top of the exposed dermal side of the skin explant. After 1 h of incubation, explants were washed to remove the loosely attached mDCs and fixed during 20 min on a drop of 4% paraformaldehyde (Sigma-Aldrich). In the case of Blebbistatin treatment, the cells were colored and pre-incubated 2 h with the drug before seeding in the ear and was further maintained during migration in the explants. In the case of MyoII WT and KO mDCs, 75,000 colored LPS-MyoII KO BMDCs were mixed with 75,000 colored LPS-MyoII WT BMDCs and added on the top of the exposed dermal side of the skin explant. After fixation, the explants were washed in PBS 2%-BSA and the lymphatic vessels were stained with Alexa Fluor 655-coupled anti-Lyve-1 antibody 1 h at 4°C. After three washes, skin explants were mounted in a microscopy slide using fluoromount-G (Invitrogen) and imaged on a Spinning disk confocal CSU X1 inverted microscope (Leica) and a ×20 dry objective (NA 0.75). For Blebbistatin experiments, mDCs overlapping or not with the lymphatic system were manually count on a SUM z-projection. For MyoII-KO experiments, a custom ImageJ macro was used to count the number of nucleus corresponding to each phenotype. Briefly, a SUM z-projection was made and, using appropriated thresholding, we detected MyoII-WT and MyoII-KO nucleus. Then, using a mask obtained from the lymphatic vessels, we counted the numbers of nucleus of MyoII-WT and MyoII-KO mDCs overlapping or not with the lymphatic system. The ratio of mDCs in the lymphatic vessels was calculated as the number of nucleus in the lymphatic vessel divided by the number of nucleus outside the lymphatic vessels.

Migration in Collagen Gels

Collagen experiments were performed as previously described (16). Briefly, mDCs were mixed at 4°C with rat tail collagen type I (Corning) at 3 mg ml^{-1} at basic pH and loaded in the custom-made chamber in polydimethylsiloxane (PDMS). The sample was incubated at 37°C for 30 min to allow gel polymerization. Then, 2 ml of DC medium containing 200 ng ml^{-1} CCL21 (R&D Systems) was added in the dish, generating a chemokine gradient that triggered directed mDC migration. When indicated, cells were pre-incubated 1 h with blebbistatin at 50 μM or Y27 at 10 μM, and then maintained in the media during their chemotaxis. Cells were imaged overnight with a DMi8 inverted microscope (Leica) at 37 °C with 5% CO_2 atmosphere and a ×10 dry objective (NA 0.40 phase). Resulting movies were processed with average subtraction, mean filter and Gaussian Blur filter to obtain cells as white round object on a dark background. Tracking was performed with Imaris software in the first 400 μm from the border of the chamber,

where the gradient is stable. Tracks of objects moving <10 μm length or lasting <10 min were removed from the analysis to avoid artifacts.

Migration in Micro-Channels

Micro-channels experiments were performed as previously described (18). Briefly, PDMS (RTV615, Neyco) was used to make microchannels of the different geometries from custom-made molds. The micro-channels were coated with bovine plasma fibronectin (10 μg ml^{-1}) (Sigma-Aldrich) for 1 h at RT and washed 3 times with PBS before incubating with DC medium for at least 1 h at 37°C and 5% CO_2 before cell loading. When indicated, this media also contained 50 μM blebbistatin or 10 μM Y27632. Migrating cells were recorded overnight with a DMi8 inverted microscope (Leica) at 37 °C with 5% CO_2 atmosphere and a ×10 dry objective (NA 0.40 phase). One image every 2 min during 16 h was recorded.

Quantification of Cell Migration in Micro-Channels

Kymographs for each channel were generated using a semi-automated ImageJ macro. For velocity measurements, kymographs from isolated migrating cells were manually extracted and analyzed using a custom Matlab program as previously described (34). For cell passage through constrictions, kymographs from each channel were analyzed using a semi-automated ImageJ macro. We focused on the passage of the first constriction encountered by the cell. The percentage of passage represents the ratio between the number of cells that passed a constriction respect to the number of cells that encountered a constriction. The passage time represents the time between the time at which the cell front reaches the constriction and the time at which the cell back exits the constriction.

Immunobloting

1.5 millions of mDCs were lysed for 30 min in 40 μl of lysis buffer containing 100 mM Tris, 150 mM NaCl, 0.5% NP-40 and a protease inhibitor cocktail tablet (Roche). 10 μl of extracts were loaded onto a 4–20% TGX gradient gel (BioRad) and transferred onto an Ethanol-activated PVDF membrane by over-night wet transfer (BioRad). The membrane was blocked, incubated sequentially with the appropriate antibodies and revealed using the SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific). Membranes were cut accordingly to the molecular weight of the protein of interest. This allowed us to evaluate different labeling in the same run. As consequence, full membranes were in most cases only fragments.

Flow Cytometry Analysis

750,000 mDCs pre-incubated 2 h with Blebbistaitin or DMSO as a control were resuspended in 50 μl of PBS 2% BSA alone or with Mouse CCL19-Fc Fusion Recombinant Protein. After 1 h of staining at 4°C, cells were washed two times and incubated for 1 h with Alexa Fluor 488-coupled anti-human Fc at room temperature. After two washes, cells were resuspended in 200 μl of PBS 2% BSA. Single cell fluorescence were measured on a Accuri flow cytometer and analyses with FCS Express 6 software. Appropriated gating was made on the SSC/FFC signal.

AUTHOR CONTRIBUTIONS

LB performed and analyzed most experiments, prepared manuscript figures and strongly participated in article writing. PS performed and analyzed collagen gels experiments and contributed to article writing. RA designed the microchannels and performed photolithography. A-ML-D provided animal models and contributed to article correction. IL wrote the code to analyze cell trajectories in collagen gels. MP participated in experiment design and contributed to article writing. PV designed the overall research and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00747/full#supplementary-material

Supplementary Figure 1 | (A,B) Analysis of mDCs migration in mouse ear explants. **(A)** Schematic representation of the experimental set-up in which *in vitro* differentiated and labeled mDCs were seeded on the dermal side of mouse ear explants. **(B)** Sum z-projection of a representative field from a skin ear explant imaged at 20X on a spinning disk. mDCs are shown in green, LVs stained with anti Lyve-1 in white. Scale bar = 30 μm. **(C)** Quantification of the ratio of mDCs overlapping with the LVs vs. those in the interstitial space. Data from two independent experiments, three ears explant per experiment and three field of view per explant. Mean and SEM are showed. Paired *t*-test was used as statistical test. **(D)** Western blot analysis of Myosin II A heavy chain expression in mDCs derived from MyoII-flox/flox/CD11c-Cre- (WT) and MyoII-flox/flox/CD11c-Cre+ (KO) mice. Histogram shows the quantification of the plot. **(E)** Directionality index of mDCs migrating in collagen gels. Gray bars correspond to random trajectories and blue bars to tracks during chemotaxis. This analysis is based on the data shown in **Figure 1E**. **(F)** Representative experiment showing the mean speed of control, blebbistatin or Y27632 treated mDCs migrating randomly in a collagen gel. Control $n = 341$, blebbistatin $n = 104$, Y27632 $n = 90$. Three independent experiments were performed. In the boxplot, the bar and the box include 90 and 75% of the points, respectively. The line inside the box corresponds to the median. The Mann-Whitney test was used as statistical test.

Supplementary Figure 2 | (A) Representative experiment of mDCs migrating in microchannels of different sizes. The figure shows the mean instantaneous speed

of untreated or blebbistatin treated mDCs in microchannel of 3, 5, and 8 μm width. $N = 91$, 109, and 178 untreated mDCs in 8, 5, and 3 μm width microchannel, respectively; $n = 53$, 85 and 66 for blebbistatin treated mDCs in 8, 5, and 3 μm width microchannels, respectively. Unpaired t-test was applied as statistical test with Welch's correction for 3 μm width microchannel. **(B)** Mean instantaneous speed of untreated or Y27632 treated mDCs in microchannel of 3, 5, and 8 μm width obtained in three independent experiments. Each dot represents the median of one experiment ($n > 30$ for each condition in each experiment). Anova with Tukey's Multiple Comparison Test was applied as statistical test. **(C)** Percentage of untreated and Y27632 treated mDCs passing through the first constriction of the chamber amongst all cells touching it. One experiment with $n = 76$, 54, 105, and 111 untreated mDCs in 1.5, 2, 3, and 4 μm width constrictions; $n = 53$, 64, 122, and 107 for Y-27632 treated mDCs in 1.5, 2, 3, and 4 μm width constrictions **(D)** Time spent in the constriction by mDCs passing the constriction in the presence or absence of Y27632. The bar and the box include respectively 90 and 75% of the points, the center corresponds to the median. One experiment with $n = 69$, 91, and 100 untreated mDCs in 2, 3, and 4 μm width constrictions; $n = 32$, 104, and 88 for Y-27632 treated mDCs in 2, 3, and 4 μm width constrictions. Unpaired *t*-test was applied for statistical test.

Supplementary Movie 1 | MyoII inhibition decreases cells speed of mDCs during chemotaxis in dense collagen gels. mDCs activated with LPS were seeded in 3 mg/ml rat tail collagen gels and exposed to 200 ng/ml CCL21. Control and blebbistatin treated cells are able to follow the chemokine gradient. Despite decreased speed, MyoII inhibition does not impact cell directionality. Bar = 100 μm.

Supplementary Movie 2 | MyoII inhibition decreases cells speed of mDCs exclusively in the 3 μm-width microchannels. The movie shows mDCs spontaneously migrating in 8 μm **(left)** or 3 μm **(right)** width microchannels. MyoII inhibition using Blebbistatin decreases mDCs speed only in the 3 μm width microchannel. Image acquired with phase contrast and a 10X objective, one image every 2 min, time indicated in h:min.

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REVIEW
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Chromatin Architecture as an Essential Determinant of Dendritic Cell Function

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Epigenetics has widespread implications in a variety of cellular processes ranging from cell identity and specification, to cellular adaptation to environmental stimuli. While typically associated with heritable changes in gene expression, epigenetic mechanisms are now appreciated to regulate dynamic changes in gene expression—even in post-mitotic cells. Cells of the innate immune system, including dendritic cells (DC), rapidly integrate signals from their microenvironment and respond accordingly, undergoing massive changes in transcriptional programming. This dynamic transcriptional reprogramming relies on epigenetic changes mediated by numerous enzymes and their substrates. This review highlights our current understanding of epigenetic regulation of DC function. Epigenetic mechanisms contribute to the maintenance of the steady state and are important for precise responses to proinflammatory stimuli. Interdependence between epigenetic modifications and the delicate balance of metabolites present another layer of complexity. In addition, dynamic regulation of the expression of proteins that modify chromatin architecture in DCs significantly impacts DC function. Environmental factors, including inflammation, aging, chemicals, nutrients, and lipid mediators, are increasingly appreciated to affect the epigenome in DCs, and, in doing so, regulate host immunity. Our understanding of how epigenetic mechanisms regulate DC function is in its infancy, and it must be expanded in order to discern the mechanisms underlying the balance between health and disease states.

Keywords: dendritic cells, epigenetics (MeSH), metabolism, inflammation, tolerance, microenvironment

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INTRODUCTION

Epigenetics refers to the regulation of gene expression by mechanisms other than changes in DNA sequence. Epigenetic mechanisms enable long-term phenotypic responses to the environment in the absence of initiating stimuli. Historically, epigenetic memory has referred to stable changes that are maintained through cell division. However, it is increasingly appreciated that dynamic changes in the epigenome, including in the absence of cell division, are equally important for proper cellular function.

Dendritic cells (DCs) are phagocytic cells of the innate immune system that reside in nearly every tissue and specialize in antigen presentation. They are rapidly responsive to stimuli including infection, inflammation, cancer, particles and cellular damage, are highly migratory, and direct the nature of ensuing immune responses by producing context-specific factors such as cytokines. As

for most cells, epigenetic mechanisms underpin lineage specification of DCs. There are several subsets of DCs, all of which are derived from a common DC progenitor (CDP). CDPs give rise to plasmacytoid DCs (pDCs) and pre-DCs, the latter of which differentiates into conventional DCs (cDC1s and cDC2s) in secondary lymphoid tissues (1–6). pDCs produce high levels of type 1 interferons (IFNs) during antiviral and anti-tumor responses. cDCs are highly-specialized antigen-presenting cells; cDC1s ($XCR1^+$) specialize in antigen cross-presentation and stimulation of $CD8^+$ T cells and Th1 responses, whereas cDC2s ($CD11b^+$ $CD172a^+$) specialize in antigen presentation to $CD4^+$ T cells and direct responses to extracellular pathogens (7–10). Additionally, during active inflammation, monocytes can acquire the function of macrophages or DCs (moDCs) (8). The transcriptional mechanisms controlling lineage commitment and DC diversity have been extensively studied. Lineage-determining factors such as PU.1 and C/EBP are significant regulators of myeloid cell differentiation. They facilitate lineage specification of hematopoietic cells by forming stable interactions with their chromatin substrates, enabling secondary factors to drive lineage-specific gene expression (11–14). The complexity of lineage-determining factors and their roles in specifying DC fate through regulation of chromatin remodeling and gene expression have been described elsewhere and is not addressed here (15, 16).

DCs are relatively rare, and thus a number of *in vitro* culture systems have been developed to study their function (17). While the cells generated in these cultures do not perfectly reflect cells found *in vivo*, their experimental use has significantly advanced our knowledge of DC biology. Human DC cultures are typically monocyte-derived and generated by culturing blood monocytes with GM-CSF and IL-4 (18). For mouse, bone marrow can be cultured with combinations of GM-CSF with or without IL-4 to give rise to heterogeneous cultures of bone marrow-derived DCs (BMDCs) that possess cDC- and macrophage-like qualities (19–22). Culturing bone marrow with FLT3L gives rise to a mixed culture containing both cDC- and pDC-like cells (23–26). More recently, the addition of Notch-ligands to the *in vitro* culture system gives rise to cells that are more phenotypically similar to cDC1s and cDC2s (27). Because of the ease of generating BMDCs and the feasibility of generating large numbers of cells, BMDCs are frequently used for biochemical studies, including those addressing epigenetic and metabolic mechanisms.

Further to differentiation, dynamic epigenetic regulation is inherent to the massive transcriptional reprogramming required to orchestrate an effective and efficient immune response (28–31). In steady-state BMDCs, transcription factors (TFs), including ATF3, IRF4, and JUNB, were discovered to serve as priming factors for genes that are rapidly induced following TLR stimulation (11). Priming factors are present at accessible promoters and enhancers in the absence of stimulation. Upon stimulation, priming factors facilitate induced gene expression, possibly by serving as docking sites for dynamic factors or by maintaining chromatin accessibility of regulatory elements for other factors (11, 32). Epigenetic regulation of gene expression is also important for communicating context. Context is inferred by cell surface receptors such as pattern recognition receptors (PRRs) and cytokine/chemokine/nutrient receptors, which detect environmental stimuli. Downstream of such receptors, receptor-specific signal transduction pathways lead to the activation of dynamic TFs, including EGR1, EGR2, NF-κB, and STATs, to mediate context-specific gene expression reprogramming (11, 15, 28, 32, 33). For example, lipopolysaccharide (LPS) stimulation of DCs leads to a signaling cascade downstream of Toll-like receptor 4 (TLR4) that results in NF-κB activation and translocation into the nucleus. NF-κB activates the transcription of thousands of LPS-response genes necessary to orchestrate inflammation (22). Similarly, type I IFNs stimulate STAT1 activation through their receptor, IFNAR. IFNAR activation leads to the activation of interferon signaling genes (ISGs) that include antiviral response genes (34). The ability of these coordinated networks of transcription factors to drive programs of gene expression is intimately linked to the accessibility to regulatory regions such as enhancers and promoters, which is determined by the chromatin landscape.

Integration of context-specific gene expression into epigenetic memory is necessary for DCs to communicate context to other cells once they have migrated away from the site of initial stimulation. The extent to which dynamic changes occurring in the chromatin landscape following stimulation remain stable in rapidly responding, short-lived immune cells such as DCs is not well-understood. While activating TF networks are relatively well-studied in DCs, less is known about the impact of chromatin modifying factors on DC function. Here, we discuss epigenetic mechanisms that have been implicated in the regulation of DC biology, with emphasis on function over differentiation.

EPIGENETIC MODIFICATIONS

DNA methylation, histone modifications and chromatin accessibility are the most well-studied mechanisms that regulate gene expression (35–37). Implicated regulatory proteins are known as "readers," "writers," or "erasers" that detect, deposit or remove histone modifications, respectively. Histone modifications and associated regulatory proteins are continually being identified and our understanding of the mechanisms by which they regulate gene expression are continually refined [**Table 1**; (44, 45)]. ATAC-seq, (Assay for Transposase Accessible Chromatin coupled to sequencing) gives an overall picture of chromatin accessibility irrespective of specific modifications and can be performed on few cells (46). Recently, a fairly comprehensive atlas of chromatin accessibility of 86 immune cells, including 5 DC subsets, was reported (47). These data provide key insights to the overall differences in the chromatin landscape among immune cells and serve as a foundation to more extensively study the mechanisms underlying the diverse and dynamic chromatin architecture in immune cells.

DNA Methylation

DNA methylation of cytosine residues (5-methylcytosine; 5mC) occurs in the context of CpG dinucleotides and is mediated by the family of DNA methyltransferases (DNMTs) (36, 48, 49). Sites of DNA methylation are relatively stable, and are propagated through DNA replication during cell division.

TABLE 1 | Enzymes mediating epigenetic modifications.

Enzyme family	Examples	Catalyzed residue(s)*	Transcriptional response
DNA Methyltransferase (DNMT)	DNMT1	Cytosine	Repression (Activation)
	DNMT3a		
	DNMT3b		
DNA Demethylase	TET1-3	5-methylcytosine (5mC)**	Activation
Histone Deacetylase (HDAC)	HDAC1-11	K residues, specificity unknown	Repression
	SIRT1	H1K26; H3K9, K14, K56; H4K16	
	SIRT2	H3K56; H4K16	
	SIRT3	H4K16	
	SIRT4-5	None	
	SIRT6	H3K9, K56	
	SIRT7	H3K18	
Histone Acetyltransferase (HAT)	HAT1	H2AK5; H4K5, H4K12	Activation
	p300	H2AK5; H2BK5, K12, K15, K20; H3K9, K14, K18, K23, K27; H4K5	
	CBP	H2AK5; H2BK12, K15; H3K18, K23, K27	
	hGCN5	H3K9, K14, K18, K23	
	Tip60	H2AK5, H3K14, H4K5	
	PCAF	H3K14	
	SRC-1	H3K9, K14	
	OGA	H3K14	
	CLOCK	H3K14	
	hMOF	H4K16	
	ATF2	H2BK5, K12, K15; H4K5	
Histone Methyltransferase (HMT)	KMT1A-B	H3K9	Repression
	KMT1C	H3K9, H3K27, H3K56	
	KMT1D	H3K9, H3K27	
	KMT1E-F	H3K9	
	KMT2A-G	H3K4	Activation
	KMT2H	H3K4, H3K36	
	KMT3A	H3K36	
	KMT3B	H3K36, H4K20	
	KMT3C	H3K4, H3K36	
	KMT4	H3K79	
	KMT5A-C	H4K20	Repression
	KMT6	H3K9, H3K27	
	KMT7	H3K4	Activation
	PRMT5	H3R8	Repression
	PRMT6	H3R2	
	CARM1	H3R2, R17, R26	Activation
	PRMT1	H4R3	
Lysine Demethylase (KDM)	KDM1A	H3K4, H3K9	Repression
	KDM1B	H3K4	
	KDM2A	H3K36	
	KDM2B	H3K36, H3K4	
	KDM3A-B	H3K9	Activation
	JMJD1C	H3K9	
	KDM4A	H3K9, H3K36, H1.4K26	Activation/ Repression
	KDM4B	H3K9, H3K36, H1.4K26	
	KDM4C	H3K9, H3K36, H1.4K26	
	KDM4D	H3K9	Activation
	KDM5A-D	H3K4	Repression
	KDM6A	H3K27	Activation
	KDM6B	H3K27	
	KDM7A	H3K9, H3K27	
	KDM8	H3K36	Repression
	PHF8	H3K9	Activation
	PHF2	H3K9	
	NO66	H3K4, H3K36	Repression
E3 ligase activity	RING1A	H2AK119ub1	Repression
	RING1B		

**Lysine (K), arginine (R). **TET catalyzes 5mC to 5-hydroxymethylcytosine (5hmC), which will be repaired by thymine-DNA glycosylase (TDG) to yield non-methylated cytosine. Enzyme families reviewed in Jones (36), Di Croce and Helin (38), Seto and Yoshida (39), Keating and El-Osta (40), Kampranis and Tsichlis (41), D'Oto et al. (42), and Kohli et al. (43).*

De novo methylation is mediated by DNMT3A/B whereas the reliable transmission of DNA methylation from a mother cell to a daughter cell depends on DNMT1 linked to the replication machinery (50). CpG-rich regions, termed CpG islands, are typically unmethylated but can be aberrantly methylated in cancer and during aging (51, 52). The relationship between CpG methylation and gene regulation is complex. Methylation in promoter regions leads to silencing, whereas methylation in the gene body may facilitate gene expression (36). Proteins containing methyl-CpG binding domains (MDB), C2H2 zinc fingers, or SET-RING finger-associated (SRA) domains that recognize methylated DNA generally promote gene repression, however can also mediate gene activation (53, 54).

Loss of 5mC can occur passively through cell division (where methylation is not copied) or can be actively mediated in a replication-independent manner by Ten eleven translocation (TET) hydroxylases (48). TET hydroxylases catalyze the oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) in an Fe^{2+}- and α-ketoglutarate-dependent manner (55). 5hmC can be iteratively oxidized by TET enzymes to other oxidized cytosines that are recognized and excised by thymine DNA glycosylase and replaced with an unmodified cytosine by base-excision repair. 5hmC is found in promoter gene bodies of actively transcribed genes, suggesting that it may have functions other than mediating DNA demethylation (48, 56, 57).The importance of TET enzymes and 5hmC for differentiation of lymphoid and myeloid cells is well-established; however, roles for DNA methylation and 5hmC in regulating immune cell function have been addressed predominantly in lymphoid cells (48).

Consistent with the role of DNA methylation in regulating cellular differentiation programs, several *in vitro* studies have found that DNA methylation is significantly remodeled during

DC differentiation. Cultured monocytes can differentiate to multiple lineages, depending on the cytokine cocktail provided. GM-CSF alone, or in combination with IL-4, will stimulate DC differentiation, while a GM-CSF, IL-4 and prostaglandin E2 (PGE2) cocktail will promote differentiation to monocyte-derived suppressor cells (MDSCs) (58–61). IL-4 signaling promotes DC differentiation by activating STAT6. STAT6 promotes the expression of DC-specific genes by recruiting TET2, which results in demethylation and increased DC-specific gene expression (62). PGE2 promotes MDSC differentiation by activating DNMT3A, which methylates and suppresses proinflammatory genes, thus supporting an immunosuppressive phenotype (63). The DNA methylome may serve to prime lineage-specific proinflammatory genes for rapid transcriptional activation upon encounter of appropriate stimuli (64). Though the DNA methylome is thought to be remarkably stable, at least one study has demonstrated that bacterial infection of human DCs leads to rapid DNA demethylation in the absence of cell division (65). In this case, loss of DNA methylation occurred most frequently at enhancers and was associated with the recruitment of dynamic TFs. Increased 5hmC levels were also detected, strongly implicating TET proteins in this process. Thus, surveying the genome-wide DNA methylation profile of DCs can reveal cellular adaptation patterns to extrinsic stimuli, particularly in the context of DC development and differentiation, and in the context of infection. Annotating DNA methylation to gene bodies, promoter regions or other regulatory regions may clarify the contribution of DNA methylation to gene expression programs in DCs. Furthermore, because DCs do not divide following stimulation, monitoring both 5mC and 5hmC may also shed light on dynamic changes in epigenetic control of key genes that regulate inflammatory function of DCs.

Histone Modification

The enzymatic addition or removal of chemical groups to histone tails regulates chromatin structure and therefore the location and activity of regulatory factors that control transcription. The most widely studied histone modifications are acetylation, methylation and ubiquitylation [**Table 1**; (38–43)]. The histone code refers to the combination of these modifications that collectively determines the outcome of gene expression (37). In general, transcriptional activation is associated with acetylation of lysine residues of histones, which promotes a more relaxed chromatin structure. Acetylation is mediated by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs). Histone methylation, on the other hand, is associated with both transcriptional activation and transcriptional repression. There are many described histone methyltransferases (HMTs) and lysine demethylases (KDM) that target a range of lysine and arginine residues (**Table 1**). Ubiquitination has been mostly studied in the context of the E3 ligase Really Interesting New Gene (RING) proteins that are associated with polycomb repressive complex 1 (PRC1) and deposit ubiquitin on H2A.

Profiling a set of well-studied histone marks can give an overall picture of the activity of a given gene or regulatory region. H3K36me3, H3K27Ac, and H3K4me3 are commonly enriched at active genes, whereas H3K27me3 and H3K9me3 are enriched at silenced genes. H3K4me1 is often found at enhancers while H3K4me3 is enriched at active promoters (66). An enhancer is considered "poised" if it carries H3K4me1 alone or in combination with H3K27me3, and is considered active if H3K4me1 is in combination with H3K27ac (67–70). The genome-wide histone modification profile helps determine cellular identity in part by instructing binding events at specific chromosomal loci; histone modifications can alter the accessibility of transcriptional machinery at underlying genes, or can serve as beacons to recruit chromatin remodelers to either detect, deposit, or remove these histone marks (71). Any irregularities in this system can thus threaten cellular identity, potentially initiating disease (72, 73). Further, several studies have argued these irregularities to be the result of an emerging player in chromatin dynamics: altered cellular metabolism (40).

INTERSECTION BETWEEN IMMUNOMETABOLISM AND EPIGENETICS

The enzymes that modify histones and DNA require specific metabolites as substrates and cofactors (**Figure 1**). Epigenetic modifications are therefore dependent on the availability of these metabolites and the metabolic pathways used by the cell. In turn, metabolic programming is controlled by epigenetics. Therefore, epigenetic and metabolic control of cellular function intersect at many levels.

Cellular metabolism is central to the regulation, function and activation of immune cells, including DCs. Glycolysis is a major metabolic pathway that rapidly generates energy by breaking down glucose into pyruvate in the cytosol. Pyruvate can enter the mitochondria and feed into the tricarboxylic acid (TCA) cycle, which produces reducing agents that donate electrons to the electron transport chain. This powers highly efficient energy production through a process called oxidative phosphorylation. Importantly, glycolysis and the TCA cycle generate intermediates that feed into numerous other metabolic pathways. Upon TLR stimulation, DCs shift their metabolic activity to glycolysis, and inhibiting this shift impairs DC activation (74, 75). The increase in glycolytic activity increases pyruvate, and subsequently citrate levels, to fuel fatty acid synthesis required to support the rapid membrane expansion that accompanies DC activation (75). Despite their similarity, cDC1s and cDC2s have recently been described to possess distinct metabolic phenotypes that are essential for their differing priming functions, with cDC1s displaying much greater oxidative metabolism (76). The epigenetic factor, polycomb group factor 6 (PCGF6), which has been found to maintain DC quiescence and limit DC activation by negatively regulating H3K4me3 levels, also impairs early glycolytic activity, as measured by extracellular acidification rate (77). Whether, PCGF6 partly limits DC activation by regulating chromatin accessibility of genes important for certain metabolic pathways is unknown.

How cellular metabolism and differing metabolic states affect the DC epigenome requires further investigation; however, several studies of conserved pathways in other innate immune cell types provide insight. Methylation requires methyl groups

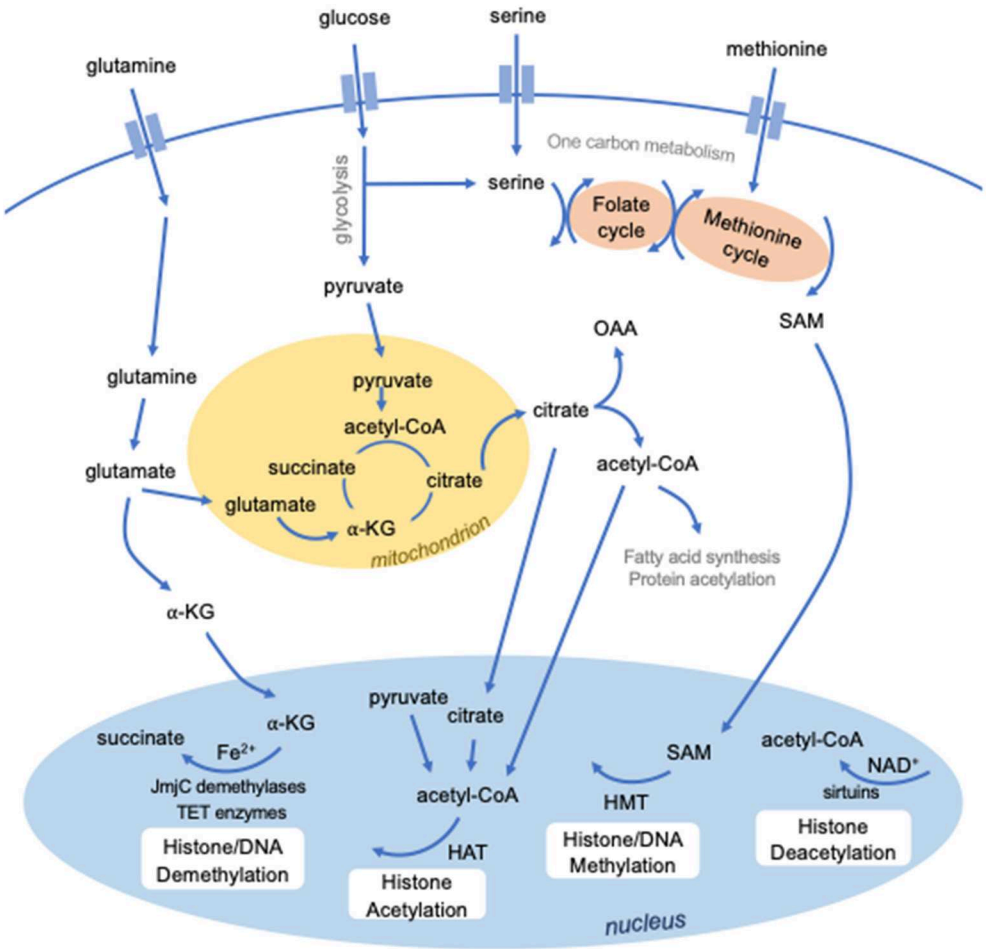

FIGURE 1 | Intersection between metabolism and epigenetics. Several metabolites are required to mediate epigenetic modifications. S-adenosylmethionine (SAM), derived from methionine and one-carbon metabolism, is used for methylation by histone methyltransferases (HMTs). Certain classes of enzymes responsible for histone (JmjC domain-containing demethylases) or DNA (TET enzymes) methylation are dependent on $Fe2^{+}$ and alpha-ketoglutarate (a-KG). Histone acetylation by histone acetyltransferases requires the metabolic intermediate acetyl-CoA, which can be derived from several sources, including pyruvate, citrate, and cytosolic acetyl-CoA. Histone deacetylation by a class of histone deacetylases known as sirtuins require NAD^{+}.

provided by S-adenosyl methionine (SAM), which is generated from ATP and methionine. Limiting SAM levels can weaken the innate immune response in *Caenorhabditis elegans* against the bacterial pathogen *Pseudomonas aeruginosa* by reducing the levels of H3K4me3 at protective bacterial response genes (78). Furthermore, demethylation requires Fe^{2+} and α-ketoglutarate (α-KG), as cofactor and cosubstrate, respectively, for JmjC domain-containing histone demethylases as well as TET enzymes. In macrophages, the α-KG/succinate ratio regulates the activity of the H3K27 demethylase JMJD3, with higher α-KG promoting JMJD3 activity at genes associated with M2 activation (79). In this instance, IL-4, which induces M2 polarization, stimulates glutaminolysis to generate α-KG to both promote JMJD3 activity as well as to suppress the NF-κB pathway by activating another α-KG-dependent enzyme, prolyl hydroxylase (79). Like succinate, several other metabolites can compete with α-KG to inhibit α-KG-dependent enzymes, including fumarate and 2-hydroxyglutarate (80, 81). Adjusting the balance of these substrates allows innate immune cells to fine-tune and modulate demethylase activity in response to external stimuli, consequently regulating their gene expression programs.

The availability of acetyl-CoA, an intermediate in several anabolic and catabolic pathways, is known to influence histone acetyltransferase activity (82). Several metabolites have also been described to activate or inhibit histone deacetylase activity (83). Importantly, a class of histone deacetylases known as sirtuins (SIRT) are dependent on the oxidizing agent NAD^{+} (84). During sepsis, SIRT1 and SIRT6 are responsible for a switch in metabolic phenotype from glycolysis during early acute inflammation to fatty acid oxidation in the late immunosuppressive phase (85). SIRT1 and endogenous NAD^{+} levels increase simultaneously during endotoxin tolerance, promoting SIRT1 binding and deacetylation at the TNFα promoter, therefore repressing *TNFA* transcription (86). These findings were demonstrated in THP-1 human promonocyte cells, murine splenocytes, and whole blood leukocytes of human sepsis patients. In contrast, short-chain fatty

TABLE 2 | Epigenetic factors that influence DC activity.

	Enzyme	Function	Known target genes in DCs	Notes	References
Promotes DC activation	KDM6B (JMJD3)	H3K27 demethylase	*Cd80*, *Cd86, CD103*		(89)
	WDR5	H3K4 methyltransferase	*IFNA*, *IFNB*		(90)
	KDM4D (JMJD2D)	H3K9 demethylase	*Il12, Il23*	Recruited by Trabid	(91)
	NuRD complex (HDAC1, HDAC2)	Histone deacetylation complex	*Tnfrsf9, Cd40, Cd80, Cd86, Cd68, Slc11a, Ciita. H2-Aa*	Recruited by Mbd2	(92)
	HDAC11	Histone deacetylase	*IL10*		(93)
Promotes DC steady-state	PCGF6	Transcriptional repressor	*Ciita*, *H2-Ab1*, *Il12a*, *Il12b*	Forms complex with KDM5C	(77)
	KDM5B	H3K4 demethylase	*Ifnb, Il6, Tnfa*	Upregulated by RSV	(94)
	HDAC2	Histone deacetylase	*Il6*	Recruited by Tet2	(95)
	G9a	H3K9 methyltransferase	*Ifna*, *Ifnb*		(96)

acids produced by the gut microbiota inhibit histone deacetylase activity (87). The most potent of these short-chain fatty acids is butyrate, which contributes to immune tolerance to commensal bacteria by inhibiting proinflammatory functions of intestinal macrophages (88). Clearly, the functions of innate immune cells are regulated by the exquisite interconnection between epigenetic and metabolic reprogramming. Further studies are required to identify the importance of metabolic-epigenetic interactions for DC function.

EPIGENETIC REGULATION OF DC FUNCTIONS

Expanding evidence suggests that epigenetic modifications contribute significantly to the regulation of DC function. Epigenetic mechanisms are implicated in the maintenance of the steady-state, responses to activating stimuli, trained immunity, and tolerance (**Table 2**). Furthermore, metabolism, nutrition, environment, and aging also impact DC function by influencing the epigenetic landscape. Ultimately, these mechanisms are important to understand as they impact immune responses to infections and cancers and contribute to inflammatory diseases such as autoimmunity and asthma.

Active Maintenance of DC Homeostasis

Maintaining DC homeostasis requires balancing of the mechanisms that repress activation and those that promote proinflammatory functions. Clues from the study of TFs suggest that active restraint of DC activation is regulated at the level of transcription. NF-κB, which is recognized to have pioneer factor activity, has been widely shown to induce inflammatory gene expression programs in part by promoting chromatin remodeling (97, 98). At steady state, NF-κB restrains DC activation and prevents DCs from inducing self-reactive cytotoxic T cell responses (99). Deficiency of the p50 subunit of NF-κB in DCs leads to the spontaneous induction of diabetes in a mouse model (99). However, NF-κB activity is also well-known to drive DC activation. One study, using genome footprinting and chromatin immunoprecipitation (ChIP), revealed that the promoter of the MHC class II transactivator *CIITA* is occupied by NF-κB (p65) at steady-state but not in activated DCs, suggesting that NF-κB relocates when DCs become activated (100). Whether the chromatin landscape dictates NF-κB binding in steady-state vs. activated state remains to be determined.

Interestingly, lineage-specific factors that contribute to the differentiation of DCs have also been described to be downregulated in response to maturation signals (101–103). For example, expression of ZBTB46, a zinc-finger DNA-binding TF, is restricted to cDCs. Downregulation of ZBTB46 accompanies TLR-stimulation and is necessary to permit activation (101, 104). Once committed, the lineage of DCs is highly stable (4, 28), therefore it is possible that sustained expression of lineage-specifying factors may serve to restrain the full maturation of DCs until the appropriate activating signals are received.

PCGF6 is a member of Polycomb Repressive Complex 1 (PRC1.6). PRC1 complexes are well-known for catalyzing the monoubiquitylation of histone H2A by a RING E3 ligase (105, 106). H2AK119ub1 leads to chromatin compaction and gene silencing. PCGF6 participates in non-canonical complexes including ones containing E2F6, which promote gene silencing by promoting H3K9 trimethylation, and others that contain KDM5C/D lysine demethylases that remove activating methyl marks at H3K4 (77, 107–112). PCGF6 and KDM5C were both found to be necessary for maintenance of the steady state (77). Following PRR stimulation, PCGF6 is rapidly downregulated, enabling DC activation. PCGF6 regulates the chromatin landscape in DCs and more specifically the levels of H3K4me3 at genes important for DC activation. Though few, these studies suggest that optimal maintenance of the steady state of DCs requires active repression of inflammation-sensitive gene loci through epigenetic silencing at steady state (**Figure 2**). Rapid relief of transcriptional and epigenetic restraints in response to stimulation is required for massive transcriptional reprogramming that supports DC activation and function.

Epigenetic Mechanisms Underpin DC Activation and Function

The chromatin landscape at steady state likely dictates the early responses to activating stimuli by regulating accessibility of genes important for DC activation. Following the transcriptional

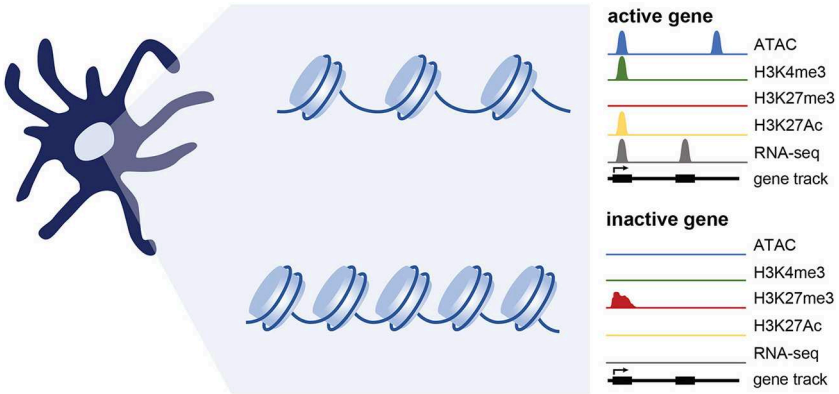

FIGURE 2 | Epigenetic changes associated with gene expression. Simplified representation of data profiles of ATAC-seq, ChIP-seq, and RNA-seq showing an active or inactive gene. Active genes are accessible (measured using ATAC-seq) and bear chromatin modifications associated with transcriptional activation such as H3K4me3 and H3K27Ac. Genes that are inactive are maintained in a repressed, less accessible state and are marked by histone modifications such as H3K27me3. While not extensively tested in DCs, genes poised for expression likely maintain accessibility, and may have a mix of activating and repressive marks.

reprogramming that accompanies DC activation, epigenetic reinforcement of gene expression becomes essential to ensure that DCs migrating to lymph nodes retain gene expression profiles to appropriately initiate T cell responses. To activate T cells, DCs must provide at least three signals: antigen presentation (signal 1), co-stimulation (signal 2), and lineage-specifying cytokine production (signal 3). The expression of proteins that constitute these signals are regulated transcriptionally, and increasing evidence suggests they are also regulated epigenetically. In steady-state splenic DCs, the expression of costimulatory molecules *Cd80* and *Cd86* is repressed by H3K27me3, which is relieved by the H3K27 demethylase KDM6B (JMJD3) during LPS stimulation (89). Furthermore, the repressive mark H3K9me3 was found to be enriched at the promoters of proinflammatory cytokines *Il12a*, *Il12b,* and *Il23* in steady-state BMDCs. Upregulation of these cytokines in LPS-activated BMDCs is largely governed by the recruitment of Trabid, a deubiquitinase that stabilizes the H3K9 demethylase KDM4D (JMJD2D) (91). Nucleosome Remodeling Deacetylase complex (NuRD) also reinforces DC activation by suppressing antigen uptake and processing (*Cd68, Slc11a*) and stimulating antigen presentation (*Ciita, H2-Aa*) (92). This occurs by stabilizing antigen-loaded MHC and by upregulation of specific costimulatory molecules and cytokines. Though these studies suggest that a dynamic epigenome is important for proper DC function, a comprehensive study focused on early and late-stage changes in the chromatin landscape following stimulation and the importance for DC function has not been reported.

Immune mediators in the inflammatory microenvironment such as cytokines, chemokines, and lipids, can temper DC responses to activating stimuli. IL-10 has long been known to potently downregulate IL-12 production (113). HDAC11 represses *IL10* and in doing so, promotes the activation and IL-12 production of primary human DCs, which is required for efficient $CD4^+$ T cell differentiation (93). STAT6, a downstream effector of IL-4 signaling, also antagonizes histone acetylation at the *Il10* promoter following LPS stimulation (114). Lipid mediators, such as prostaglandins, can also be sculptors of the epigenome in DCs. Prostaglandin I2 suppresses H3K4me3 enrichment at the *TNFA* promoter by inhibiting components of a methyltransferase complex, MLL and WDR5, from translocating into the nucleus (115). A further study by the same group found that antagonism of the cysteinyl leukotriene receptor promotes an anti-inflammatory phenotype in human moDCs by enhancing H3 acetylation at the *IL10* promoter (116). Inhibiting chromatin remodelers could be an effective therapeutic avenue for inflammatory conditions, in particular those driven by TNFα or controlled by IL-10. Together these studies demonstrate that epigenetic mechanisms significantly contribute to the activation of DCs, and importantly, that factors in the inflammatory environment that modify the epigenome may have lasting effects on DC responsiveness.

Trained Immunity and Tolerance

The response of myeloid cells can be influenced by previous exposure to inflammatory stimuli. Exposure of DCs and macrophages to low levels of endotoxin induces tolerance which decreases their sensitivity to subsequent stimuli. Exposure of cells to stimuli that increases subsequent responsiveness is termed "trained immunity" and is most commonly noted in monocytes; whether trained immunity is transferred to monocyte-derived DCs upon differentiation is not known. There is increasing evidence that epigenetic and metabolic programming underlies tolerance and training of myeloid cells (98, 117).

Tolerance in myeloid cells, including DCs, is a refractory period following proinflammatory stimulation whereby the immune system is non-responsive to subsequent threats. During sepsis, for example, tolerance serves as a protective mechanism to prevent endotoxin shock in the host. In this state, monocytes, DCs, and macrophages adopt a chromatin landscape that predominantly favors immune suppression (29, 31). This is in part accomplished by the upregulation of suppressive factors such as IL-10, PD-L1, IDO, and TGFβ, along with concomitant

silencing of IL-12 and other proinflammatory mediators. These changes in gene expression are accompanied by changes in H3K27me3, H3K27Ac, and H3K4me3 enrichment (29, 31, 118). HDAC2 activity at the *Il6* promoter during late-stage inflammation can lead to *Il6* downregulation and a subsequent return to homeostasis (95).

Training of monocytes by β-glucan stimulation leads to epigenetic and metabolic alterations that prime proinflammatory genes for enhanced expression in response to further stimulation (117). Bacillus Calmette-Guérin (BCG) exposure also trains monocytes to enhance their responses against *Mycobacterium tuberculosis* infection (119, 120). Training can occur at the level of hematopoietic stem cells, leading to unique epigenetic and metabolic signatures in macrophages arising from BCG-trained monocytes (120). BCG and β-glucan training is dependent on glycolysis induced through key metabolic regulators mTOR and HIF-1α (119, 121). Innate immune memory may also occur in microglia, myeloid cells in the brain, affecting neuropathology in murine models of stroke and Alzheimer's. HIF-1α levels are similarly increased in the trained microglia suggesting metabolic reprogramming may underlie training (122). A transcriptomics and metabolomics approach uncovered that glycolysis, glutaminolysis and cholesterol synthesis are essential metabolic pathways for inducing the trained phenotype in monocytes (123). Fumarate accumulation resulting from increased glutaminolysis leads to inhibition of histone demethylases and an increase in H3K4me3 marks at the promoters of proinflammatory cytokines. In addition, mevalonate, a metabolite from the cholesterol synthesis pathway, induces trained immunity by autocrine signaling through IGF1 receptor and subsequent mTOR activation (124). Collectively, these studies suggest that cells of the myeloid lineage undergo epigenetic and metabolic reprogramming in response to environmental stimuli that alters subsequent responses to stimuli. The extent to which environmental stimuli alters metabolic and epigenetic programming of DCs and alters their subsequent responses remains to be studied in detail.

Viral Infection

The study of antiviral immunity has provided key insights into the contribution of epigenetic mechanisms to DC activation. For instance, interferon production by human DCs can be activated or suppressed by functionally dichotomous chromatin remodelers; the H3K4-specific methyltransferase WDR5 stimulates antiviral immunity via H3K4 trimethylation at the *IFNA* and *IFNB* promoters (90), while H3K9me2 enrichment by the histone-lysine N-methyltransferase G9a at *IFNA* and *IFNB* promoters instead correlates with a decreased DC-driven antiviral response (96). Although a practical system to ensure appropriate interferon expression, certain pathogens have evolved strategies to hijack these endogenous epigenetic pathways and skew the epigenetic signature in their favor. Respiratory syncytial virus (RSV) infection can be cleared by a T_H1 cytokine profile, but RSV-infected patients often mount a T_H2 cytokine response non-conducive to efficient RSV clearance. One group found aberrant T_H2 responses to be driven by an RSV-mediated upregulation of endogenous H3K4 demethylase KDM5B in several DC types, a transcriptional repressor of T_H1-associated cytokines important for RSV clearance (94). Furthermore, during viral infection in mice, TET2 is recruited by CXXC5 to the *Irf7* promoter to induce *Irf7* hypomethylation and expression in pDCs, resulting in the onset of an antiviral response (125). Given the role of TET2 in stabilizing HDAC2 at the *Il6* promoter (as described earlier), TET2 drives dichotomous DC functions; while TET2 can recruit HDAC2 to help repress *Il6* and resolve IL-6-driven inflammation, it can also initiate an inflammatory antiviral response by hypomethylating and upregulating *Irf7* expression. Advances in both the understanding of the biochemical function of 5hmC and TET enzymes in DCs are necessary to fully appreciate the role of dynamic changes in DNA methylation for regulating gene expression during infection.

TABLE 3 | Environmental factors that shape the epigenome in DCs.

Extrinsic agent	Effect on DC function	References
Aging	Increase in global DNA hypomethylation	(126)
	Upregulation of *TNFA, IL1A, IL17RC, TLR2, Il23p19*	(127–132)
Chemicals		
Phthalates	Enhance T_H2 allergic responses	(133)
	Downregulate *IRF7*	(134)
Nutrition		
Zinc deficiency	Induces *Il6* promoter demethylation	(135)
Vitamin C	Increases NF-κB activation, IL-12p70 secretion	(136)
	Regulates TET-mediated DNA demethylation (ES cells, lymphomas)	(137, 138)
Lipid Mediator		
Prostaglandin I2	Reduces H3K4me3 enrichment at *TNFA* promoter	(115)
Cysteinyl leukotrienes	Reduces H3 acetylation at *IL10* promoter	(116)

ENVIRONMENTAL FACTORS

A hallmark of the epigenome is its proclivity to undergo extensive remodeling in response to environmental stimuli. Though understudied, accumulating evidence demonstrates that extrinsic factors (in addition to microbes and inflammatory mediators), such as nutrients, chemicals and even aging, can manipulate DC function by altering the epigenetic landscape [**Table 3**; (139)].

Chemicals and Nutrients

Phthalates, endocrine-disrupting chemicals ubiquitous in the plastic industry, have been shown to possess adjuvant-like properties that enhance T_H2 allergic responses (133). Phthalates were found to downregulate *IRF7* expression in human pDCs by inhibiting H3K4-specific methyltransferase WDR5 translocation into the nucleus (134). Nutrients from the diet are also

known to affect immune cell function through epigenetic regulation. For example, recent estimates suggest a notable zinc deficiency in 65% of the senior population (>65 years old) (140). Zinc deficiency can inappropriately enhance inflammatory responses (141); zinc deficiency was found to correlate with *Il6* promoter demethylation in THP-1 cells, which led to increased IL-6 production and inflammation (135). Several studies have also established vitamin C as a modulator of DNA demethylation (137, 138). Vitamin C can directly regulate TET-mediated DNA demethylase activity in lymphoma and ES cells. Since vitamin C treatment has been shown to increase NF-κB activation and enhance IL-12p70 secretion by BMDCs (136), vitamin C may promote inflammation by demethylation of genes important for DC activation. As we continue to better understand the mechanisms by which nutrition and metabolism regulate cellular physiology, more links are likely to become apparent between these small molecules and epigenetics.

Aging

Immune aging or "inflammaging" refers to the observed increase in proinflammatory cytokine expression, such as TNFα, by aged innate cells in the absence of acute infection or stimulation (142). Transcriptional dysregulation in many cell types, including non-immune cells, has been shown to correlate with stochastic epigenetic modifications incurred with age, a process known as "epigenetic drift" (143). An early study found a positive correlation between age and global DNA hypomethylation (126), with several later studies reporting demethylation and concomitant dysregulation at key proinflammatory genes, including *TNFA* (127, 128), *IL1A* (129), *IL17RC* (130), and *TLR2* (131). Splenic T cells from aged C57BL/6 mice (>22 months old) show elevated levels of IL-17 secretion (144). Accordingly, increased IL-17 production is also observed in many autoimmune diseases (145), therefore epigenetic drift in DCs may underlie increased age-related incidences in autoimmunity. Indeed, the activation marker H3K4me2 is enriched at the *Il23p19* promoter in aged DCs (132), and IL-23 production is known to play a pivotal role in the maintenance and expansion of T_H17 immune responses (146). Inflammation ultimately has the capacity to influence epigenetic regulation (147) and therefore may impact age-associated epigenetic changes in immune and non-immune cells. The interconnectivity of these processes likely underlies long-term immune functionality and organismal health.

INFLAMMATORY DISEASES

DCs are an important driver of the inflammation associated with autoimmune disease and allergic asthma. In particular, histone demethylases and hydroxylases containing the JmjC domain, including KDM5C, JMJD2D, and JMJD3, appear to play a significant role in DC-mediated pathogenesis. KDM5C is an important regulator of the steady-state and activation of murine DCs (77). TRABID promotes experimental autoimmune encephalitis (EAE) by stabilizing JMJD2D at the *Il12* promoter, enhancing IL-12 production and immunopathology (91). However, JMJD3 inhibition limits EAE pathology and promotes a tolerogenic DC profile characterized by the reduced expression of CD80/86, and reduced secretion of proinflammatory cytokines IL-6, IFN-γ, and TNFα (89). Several diseases have been linked to aberrant DC methylation profiles in DCs. DNA hypermethylation at the *IRF8* promoter has been noted in Ocular Behcet's Disease (148) and Koyanagi-Harada Disease (149). In both cases, pharmacological DNA demethylation suppressed proinflammatory cytokine production by patient-derived DCs *ex vivo*. In contrast, genome-wide DNA demethylation was observed in the pDCs of patients with systemic lupus erythematosus (SLE), resulting in increased *IFNA* expression which could contribute to SLE onset (150).

Epigenetic modifications have also been described in asthma (151). Upon allergen recognition in the lung, lung-resident DCs upregulate chemokine receptor CCR7, allowing for their migration to the mediastinal lymph nodes, where they prime T cells and promote allergic inflammation. Although several lung-resident DC subsets exist (including cDCs and moDCs), *Ccr7* upregulation is relatively cDC-specific (152). H3K27me3 enrichment was found at the *Ccr7* promoter in moDCs, but not cDCs, suggesting that some lineage specific functions of DCs may be epigenetically determined (153). Mouse studies also suggest asthma risk to be an inherited characteristic partially mediated by an altered DC epigenome. Adoptive transfer experiments in mice identified DCs to be the "carrier" of asthmatic susceptibility; DCs transferred from neonates of asthmatic mothers to neonates of non-asthmatic mothers increased asthma susceptibility in the recipients, indicating a functional skew in DCs early in life that promote allergic responses (154). Donor and recipient mice were genetically identical, suggesting the observed functional skew to be epigenetically regulated. Indeed, the DC methylomes of neonates from asthmatic mothers differed significantly from neonates of healthy mothers, and approximately 50% of the differentially methylated genes belong to allergy and asthma pathology networks (155). Thus, allergen exposure early in life results in alternative epigenetic regulation of key genes that contribute to allergic responses. Thus, the extent to which inflammatory genes are epigenetically primed in DCs likely contributes to inflammatory disease incidence and severity.

SUMMARY AND PERSPECTIVES

Because DCs are fast-acting and short-lived, the contribution of epigenetic mechanisms to DC responsiveness and function has been overlooked. However, there is growing appreciation of the importance of epigenetic mechanisms in controlling dynamic, and even short-lived, cellular responses. The past decade has seen exciting advancements in our understanding of how the environment impacts immunobiology at the epigenome level. Significant steps have been taken to understand how the chemicals and nutrients in our environment influence the immune system, as well as the mechanisms by which the aging process contributes to age-related inflammation.

The development and use of low-input techniques are necessary to expand epigenetic studies to different *in vivo*-derived DC populations (46, 156, 157). Further studies are needed to expand our knowledge of the mechanisms that regulate the epigenome in DCs and the consequences for healthy and pathological inflammation. DC function is highly influenced by the local environment in which it is stimulated. Thus, environmental factors that shape the epigenome of DCs at steady state are likely to have lasting effects on DC function. Insightful discoveries on the effects of local nutrition, metabolite availability, and inflammation on the epigenetic landscape in DCs will further our understanding of the dynamic changes in gene expression that support or interfere with host immunity.

AUTHOR CONTRIBUTIONS

GB, MC, HG, and CK contributed by researching and writing manuscript and creating figures and tables.

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IRE1α Activation in Bone Marrow-Derived Dendritic Cells Modulates Innate Recognition of Melanoma Cells and Favors CD8$^+$ T Cell Priming

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The IRE1α/XBP1s signaling pathway is an arm of the unfolded protein response (UPR) that safeguards the fidelity of the cellular proteome during endoplasmic reticulum (ER) stress, and that has also emerged as a key regulator of dendritic cell (DC) homeostasis. However, in the context of DC activation, the regulation of the IRE1α/XBP1s axis is not fully understood. In this work, we report that cell lysates generated from melanoma cell lines markedly induce XBP1s and certain members of the UPR such as the chaperone BiP in bone marrow derived DCs (BMDCs). Activation of IRE1α endonuclease upon innate recognition of melanoma cell lysates was required for amplification of proinflammatory cytokine production and was necessary for efficient cross-presentation of melanoma-associated antigens without modulating the MHC-II antigen presentation machinery. Altogether, this work provides evidence indicating that *ex-vivo* activation of the IRE1α/XBP1 pathway in BMDCs enhances CD8$^+$ T cell specific responses against tumor antigens.

Keywords: IRE1α, XBP1s, UPR, dendritic cell, melanoma, cross-presentation

INTRODUCTION

Dendritic cells (DCs) are an heterogeneous family of leukocytes competent to instruct antigen-specific immune responses (1). Based on surface markers, location, ontogeny, and function, these cells can be divided into plasmacytoid DCs (pDCs) and conventional DCs (cDCs), which are sub-classified into cDC1 and cDC2 subtypes (2). cDC1s express the surface markers XCR1, DNGR-1, and CD103 in non-lymphoid organs, and require the transcription factors Batf3 and Irf8 for development (3–6). On a functional level, cDC1s are highly efficient at priming CD8$^+$ T cell responses *in vivo* to cell-associated antigens through a process termed "cross-presentation" (7). On the other hand, cDC2s

express the surface markers CD11b and CD172a (SIRPα), the transcription factors Irf4, Klf4, and Notch2 are recognized for modulating $CD4^+$ T cell responses (2, 4, 5). In inflammatory settings, blood monocytes can also differentiate into antigen presenting cells that resemble $CD11b^+$ DCs and that have been referred to as monocyte-derived DCs (8). Cell equivalents of cDCs/pDCs and monocyte-derived DCs can be generated upon *ex-vivo* treatment with FMS-like tyrosinase kinase 3 ligand (FLT3L) or granulocyte-macrophage colony-stimulating factor (GM-CSF), respectively (9, 10). Remarkably, the process of antigen cross-presentation, which is essential for eliciting cytotoxic T cell immunity against tumors, can be efficiently executed by cDC1s, but also by GM-CSF derived DCs through different transcriptional programs (11).

The remarkable ability to evoke T cell immunity have turned DCs into prominent candidates in the generation of cell-based vaccines, particularly in the field of cancer immunotherapy (12). In light of these findings, the intracellular mechanisms governing the immunogenic function of DCs, and in particular those safeguarding cellular function and homeostasis, are matter of extensive research in cancer immunology.

Although it is well-described that microbes and danger signals are potent elicitors of DC activation, emerging evidence indicates that DCs are also sensitive to a broad variety of stress signals for fine-tuning an activated profile (13). A relevant cellular stress-sensing pathway in DC biology is the unfolded protein response (UPR), which is the adaptive cellular mechanism responsible to maintain the fidelity of the cellular proteome (14). The UPR is triggered by accumulation of misfolded proteins in the ER and it is controlled by three ER-resident signal transducers: inositol requiring enzyme 1 (IRE1) alpha and beta, protein kinase R-like ER kinase (PERK) and activating transcription factor 6 (ATF6) alpha and beta (14, 15). The UPR sensors control the expression of genes involved in the recovery of ER homeostasis and also coordinate the execution of cell death under conditions of irrevocable ER stress (14, 16, 17). The IRE1α arm of the UPR is highly conserved among species and it is the most characterized branch in immunity (18). IRE1α is an enzyme containing a serine/threonine kinase domain and an endonuclease domain. In response to the accumulation of misfolded proteins in the ER, IRE1α dimerize, and trans-autophosphorylate activating its endonuclease domain, which performs an unconventional splicing reaction of the *Xbp1* (X-box binding protein) mRNA, generating the transcription factor XBP1 spliced (XBP1s), a major regulator of ER biogenesis (16). In addition, under certain conditions of chronic ER stress or functional loss of XBP1, IRE1α endonuclease initiates the cleavage of additional mRNAs of diverse nature, in a process named "Regulated IRE1 Dependent Decay" or RIDD (19). RIDD was originally proposed to reduce the ER folding load by alleviating the detrimental effects of ER stress.

The dual function of IRE1α endonuclease has emerged as a relevant regulator of DC homeostasis and function. On one hand, XBP1s is constitutively expressed by DC subsets and high expression of XBP1s is a hallmark of cDC1s (20–22). In addition, cDC1s are highly sensitive to changes in IRE1α signaling; as it is reported that RIDD regulates cDC1 survival in mucosal tissues and curtails their ability to cross-present dead cell-associated antigens (21, 22). Whereas, these studies have uncovered a crucial role for the IRE1α/XBP1s axis in non-activated DCs, it remains to be addressed the contribution of the pathway in the functionality of the different DC lineages upon inflammation. This is a relevant aspect considering that innate recognition is a well-described inducer of DC activation (23) and because several pattern recognition receptors (PRRs) induce IRE1α activation for amplification of proinflammatory cytokines (24–28). Interestingly, in the field of tumor therapy, the role of the IRE1α/XBP1s axis in DCs has shown distinct effects depending on whether the pathway is targeted *ex-vivo* or during the course of tumor growth. On one hand, in models of ovarian cancer it has been reported that XBP1s signaling in tumor-infiltrating DCs curtails their ability to trigger anti-tumor T cell immunity, which in turn promotes tumor growth (29). However, enforced expression of XBP1s in *ex-vivo* generated DCs has shown opposite effects, as it potentiates the efficacy of DC-based vaccines in prophylactic and therapeutic settings (30, 31). Thus, the relevance of IRE1α/XBP1s signaling in DCs has not been fully elucidated and it appears to be dependent on the type of DC targeted, on the experimental setting (*in vivo* or *ex-vivo*) and inflammatory context.

In this study, we report that lysates derived from melanoma cell lines are efficient elicitors of the IRE1α-dependent XBP1s branch of the UPR in bone marrow derived DCs (BMDCs), which favors cross-presentation of a melanoma-associated antigen. Pharmacological blockade of IRE1α endonuclease in BMDCs stimulated with melanoma cell lysates impairs cross-presentation of antigens, without interfering with the MHC-II pathway. Furthermore, BMDCs expressing a mutant isoform of IRE1α that lacks the endonuclease domain were less efficient at inducing $CD8^+$ T cell proliferation to a melanoma-associated antigen *in vivo*. Our data indicates that activation of the IRE1α/XBP1s axis in BMDCs *ex-vivo* is required to endure $CD8^+$ T cell priming to melanoma antigens. Knowledge derived from this study may be considered in the design of DC-based vaccines for cancer immunotherapy.

Abbreviations: ATF6, activating transcription factor 6; BM, bone marrow; DC, dendritic cell; cDC, conventional DC; cDC1, conventional DC type 1 ($XCR1^+$ or $CD24^+$ DC); cDC2, conventional DC type 2 ($SIRP\alpha^+$ DC); ER, endoplasmic reticulum; ERAI, ER stress-activated indicator; Flt3L, FMS-related tyrosine kinase 3 ligand; FP, fluorescent protein; GM-CSF, granulocyte macrophage colony-stimulating factor; IRE1, inositol-requiring enzyme 1; KO, Knock-out; MEL, Human melanoma cell line lysates; MHC class I, major histocompatibility class I; pDC, plasmacytoid DC; PERK, protein kinase R-like ER kinase; RIDD, regulated IRE1-dependent decay; TRP-1, Tyrosinase-Related Protein 1; UPR, unfolded protein response; XBP1s, spliced XBP1; XBP1u, unspliced XBP1.

RESULTS

Innate Recognition of Melanoma Cell Lysates Elicits Activation of IRE1α Endonuclease and the Splicing of *Xbp1* mRNA in BMDCs

Previous reports have demonstrated that IRE1α activation is a key regulator of cDC1 function and survival in steady state (21, 22). In inflammation, it has been shown that myeloid

cells activate the IRE1α/XBP1s axis in response to microbial ligands of Toll-Like Receptors (TLR), RIG-I-like receptors but also with molecules expressed by tumors (25–29, 32, 33). In this context, we sought to investigate if DCs differentially activate the IRE1α/XBP1s axis during recognition of innate stimuli of diverse origin. For this purpose, we generated *in vitro* cultures of bone marrow (BM) cells cultured in presence of the cytokine FLT3L, which is a culture that generates an heterogeneous mix of cell equivalents of cDC1, cDC2, and pDCs (referred to as "FL-DCs") (**Supplemental Figure 1B**) (10). We included lipopolysaccharide (LPS) as a microbial stimulus, house dust mite extract (HDM) as a model allergen, and a cell lysate generated from human melanoma cell lines (referred to as "MEL"), as a tumor-related stimulus. MEL has proven to be a clinically effective stimulus in DC vaccines in patients with advanced melanoma, and it is generated by cycles of freeze-thaw of three established human melanoma cell lines (34, 35). We investigated whether LPS, HDM, or MEL lysates induced the splicing of *Xbp1* mRNA (*Xbp1s*) by FL-DCs (**Figure 1A**). As a positive control we included the pharmacologic ER-stress inducer tunicamycin (TM). Data in **Figure 1A** shows that MEL lysate preferentially induced *Xbp1s* mRNA in FL-DCs compared to LPS and HDM, a feature that was also observed in qPCR analysis (**Figure 1B**). The cancer cell lysate also induced expression of additional targets of the UPR in FL-DCs such as the ER chaperone *BiP* (**Figure 1B**) and showed a trend in the induction of *CHOP*, a transcriptional regulator activated downstream of PERK (**Figure 1B**). Of note, we confirmed that MEL lysates do not contain viable mRNA that could potentially interfere with these assays (**Supplemental Figure 1A**). Thus, these data indicate that melanoma cell lysates elicit efficient activation of XBP1s and certain members of the UPR in FL-DCs.

To confirm the activation of the IRE1α arm of the UPR in DC subsets activated with MEL by an independent experimental approach, we generated FL-DCs from the ERAI reporter mice (36). This transgenic mice line reports on IRE1α endonuclease activity by expressing a partial sequence of human XBP1 that includes the IRE1α splicing sites, fused to Venus fluorescent protein (VenusFP) (36). Stimulation of FL-DC cultures from ERAI mice with increasing doses of MEL lysates revealed a dose-dependent effect in the induction of VenusFP in cDC1 equivalents (referred to as "cDC1 FL-DC") (**Figure 1C**). However, MEL stimulation also increased VenusFP expression in cDC2 equivalents (referred to as "cDC2 FL-DC") but not in pDC equivalents (referred to as "pDC FL-DC") (**Figure 1D**), demonstrating that only conventional DCs activate IRE1α endonuclease upon MEL recognition.

Next, considering that MEL is a melanoma cell lysate of human origin, we sought to investigate whether the factor driving XBP1s in FL-DCs might also be present in murine melanoma cells. As shown in **Figure 1E,** stimulation with lysates generated from B16-F10 melanoma cells led to enhanced VenusFP expression in FL-DCs to a similar extent than the human lysates, indicating that the ability to trigger XBP1s is not due to recognition of a xenogeneic factor. Induction of XBP1s by B16 lysates was also confirmed by qPCR analysis (**Figure 1F**). Furthermore, we also noticed that VenusFP fluorescence in FL-DCs was triggered by melanoma cell lysates but it was not induced by a human-derived blood leukocyte lysate (**Figure 1E**), suggesting that the factor responsible for XBP1s activation is expressed by cancer cells. Finally, we sought to investigate whether activation of XBP1s triggered by the melanoma lysate was a general feature across DC subtypes. As illustrated in **Figure 1G**, BMDCs cultured in presence of GMCSF ("GMCSF-BMDCs"), which are an heterogeneous culture of antigen presenting cells phenotypically different to FL-DCs (**Supplemental Figure 1B**) (37), also induce the expression of *Xbp1s* and *Bip* upon stimulation with B16 lysates, indicating that several DC subtypes can activate the IRE1α/XBP1s axis upon melanoma cell recognition. Altogether, our data indicates that melanoma cell lysates elicit efficient activation of IRE1α endonuclease and *Xbp1s* mRNA in cultures of BMDCs.

Melanoma Cell Lysates Induce XBP1s, but Not RIDD

The ability of melanoma lysates to activate IRE1α and XBP1s prompted us to investigate whether these compounds might also trigger canonical RIDD. Data in **Figure 2A** illustrates that MEL stimulation in FL-DCs showed a trend in the expression of the XBP1s target gene *Erp44.* The induction of the additional XBP1s target gene *Sec61a* did not reach statistical significance, indicating that MEL lysates do not induce the full XBP1s transcriptional program. Furthermore, MEL-stimulated FL-DCs did not reduce the expression levels of *Bloc1s1*, an archetypical RIDD target or *Tapbp*, a RIDD target in DCs that interferes with the MHC-I antigen presentation pathway (19, 21). These data indicates that RIDD is not induced upon stimulation with melanoma cell lysates. Furthermore, we observed that in addition to MEL, cell lysates generated from ovarian carcinoma cell lines (OvCa) and gallbladder cancer cell lines (GBCa) induced expression of VenusFP in FL-DCs (**Figure 2B**). Thus, this evidence indicates that lysates derived from various cancer cell types contain factors that induce *Xbp1s* mRNA in DCs.

Pharmacological Inhibition of IRE1α Endonuclease Decreases the Production of Proinflammatory Cytokines in FL-DCs Stimulated With Tumor Cell Lysates

It has been previously reported that IRE1α couples innate recognition with the induction of inflammatory responses (15, 25, 28). To address the contribution of the IRE1α/XBP1s axis in innate recognition of MEL, we used 4μ8C, a selective inhibitor of the IRE1α endonuclease domain (38). Dose titration of 4μ8C in FL-DCs efficiently inhibited XBP1s in response to TM, without affecting survival or overall DC subset composition (**Figure 3A** and **Supplemental Figures 2A,B**). To monitor DC maturation, FL-DCs were pre-incubated with 4μ8C or control vehicle and were subsequently stimulated with MEL, and expression of costimulatory molecules was quantified by flow cytometry (**Figures 3B,C**). Treatment with 4μ8C did not alter surface expression of MHC-II, or the costimulatory molecules CD80, CD86, and PD-L1 in MEL-activated cDC1 and cDC2 FL-DCs (**Figures 3B,C**). However, we noticed that FL-DCs stimulated

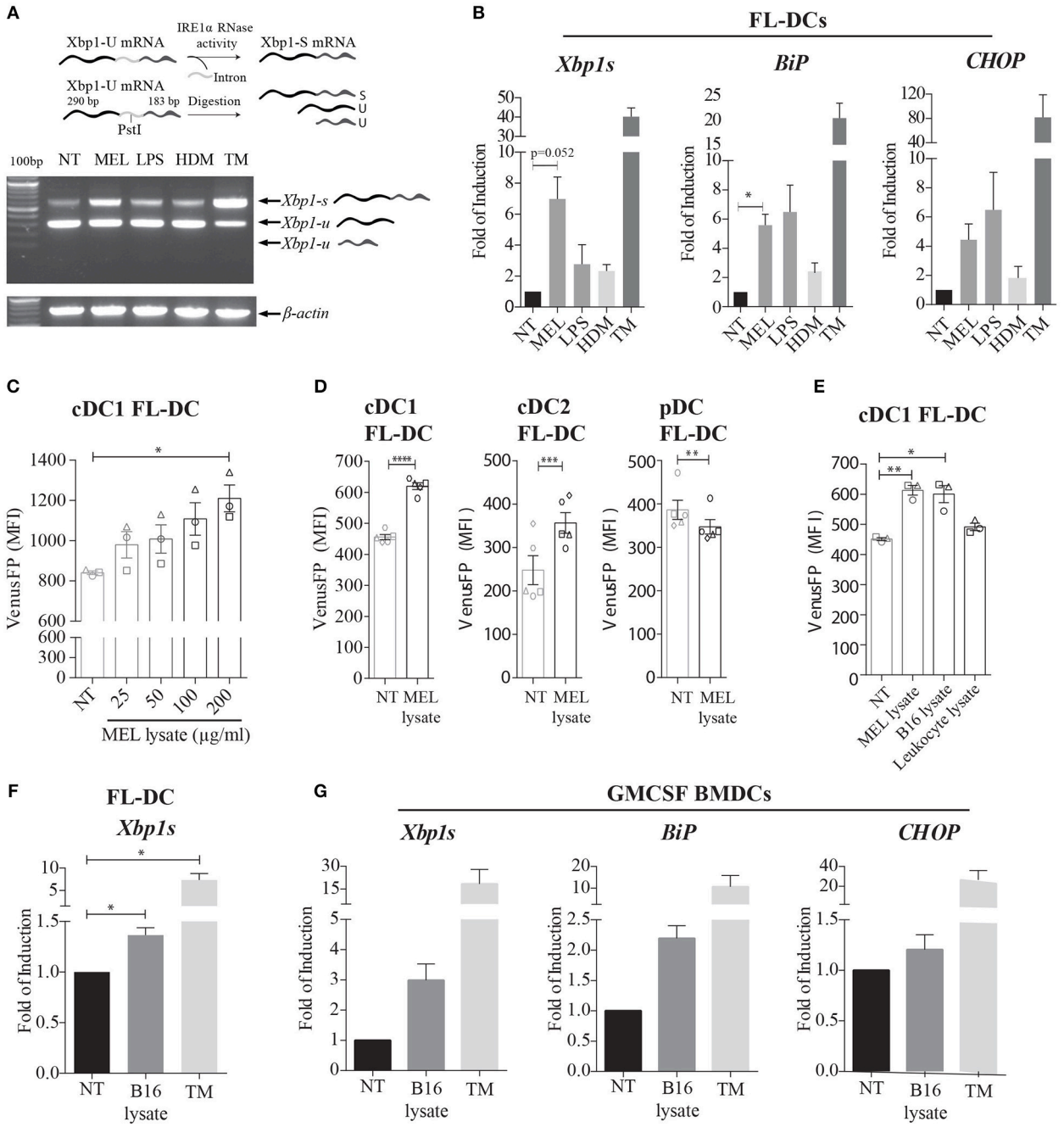

FIGURE 1 | Human and murine melanoma cell lysates induce expression of XBP1s and additional members of the UPR in murine BMDCs. **(A)** FL-DCs were left untreated (NT) or stimulated with 100 μg/ml cell lysate from human melanoma cell lines (MEL), 100 ng/ml lipopolysaccharide (LPS), 50 μg/ml house dust mite extract (HDM), or 1 μg/ml tunicamycin (TM) for 8 h. Expression of *Xbp1s* was determined by a RT-PCR protocol for *Xbp1s* and *Xbp1u* that includes a digestion step with the restriction enzyme PstI. The Pst I digestion site in the intron of *Xbp1u* mRNA allows the distinction between *Xbp1s* and two fragments of *Xbp1u* mRNA. A representative scheme is illustrated. Data is representative of three independent experiments. **(B)** FL-DCs were stimulated as in **(A)** and expression of *Xbp1s*, *BiP*, and *CHOP* mRNA was measured by qPCR relative to *L27* expression, and depicted as fold of induction to the NT condition. Data in graphs depicts three independent experiments. **(C)** FL-DCs generated from ERAI mice were left untreated (NT) or stimulated with 25, 50, 100, and 200 μg/ml of MEL for 16 h for the quantification of VenusFP expression. Data in graphs depicts the MFI of cDC1 FL-DC (XCR1$^+$) of three independent experiments. **(D)** ERAI FL-DCs were NT or stimulated with 100 μg/ml MEL for 24 h for the quantification of VenusFP expression. Data in graphs depicts the MFI of cDC1 FL-DC (XCR1$^+$), cDC2 FL-DC (SIPRα$^+$), and pDC FL-DC (B220$^+$). **(E)** ERAI FL-DCs were NT or stimulated with 100 ug/ml MEL, 100 ug/ml human leukocyte cell lysate or 100 ug/ml B16F10 murine melanoma cell lysates (B16 lysate) for 24 h to evaluate VenusFP expression. Data in graphs depicts the MFI of cDC1 FL-DC (XCR1$^+$) of three independent experiments. **(F)** FL-DCs were left untreated (NT) or stimulated with 100 μg/ml B16 lysate or 1 μg/ml TM for 8 h and expression of XBP-1s was measured by qPCR. Data in graphs depicts

(Continued)

FIGURE 1 | three independent experiments. **(G)** GMCSF BMDCs were left untreated (NT) or stimulated with 100 μg/ml B16 lysate or 1 μg/ml TM for 8 h and expression of *XBP-1s*, *BiP,* and *CHOP* mRNA was measured by qPCR relative to *L27* expression, and depicted as fold of induction to the NT condition. Data in graphs show two independent experiments. For **(C–E)**, each symbol in the graph represents data derived from one independent experiment. For all error bars represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (paired Student's *t*-test).

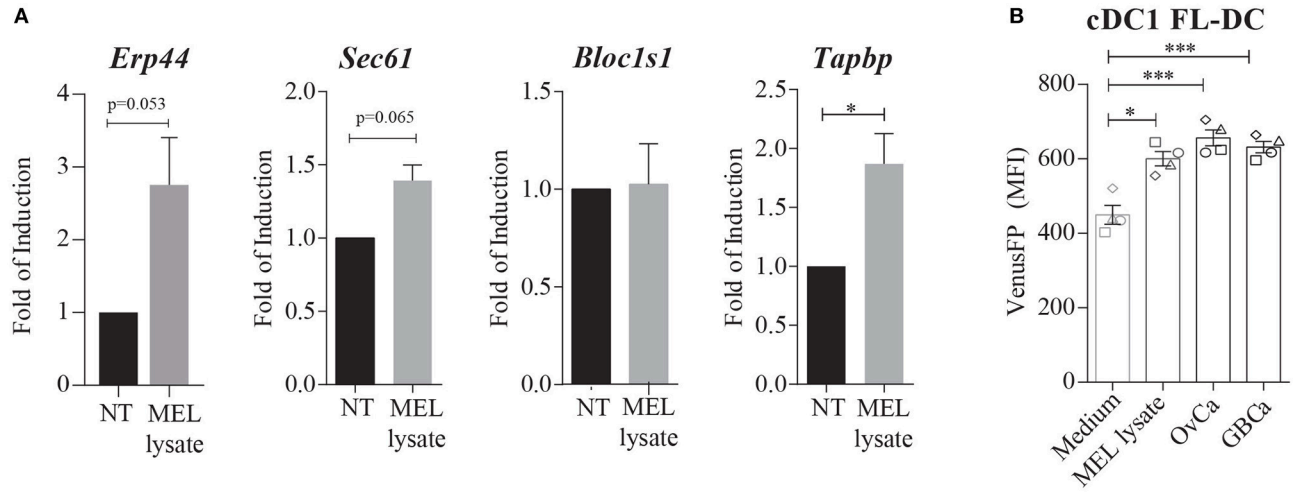

FIGURE 2 | Melanoma cell lysates induce activation of XBP1s and XBP1s-dependent genes, but not RIDD. **(A)** FL-DCs were left untreated (NT) or were stimulated with 100 μg/ml MEL for 8 h. Expression of *Erp44, Sec61a*, *Bloc1s1*, and *Tapbp* mRNA was measured by qPCR relative to *L27* expression, and depicted as fold of induction to the NT condition. Data in graphs depicts three to five independent experiments. **(B)** Expression of VenusFP in FL-DCs generated from ERAI mice and stimulated with AIM-V medium (control medium) 100 ug/ml MEL, 100 ug/ml human ovarian cancer lysate (OvCa) and 100 ug/ml human gallbladder cancer cells lines (GBCa) for 24 h. Data in graphs depicts the MFI of cDC1 FL-DCs ($XCR1^+$) and each symbol in the graph represents data derived from one independent experiment. For all error bars represent mean ± SEM. *$p < 0.05$, ***$p < 0.001$ (paired Student's *t*-test).

with MEL in presence of 4μ8C produced lower levels of the cytokines IL-6, TNF-α, and IL-10 compared to control vehicle (**Figure 3D**). In addition, the production of IL-12p40, a subunit shared by IL-12 and IL-23, was markedly inhibited by 4μ8C treatment in MEL-stimulated cDC1 FL-DCs (**Figure 3E**). These data indicates that pharmacological inhibition of IRE1α endonuclease activity in FL-DCs decreases optimal production of IL-6, TNF, IL-10, and IL-12p40 to tumor cell lysates.

Inhibition of IRE1α Endonuclease Activity Does Not Interfere With Endogenous MHC Class I Presentation and Cross-Presentation in Non-activated FL-DCs

Considering that tumor cells are a relevant source of stimuli for priming cytotoxic T cell responses (39); and that our results indicate that melanoma cell lysates induce the IRE1α/XBP1s axis, we investigated whether this UPR branch could regulate the ability of DCs to activate $CD8^+$ T cells upon MEL recognition. To address this issue, we first sought to investigate if acute blockade of IRE1α endonuclease modulated antigen presentation via MHC Class I in resting conditions. This aspect is relevant considering that DCs constitutively activate XBP1s *in vivo* and that genetic ablation of XBP1 in cDC1s leads to the induction of compensatory RIDD in steady state, which prevents the cross-presentation of dead cell-associated antigens (21, 22). Furthermore, it is well-described that genetic ablation of UPR members results in compensatory adaptive mechanisms within the entire UPR pathway (21, 22, 40, 41). We observed that 4μ8C treatment led to a mild reduction in expression of surface levels of MHC Class I, which did not reach significance in cDC1 FL-DCs (**Figure 4A**). These results prompted to investigate if 4μ8C treatment also resulted in reduced presentation of MHC-I/peptide complexes to $CD8^+$ T cells. To this end, FL-DCs were pretreated with 4μ8C- or control vehicle and were subsequently pulsed with various doses of synthetic $OVA_{257-264}$ peptide (which does not require processing by the MHC-I antigen presentation machinery). After the incubation period, cells were fixed and cultured with OT-I T cells (expressing a transgenic, MHC Class I-restricted, TCR specific for $OVA_{257-264}$ derived from ovalbumin, OVA). As shown in **Figure 4B**, 4μ8C treatment did not affect the ability of FL-DCs to present $OVA_{257-264}$ to OT-I T cells; as measured by expression of the early T cell activation marker CD69. Thus, although 4μ8C treatment resulted in modest reduction of surface MHC-I expression, this effect is not sufficient to inhibit the presentation of specific MHC Class I-peptide complexes leading to T cell activation.

To evaluate if IRE1α via XBP1s modulates the processing route of endogenous antigens in MHC Class I, we generated FL-DCs from CD11c-DOG mice. This is a transgenic mice line that expresses OVA under control of the CD11c promoter, allowing

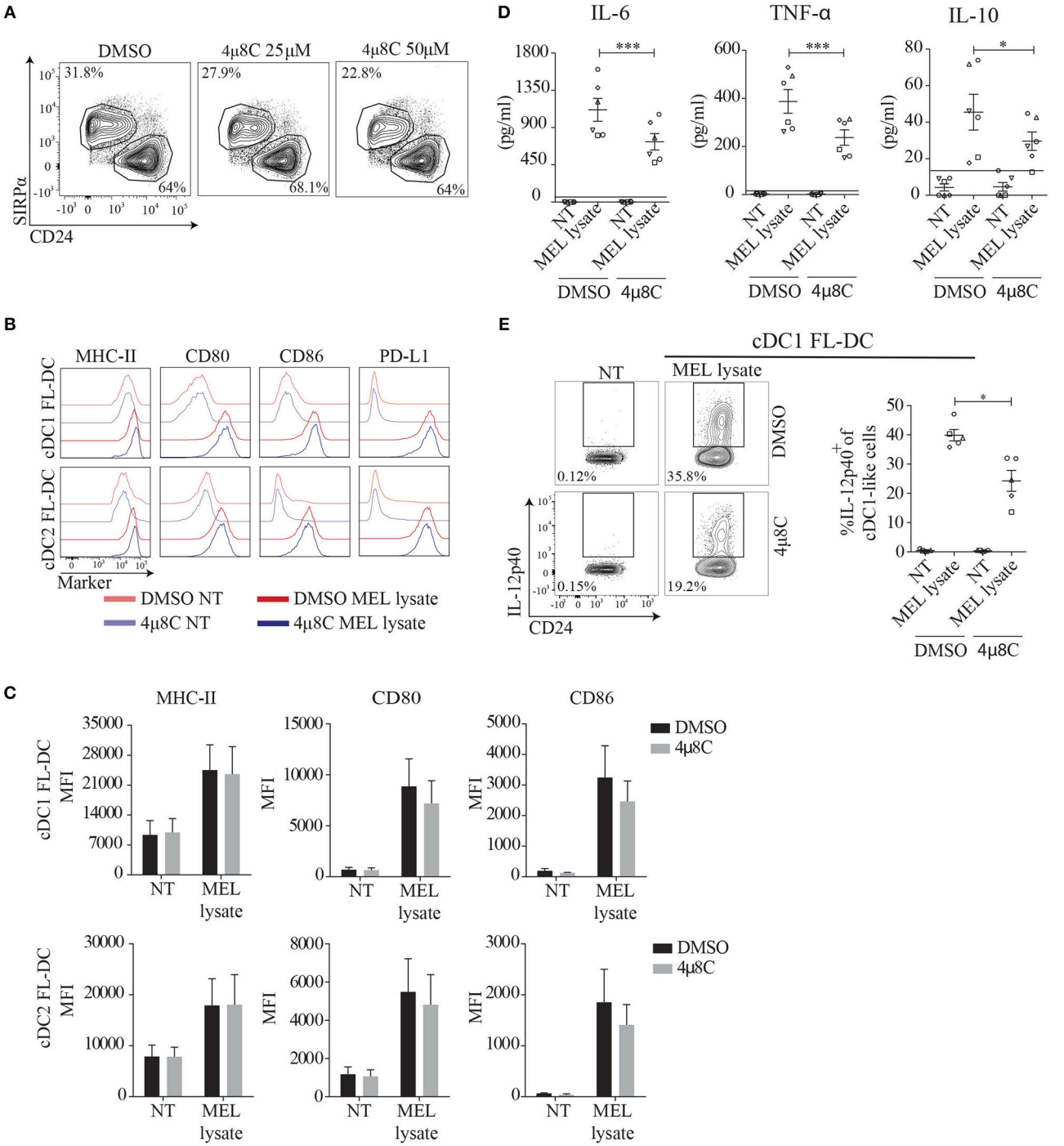

FIGURE 3 | Inhibition of the IRE1α endonuclease domain by the aldehyde 4μ8C does not affect BMDC cellularity or expression of costimulatory molecules, but reduces the production of cytokines upon MEL stimulation. **(A)** FL-DCs were stimulated with increasing doses of 4μ8C or DMSO and composition of DC subtypes was monitored 24 h post treatment. **(B,C)** FL-DCs were pretreated with 20 μM 4μ8C or DMSO for 2 h and stimulated with 100 μg/ml MEL for additional 16 h. Expression of MHC-II, CD80, CD86, and PD-L1 were measured by flow cytometry. Histograms shown in **(B)** are one representative experiment out of five of cDC1 FL-DC ($CD24^+$) and cDC2 FL-DC ($SIPR\alpha^+$) generated in cultures and graphed in **(C)**. **(D)** FL-DCs were pretreated with 20 μM 4μ8C or DMSO for 5 h and stimulated with 100 μg/ml MEL for additional 16 h. TNF-α, IL-6, and IL-10 were quantified by cytometric bead array. **(E)** FL-DCs were pretreated with 50 μM 4μ8C or DMSO for 5 h and stimulated with 100 μg/ml MEL for additional 16 h. IL-12p40 was analyzed by intracellular staining. Contour plots and graphs are for cDC1 FL-DC ($CD24^+$) generated in cultures. For **(D,E)**, each symbol in the graphs represents data derived from one independent experiment. For all error bars represent mean ± SEM. $^*p < 0.05$, $^{***}p < 0.001$ (paired Student's *t*-test).

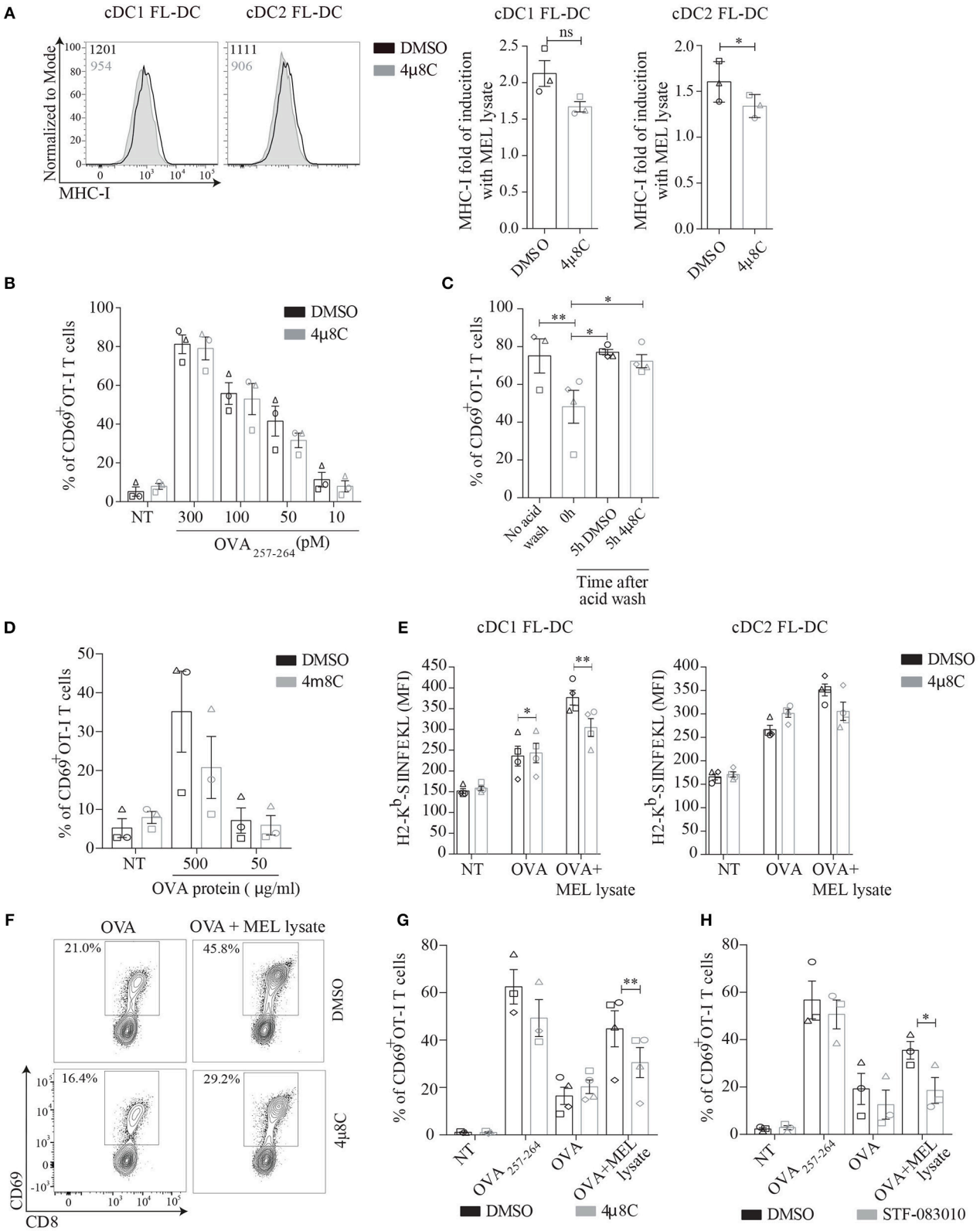

FIGURE 4 | MEL adjuvant function in MHC-I cross-presentation is reduced by inhibition of IRE1α signaling in BMDCs. **(A)** FL-DCs were incubated with 50 μM 4μ8C or DMSO for 6 h and MHC-I expression was measured of cDC1 FL-DC (XCR1$^+$) and cDC2 FL-DC (SIPRα$^+$) by flow cytometry. Data in graph depicts of three independent experiments. **(B)** FL-DCs were incubated with 50 μM 4μ8C or DMSO for 6 h and then were pulsed with increasing doses of SIINFEKL peptide for the

(Continued)

FIGURE 4 | last 20 min of culture. Then cells were counted, fixed and 5×10^4 FL-DCs were cultured with 5×10^4 OT-I T cells. OT-I activation was quantified by expression of CD69. Data in graph shows three independent experiments. **(C)** FL-DCs from DOG mice were incubated with an acid wash solution (see section Materials and Methods) to remove OVA peptides from surface MHC-I molecules. Then cells were incubated in presence of 50 μM 4μ8C or DMSO for 5 h in complete medium and were fixed and cultured with OT-I T cells as in **(B)**. Data is representative of three to four independent experiments. **(D)** FL-DCs were incubated with 50 μM 4μ8C or DMSO for 6 h and then pulsed with increasing concentrations of OVA protein for the last 5 h. Cells were counted, fixed and cultured as in **(B).** Data in graph shows three independent experiments. **(E)** FL-DCs were incubated with 20 μM 4μ8C or DMSO for 5 h and then stimulated with 250 μg/ml OVA or 250 μg/ml OVA plus 100 μg/ml MEL for 16 h. MHC-I/SIINFEKL complex were measured of cDC1 FL-DC ($XCR1^+$) and cDC2 FL-DC ($SIPR\alpha^+$) by flow cytometry using 25.D1-16 antibody ($H\text{-}2K^b$-SIINFEKL). **(F)** FL-DCs were incubated with 50 μM 4μ8C or DMSO for 6 h and then pulsed with 200 μg/ml OVA or 200 μg/ml OVA plus 100 μg/ml MEL for the last 5 h. Alternatively cells were pulsed with 100 pM SIINFEKL peptide for the last 20 min. Cells were counted, fixed and cultured as in **(B)**. **(G)** Data in graph shows three to four independent experiments of **(F)**. **(H)** FL-DCs were incubated with 60 μM STF or DMSO and then treated as in **(B).** Data in graph shows three independent experiments. Each symbol in the graphs represents data derived from one independent experiment. For all error bars represent mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ (paired Student's *t*-test).

constitutive expression of cytosolic OVA protein in DCs (42). FL-DCs from CD11c DOG mice were treated with acid wash to remove OVA peptides from MHC Class I molecules at the cell surface (43). After treatment with acid wash, cells were allowed to recover for 5 h in presence of 4μ8C or control vehicle and the generation of newly formed MHC Class I/ $OVA_{257-264}$ peptide complexes was quantified upon culture with OT-I T cells (**Figure 4C**). CD11c DOG FL-DCs that recovered in presence of 4μ8C displayed a similar capacity to activate OT-I cells than cells that recovered in presence of control vehicle. These data indicates that acute blockade of IRE1α endonuclease does not inhibit processing of cytosolic antigens and loading onto MHC Class I molecules (**Figure 4C**). Finally, to account for cross-presentation in steady state, 4μ8C-treated FL-DCs were pulsed with different doses of soluble OVA protein for 5 h, and cells were fixed and cultured with OT-I cells for quantification of CD69 expression (**Figure 4D**). No significant differences were observed between 4μ8C treatment and control vehicle in the ability to cross-present soluble OVA protein by resting FL-DCs. Altogether, these data indicates that pharmacological inhibition of IRE1α endonuclease with the aldehyde 4μ8C does not impinge on endogenous MHC Class I presentation and cross-presentation of OVA in absence of innate stimulation.

Innate Recognition of MEL Lysates Via the IRE1α/XBP1s Axis Favors Cross-Presentation of Antigens to $CD8^+$ T Cells

We investigated whether IRE1α activation in response to melanoma cell lysates promoted cross-presentation of OVA. For this purpose, FL-DCs were pre-incubated with 4μ8C or control vehicle and pulsed with OVA or OVA plus MEL and the quantification of MHC-I/OVA $OVA_{257-264}$ peptide complexes was quantified using the antibody 25.D1-16, that recognizes the $H\text{-}2K^b$-SIINFEKL complex (44) (**Figure 4E**). No effect of 4μ8C on 25.D1-16 staining was observed in FL-DCs pulsed with OVA alone, in agreement with results shown in **Figure 4D**. However, in presence of MEL lysates, 4μ8C treatment reduced surface expression of SIINFEKL-loaded MHC-I molecules in FL-DCs, an effect that was particularly noticeable in cDC1 FL-DCs (**Figure 4E**). These results indicate that pharmacological inhibition of IRE1α endonuclease activity decreases the cross-presentation of MEL-associated antigens. To functionally test for cross-presentation, FL-DCs were incubated with 4μ8C or control vehicle, and pulsed with OVA or OVA plus MEL, and then fixed prior to culture with OT-I T cells (**Figures 4F,G**). FL-DCs stimulated in presence of MEL-OVA increased the cross-presentation of OVA as indicated by augmented CD69 expression, in comparison with FL-DCs pulsed with OVA in absence of MEL. However, the adjuvant effect of MEL in augmenting OT-I T cell activation was consistently reduced in FL-DCs treated with 4μ8C, suggesting that IRE1α activation upon recognition of MEL lysates favors $CD8^+$ T cell activation. Furthermore, to confirm that this effect is specifically attributed to IRE1α activity, we included an additional IRE1α endonuclease inhibitor (STF-083010), which possesses demonstrated *in vivo* activity (45). STF-083010 inhibited XBP1s induced by TM without affecting global viability (**Supplemental Figures 2C,D**). Similar to the effects noticed with 4μ8C (**Figures 4F,G**), treatment with STF-083010 also reduced the cross-presentation of OVA by MEL-stimulated FL-DCs (**Figure 4H**). To sum up, these data indicates that activation of IRE1α endonuclease contributes to decoding the adjuvant effect of MEL lysates for cross-presentation of antigens.

Inhibition of IRE1α Endonuclease Function Selectively Prevents Cross-Presentation of a Melanoma-Associated Antigen Without Impairing Presentation of Tumor Antigens in MHC Class II

To extend our findings to a more physiological setting, we analyzed the cross-presentation of an antigen intrinsic to melanoma cells and investigated the dependence of IRE1α/XBP1s axis in this process. To this end, we isolated $CD8^+$ T cells from pmel-1 transgenic mice, which bear a MHC Class I-restricted, transgenic TCR specific for the human and murine melanocyte antigen $gp100_{25-33}$ (46). We verified that MEL lysates contained sufficient amounts of the gp100 antigen, which could only be cross-presented to pmel-1 T cells via a BMDC (**Supplemental Figure 3A**). Furthermore, we demonstrate that both, MEL lysates and B16 lysates contained antigens for cross-presentation to pmel T cells, showing a higher efficiency for the human lysate over the murine counterpart (**Supplemental Figure 3B**). These data is consistent with reported work demonstrating that the pmel-1 TCR recognizes the human $gp100_{25-33}$ peptide with greater

efficiency than the mouse $gp100_{25-33}$ peptide, due to a more efficient binding of the human sequence to H-2D^b (46). These data confirms that MEL lysates are a suitable source of antigen for cross-presentation studies to pmel T cells. We first tested if IRE1α was required for engulfment of MEL lysates, and observed that 4μ8C-treated cells acquire similar amounts of MEL-labeled material over a period of time compared to the control condition (**Supplemental Figure 3C**), indicating that inhibitor treatment does not affect antigen uptake. Then, we interrogated if MEL-stimulated FL-DCs with an active IRE1α/XBP1s axis were more competent to activate pmel T cells than FL-DCs with the pathway inhibited. Whereas, 4μ8C did not impair MHC Class I presentation of the human $gp100_{25-33}$ peptide, inhibition of IRE1α endonuclease in MEL-stimulated FL-DCs resulted in reduced activation of pmel T cells (**Figure 5A**). Furthermore, 4μ8C treatment also reduced the ability of MEL-stimulated FL-DCs to trigger pmel T cell proliferation and IFN-γ production (**Figure 5B** and **Supplemental Figure 3D**). To extend these findings to additional DC subtypes, we included GMCSF-BMDCs as source of antigen presenting cells and noticed a similar effect than that observed for FL-DCs (**Figures 5C,D**), indicating that blockade of IRE1α endonuclease activity broadly impacts on the ability of various subtypes of BMDCs to cross-present a melanoma-associated antigen for CD8$^+$ T cell activation.

Finally, we investigated if 4μ8C treatment also inhibited the presentation of a melanoma-associated antigen via MHC Class II. To this end, we isolated CD4$^+$ T cells from TRP-1 mice, which express a MHC Class II-restricted, transgenic TCR specific for the tyrosinase-related protein 1 antigen present in melanoma (47). In contrast to the observations noticed with pmel CD8$^+$ T cells, 4μ8C treatment did not impair the proliferation of TRP-1 CD4$^+$ T cells. These data indicates that inhibition of IRE1α endonuclease activity does not influence antigen presentation on MHC Class II (**Figure 5E**). To sum up, we conclude that activation of the IRE1α/XBP1s axis favors DC activation for CD8$^+$ T cell activation to melanoma-associated antigens but it is dispensable for CD4$^+$ T cell priming.

IRE1α Endonuclease Activity Potentiates the Cross-Presentation Abilities of GMCSF-BMDCs *in vivo*

To obtain insights on the function of IRE1α endonuclease activity by an independent approach, we generated DC cultures from BM of IRE1^{trunc} DC mice, which is a crossed mice line between *Itgax*-Cre mice that express Cre recombinase under the promoter of the *Cd11c* gene (48) and *Ern1*$^{fl/fl}$ mice, which have loxP sites flanking exons 20 and 21 of the gene (49). IRE1^{trunc} DC mice harbor a truncated IRE1 isoform that possesses preserved kinase function but impaired endonuclease activity (49). We validated the model by generating FL-DCs and GM-CSF DCs from BM of IRE1^{trunc} DC mice and Ctrl littermates, which correspond to *Ern1*$^{fl/fl}$ mice lacking the Cre recombinase (**Figures 6A,B**). Remarkably, we observed that FL-DCs from IRE1^{trunc} DC mice do not express the truncated IRE1α isoform and expressed similar amounts of WT IRE1α protein than Ctrl counterparts (**Figure 6A**). This data indicates that FL-DC cultures do not mediate efficient Cre-dependent excision of the loxP-flanked sites in the *Ern1*$^{fl/fl}$ gene and therefore, are not a suitable model to study loss of IRE1α endonuclease function. However, in cultures of GMCSF-BMDCs from IRE1^{trunc} DC mice, we observed the presence of the truncated IRE1α isoform, although the expression levels of the truncated protein were highly variable among BM cultures derived from independent mice (**Figure 6B**, line 2,4,6). There was also considerable expression of the WT isoform of IRE1α protein remaining in these cultures, which differs with previous observations with splenic DC counterparts (22). Thus, IRE1^{trunc} GMCSF-BMDCs are a model of DCs expressing a mix of WT and truncated isoforms of IRE1α. We verified that IRE1^{trunc} GMCSF-BMDCs developed normally and that expressed normal levels of CD11c and MHC-II, along with surface markers associated to conventional DCs (CD135, FLT3; receptor for FLT3L) and to monocyte-derived macrophages (CD115), which were previously reported in these cell cultures (37) (**Figure 6C**).

To test the function of IRE1^{trunc} GMCSF-BMDCs *in vivo*, IRE1^{trunc} or Ctrl cells were stimulated with B16 lysates plus OVA and were then adoptively transferred into B6 mice that receive OT-I T cells labeled with the proliferation dye Cell Trace Violet the day before. OT-I T cell proliferation was monitored on day 5 in spleen (**Figure 6D**). Adoptive transfer of GMCSF-BMDCs from Ctrl mice elicited a high degree of CD8$^+$ T cell activation, as indicated by the proliferation profile of OT-I T cells in spleen. In contrast, adoptive transfer of IRE1^{trunc} GMCSF-BMDCs resulted in a mild but consistent reduction in the frequencies of proliferating OT-I T cells (**Figure 6E**), which accounted for a 15% reduction in frequencies of proliferating OT-I T cells. These results are consistent with results depicted in **Figure 5D** and indicate that IRE1α endonuclease function potentiates the cross-presentation of tumor cell associated antigens by *ex-vivo* generated DCs.

DISCUSSION

The intracellular mechanisms responsible to promote immunogenic DC function in cancer are matter of intense investigation. In this work, we report that recognition of melanoma cell lysates induces efficient activation of the IRE1α/XBP1s axis in BMDCs, which in turn increases cross-presentation of melanoma-associated antigens. Our findings indicate that MEL stimulation induces expression of the canonical UPR member BiP and efficiently triggers XBP1s in absence of RIDD. Further experiments will be necessary to elucidate the nature of the XBP1s-activating factor present in melanoma cell lysates, which is expressed in melanoma cells from human and mice origin, and it is also found in additional cancer cell lines such as ovarian and gallbladder cancer. In this context, it is plausible that activation of the IRE1α/XBP1s axis by MEL occurs downstream of PRR recognition, as it is known that innate immune sensing intersect with the UPR at various points for optimal activation of NF-kB, IRF-3, or JNK (26, 28, 50). On one hand, STING activation couples to the UPR (51) and signaling via TLR2 and TLR4 activate XBP1s

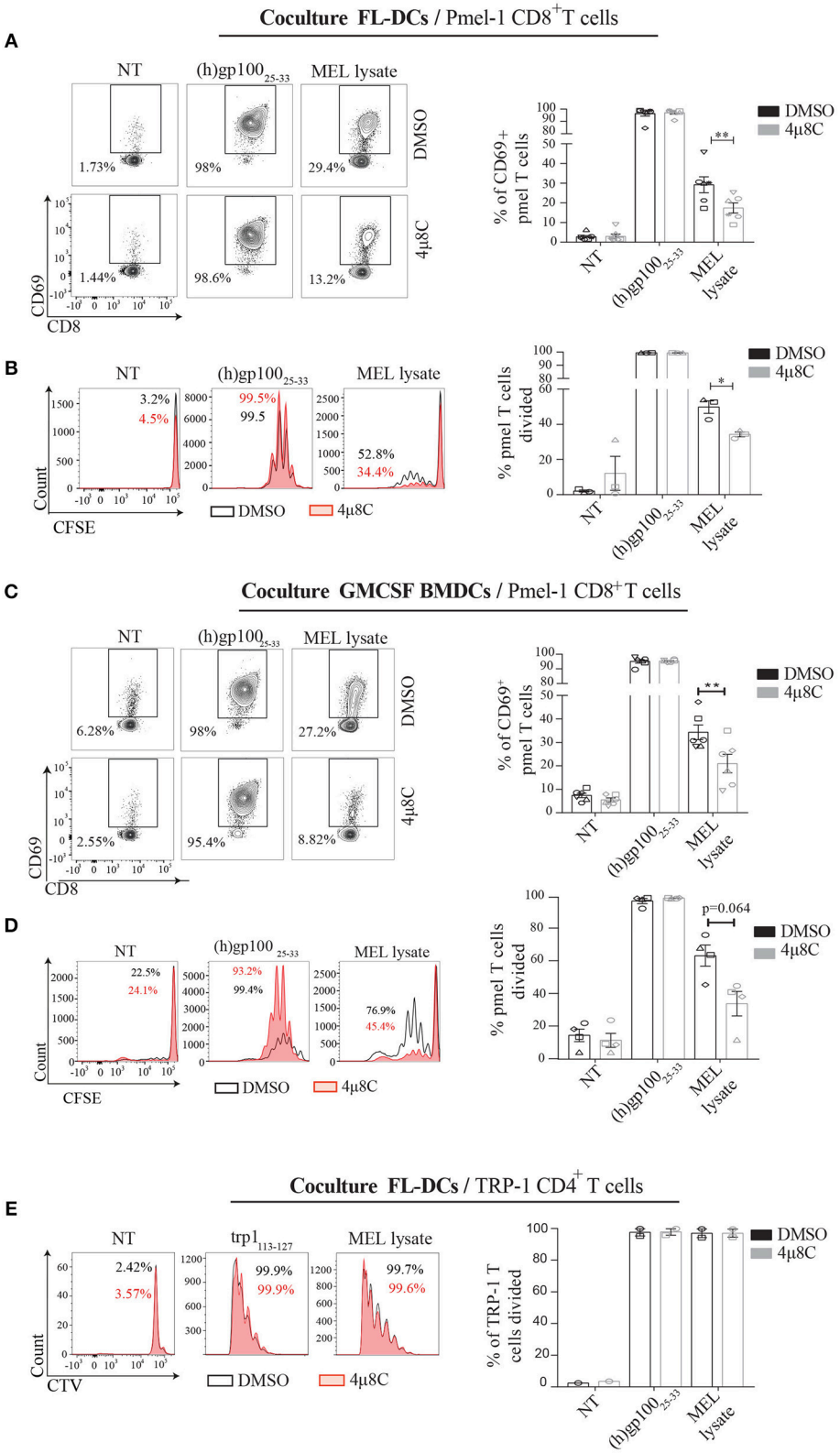

FIGURE 5 | Inhibition of IRE1α endonuclease function reduces the cross-presentation of a melanoma-endogenous antigen *in vitro*. **(A)** FL-DCs were preincubated with 50 μM 4μ8C or DMSO for 6 h and pulsed with 100 μg/ml MEL for the last 5 h of culture. Alternatively, cells were pulsed with 2.5 μM human gp100 peptide for the last 20 min of culture. Cells were counted, fixed and 5 × 10^4 FL-DCs were cocultured with 5 × 10^4 pmel-1 $CD8^+$ T cells. Pmel-1 $CD8^+$ T cell activation

(Continued)

FIGURE 5 | was quantified by expression of CD69 on day 1 through flow cytometry. Data in graph shows seven independent experiments. **(B)** FL-DCs were treated as in **(A)** but were not fixed and 2×10^4 FL-DCs were cultured with 5×10^4 CFSE-labeled pmel-1 $CD8^+$ T cells. Proliferation was quantified on day 3 by flow cytometry. Data in graph shows three independent experiments. **(C)** GM-CSF BMDCs were treated and cocultured as in **(A).** Data in graph shows six independent experiments. **(D)** GM-CSF BMDCs were treated and cocultured as in **(B).** Data in graph shows four independent experiments. **(E)** FL-DCs were treated as in **(B)** but were cultured with 5×10^4 CellTrace Violet-labeled CTV = $CD4^+$ T cells isolated from Trp1 mice. Proliferation was measured on day 5 by flow cytometry. Data in graph shows two independent experiments of **(A)**. Each symbol in the graphs represents data derived from one independent experiment. For all error bars represent mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ (paired Student's *t*-test).

via reactive oxygen species (ROS) for exacerbation of cytokine production in macrophages (25). In particular, it has been demonstrated that XBP1s binds to the promoter regions of the *Tnf* and *il-6* genes, providing direct evidence linking the UPR to transcriptional activation of cytokines (25). In fact, most of what is currently known on XBP1s function in the regulation of cytokine production emerges from studies in macrophages (25, 52), and it is not clearly understood if similar mechanisms are applicable to DCs. We observe that pharmacological inhibition of IRE1α endonuclease decreases the production of IL-6, TNF, IL-10, and IL-12p40, by FL-DCs to MEL stimulation, which is reminiscent to data previously reported in XBP1 KO macrophages (25). If TLR-dependent XBP1s activation is a conserved feature across macrophages and DCs, then it would be highly plausible that TLR4 signaling accounted for IRE1α/XBP1s activation in MEL-activated FL-DCs, as it has been previously reported that the melanoma cell lines used in this study express the endogenous TLR4 ligand HMGB1 (34). On a mechanistic basis, it is plausible that XBP1s transcriptionally activate expression of *Tnf* and *Il6* genes, although we do not provide formal evidence of this process in this study. Furthermore, on the basis of the presented experiments, we cannot exclude an XBP1s-independent function of IRE1α endonuclease, as it has been recently reported that the enzyme may degrade certain microRNAs still in absence of canonical RIDD (53). Additional parameters, including upregulation of costimulatory molecules, remained unaffected upon pharmacological blockade of IRE1α endonuclease, indicating that the pathway regulates a particular aspect of the transcriptional program of MEL-activated DCs. Thus, our data shows that the IRE1α/XBP1s axis in BMDCs adjusts the magnitude of cytokine production upon innate recognition of cancer cell lysates.

The endonuclease domain of IRE1α is reported to have dual functions in MHC-I antigen presentation, which may be dependent on the cell lineage, pathological setting or the extent of ER stress that can be tolerated by a particular cell type (41). On one hand, IRE1α via XBP1s has shown to regulate expression of several members of the MHC-I antigen presentation machinery such as calnexin, calreticulin, and Erp57 in HEK $293T^{DAX}$ cells (54). On the other hand, induction of RIDD in DCs (by means of XBP1 genetic ablation) results in reduced cross-presentation of dead cell-associated antigens *in vivo* (21). Our data shows that acute blockade of IRE1α endonuclease in non-activated FL-DCs does not impair their ability to present cytosolic OVA via MHC-I nor to cross-present OVA protein to OT-I T cells although it modestly reduces surface expression of MHC-I. One possibility accounting for these findings may be that BMDCs express additional regulatory mechanisms to ensure efficient antigen presentation. However, in contexts of DC activation, we demonstrated that XBP1s induction in MEL-stimulated BMDCs promotes their ability to cross-present antigens. Although the magnitude of this response is discrete, it suggests that activation of the IRE1α/XBP1s pathway may be relevant to induce $CD8^+$ T cell responses to tumor-derived signals. The intracellular mechanisms by which XBP1s leads to increased cross-presentation of melanoma cell-associated antigens *in vitro* remain to be elucidated, although we show that this effect is independent of antigen uptake and that pharmacological blockade of IRE1α reduces the expression of specific MHC Class I/ peptide complexes at the cell surface.

Importantly, in this work we studied BMDCs from $IRE1^{trunc}$ DC mice. This genetic model of IRE1α endonuclease ablation was proven not to be useful for the study of FL-DCs, which prevented further studies in the cDC1 lineage of DCs. At present it is unclear as to why FL-DCs did not carry out Cre-mediated excision of the *Ern1*-floxed gene but it may be related to the immature stage of FL-DCs found in these cultures (55). Future studies using recently reported protocols for the generation of more authentic cDC1s will be valuable to translate these findings to DC subtypes that may be useful in clinical settings (55). However, in experiments using GMCSF-BMDCs from $IRE1^{trunc}$ mice, which expressed the truncated IRE1α isoform, we noticed that these cells were less competent to induce proliferation of antigen specific $CD8^+$ T cells in the spleen. Although this effect was not severe, it is unclear if the presence of a remaining pool of the WT IRE1α isoform noticed in these cultures accounted for the discrete differences. Future studies using additional technologies of genetic editing such as CRISPR-Cas9 could help circumvent this issue and provide a full picture on the role of the pathway in melanoma tumor growth, cytotoxic T cell responses *in vivo* and $CD8^+$ T cell memory.

At present, it remains to be further investigated the mechanisms that intersect the IRE1α/XBP1s pathway with the MHC-I antigen presentation and cross-presentation route. In fact, cell biological processes known to enhance the efficiency of cross-presentation such as restraining phagolysosome fusion upon TLR signaling (23) have not been explored as consequence of UPR activation. Future studies will unveil the molecular mechanisms linking the IRE1α arm of the UPR with the MHC-I antigen presentation machinery in contexts of innate recognition.

Finally, an aspect that should not be ignored is that activation of the IRE1α/XBP1s axis in DCs does not predictably lead to enhanced T cell activation. It is reported that XBP1 KO $CD11b^+$ DCs infiltrating ovarian cancer tumors are more efficient to activate anti-tumor $CD8^+$ and $CD4^+$ T cell responses and can control tumor growth (29). Although these and our

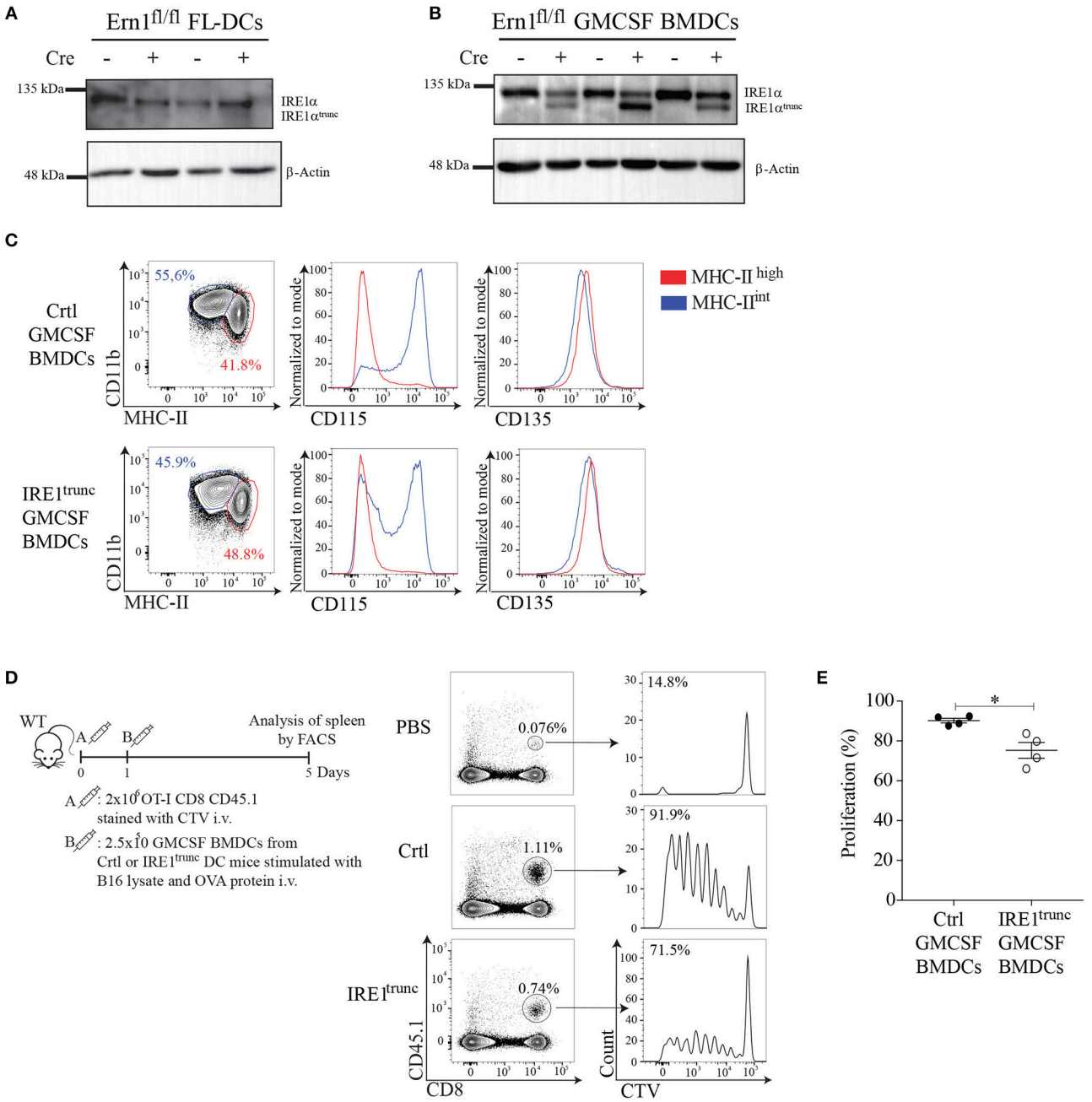

FIGURE 6 | IRE1α endonuclease activity in GMCSF-BMDCs promotes cross-presentation of tumor-associated antigens *in vivo*. **(A,B)** Western Blot analysis of IRE1 levels in FL-DCs and GMCSF BMDCs of IRE1^{trunc} or ctrl DC mice. **(C)** Phenotype of GMCSF BMDCs from IRE1^{trunc} or ctrl DC mice at day 8 of culture (gate on CD11c^{+} cells). **(D)** *In vivo* proliferation of OT-I CD8 T cells (CD45.1^{+}). 2 × 10^{6} CD8 T cells stained with CellTrace Violet (CTV) were adoptively transferred into congenic mice. One day later mice were injected i.v. with 2.5 × 10^{5} GMCSF BMDCs, from IRE1^{trunc} or ctrl DC mice pulsed with 100 μg/ml B16 lysate plus 200 μg/ml OVA. Histograms represent the proliferation of transferred cells (CD8^{+} CD45.1^{+}) in the spleen. **(E)** The graph represents the percentage of proliferation of CellTrace Violet-labeled cells. Each symbol in the graph represents data from an individual mouse. Error bars represent mean ± S.E.M. *$p < 0.05$ determined by Mann–Whitney test.

findings may seem at first glance contradictory, there are aspects to be considered. These include the immunostimulatory or immunosuppressive properties of different cancer cell preparations. This is a highly relevant issue considering that, whereas the conditioned media of ovarian cancer tumors is highly immunosuppressive and curtails T cell proliferation (29), we show that melanoma cell lysates act as adjuvants for cross-presentation. At present, it is not fully understood what dictates

the immunogenicity vs. the immunosuppressive properties of preparations from different cancer cell types and in fact, several variables such as the stage of tumor progression, the use of cell lines vs. implanted tumors, the nature of the cancer cell, the amount/type of danger signals expressed by each cancer type could influence this outcome. Furthermore, a possibility is that IRE1α and XBP1s may control different cell biological processes in DCs according to an immunogenic or an immunosuppressive environment. Additional aspects on the role of the IRE1α/XBP1s in promoting tumor cell growth or tumor rejection may also be associated with the extent of ER stress imposed by the tumor microenvironment, which cannot be recapitulated by in *in vitro* approaches. Finally, the functionality of the IRE1α/XBP1s axis in different DC lineages may also play a role, considering that not all tumors are able to recruit the DC subtypes responsible to mediate cytotoxic responses *in vivo*. This is relevant considering that cDC2s, in contrast to cDC1s, are not sensitive to XBP1 loss in resting conditions (21). In the present study, we present evidence that are consistent with previous data showing that enforced XBP1s expression potentiates antitumor T cell immunity of DC vaccines generated *ex vivo* (30, 31). Altogether, our findings support the notion that activation of the IRE1α/XBP1s pathway may be relevant for improving the immunogenic efficacy of DC-based vaccines in melanoma.

MATERIALS AND METHODS

Mice

Wild-type C57BL/6, *Itgax*-Cre mice (48), *Ern1*$^{fl/fl}$ mice (49), *Ern1*$^{fl/fl}$ x *Itgax*-Cre mice (IRE1^{trunc} DC mice), Pmel-1 (46), Trp-1 mice (47), and ERAI mice (36) were bred at Universidad de Chile. OT-I mice (56) CD11c.DOG mice (42) were bred at Fundación Ciencia & Vida. All mice were on a C57BL/6 background and Trp-1 mice were on a RAG$^{-/-}$ background. For all experiments, mice between 5 and 20 weeks of age were bred in specific pathogen-free conditions. All animal experiments were performed in accordance with institutional guidelines for animal care and were approved by the Ethical Review Committees at University of Chile and Fundación Ciencia & Vida.

Medium and Reagents

Culture medium was RPMI 1640 GlutaMAX™ (Gibco) supplemented with penicillin, streptomycin (Hyclone), 2-mercaptoethanol (Gibco) and 10% heat-inactivated fetal bovine serum (FBS) (Corning). FACS Buffer was PBS 1X (Gibco), supplemented with 1% FBS and 2 mM EDTA (Ambion). Cytometric bead array (CBA) Mouse Inflammation Kit was purchased from BD Biosciences. IRE1 Inhibitor III, 4μ8C (38) was from EMD Millipore. STF-083010 (45), Tunicamycin (TM), lipopolysaccharide (LPS), PMA and Ionomycin were from Sigma-Aldrich. House Dust Mite (*D. pteronyssinus*) was purchased from GreerLabs. $OVA_{257-264}$ peptide (SIINFEKL) was purchased from Invivogen. Soluble Low Endo Ovalbumin was purchased from Worthington Biochemical. Human gp100 peptide ($hgp100_{25-33}$, KVPRNQDWL) and Mouse TRP-1 peptide (TRP-$1_{106-130}$, SGHNCGTCRPGWRGAACNQKILTVR) were purchased from Genetel Laboratories LLC. Brefeldin A was from eBiosciences.

Cell Lines, Melanoma Lysates, and Supernatants

The human melanoma lysates (MEL) was derived from 3 allogeneic melanoma cell lines (Mel1, Mel2, and Mel3), which were isolated and purified from metastasic lymph nodes (35). Identity of cell lines was confirmed by Short Tandem Repeat (STR) DNA profiling analysis (not shown). Briefly, the lysates were made from a mix of equal amounts of cell lines, taken to a final concentration of 4×10^6 cells/ml, in eppendorf tubes. Cells were lysed through 3 cycles of freeze–thaw in liquid nitrogen. The protein concentration was estimated by Bradford's method using a biophotometer (Eppendorf). The human gallbladder cancer lysates (GBCa) (57), human ovarian cancer cell lysates from SKOV3 cell lines (ATCC) (OvCa), leukocyte lysed from PBMC and B16.F10 cell line lysate (B16 lysate) were lysed using the same method.

Flow Cytometry and Cell Sorting

Antibodies for flow cytometry were purchased from BD Pharmigen, BD Horizon™, eBioscience, Biolegend or Miltenyi Biotec and the viability dye LIVE/DEAD® Fixable Aqua (Thermofisher Scientific) was used for discriminating dead cells from analysis. Depending on the experiment, cells were stained with the following antibodies in presence of CD16/31 (Fc Block): CD11b (M170), CD86 (GL-1), I-A/I-E (M5/114.15.2), XCR1 (ZET), CD80 (16-10A1), PD-L1 (MIH5), CD8α (53.6.7), CD172α (P84), CD3ε (145-2C11), B220 (RA3-6B2), CD103 (2E7), CD11c (N418), CD69 (IM7), H-2K^b (AF6-88.5), CD115 (AFS98), CD24 (M1/49), CD45.1 (A20), CD135 (A2F10), and Streptavidin. Acquisition and analysis of labeled cell suspensions was performed on FACSVerse and LSR Fortessa (BD Biosciences) and subsequent analysis of data was made with FlowJo10 software (FlowJo, LLC). Cell sorting was performed on FACS Aria III (BD Biosciences).

Generation of Mouse Flt3L and GM-CSF BMDCs

BMDCs were differentiated from femurs and tibias of C57BL/6 mice. FL-DCs (10) were generated by culturing BM cells in culture media in the presence of 150 ng/ml of human recombinant Flt3L (Peprotech) for 7–8 days. GM-CSF DCs (58) were generated by culturing BM cells in the presence of 20 ng/ml mouse recombinant GM-CSF (Biolegend) for 8 days. Fresh culture medium with cytokine was added on day 3, and on day 6 the medium was refreshed.

BMDCs Activation

2×10^5 FL-DCs were pretreated with 20 μM 4μ8C or DMSO for 2 h and stimulated with 100 μg/ml MEL for 16 h. Expression of MHC-II, CD80, CD86, and PD-L1 was measured by flow cytometry. For CBA, 2×10^5 FLT3-L BMDCs were incubated for 6 h with DMSO or 4μ8C 20 μM, and then stimulated with MEL 100 μg/ml for 16 h. After incubation, cells were centrifuged and supernatant was collected. For activation of ERAI FL-DCs,

2×10^5 cells were stimulated with 100 μg/ml of the following lysate preparations: MEL, B16 lysate, Leukocyte lysate, OvCa, and GBCa for 24 h. Expression of VenusFP was measured by flow cytometry. For MEL titration, 2×10^5 FL-DCs were not treated or stimulated with increasing amounts (2, 50, 100, and 200 μg/ml) of MEL. Expression of VenusFP was measured after 16 h by flow cytometry. For MHC-I staining, FL-DCs were incubated with 50 μM 4μ8C or DMSO for 6 h and MHC-I expression was measured by flow cytometry.

Quantification of Cytokine Production

For CBA analysis, 2×10^5 Flt3L BMDCs were incubated for 22 h with 20 μM 4μ8C or DMSO, and stimulated with MEL 100 μg/ml for the last 16 h of culture. After incubation, supernatant was collected for cytokine analysis. For intracellular staining of the IL-12p40 subunit, 2×10^5 FL-DCs were stimulated with 50 μM 4μ8C or DMSO at 37°C for 22 h, followed by stimulation with 100 μg/ml of MEL lysates for the last 16 h of culture. During the last 4 h of stimulation, Golgi Plug 1X (BD Biosciences) was added to the wells. After extracellular staining, BMDCs were fixed and permeabilized using the Cytofix/Cytoperm™ fixation/permeabilization kit (BD Biosciences). For IL-12p40 staining, cells were labeled with the IL-12/IL-23 p40 eFluor® 660 antibody (C17.8; eBioscience). For detection of IFNγ, $CD8^+$ T cells were collected on day 3 of coculture and were stimulated with 0.25 μM PMA and BFA 1x for 4 h. After extracellular staining, T cells were fixed and permeabilized with the Foxp3/Transcription Factor Fixation/Permeabilization Kit (eBioscience), and cells were labeled with IFNγ PE (XMG1.2, eBioscience).

PCR, qPCR, and Primers

RNA was obtained from Flt3L BMDCs using the TriPure isolation reagent (Roche, Sigma Aldrich) following the manufacturer's instructions. Complementary DNA (cDNA) was made using the M-MLV Reverse Transcriptase kit (Invitrogen, Thermo Fischer Scientific) and SYBR green-based qPCR was performed using MX3005P (Stratagene, Agilent Techonologies). XBP-1 splicing analysis by conventional PCR as described previously (59). Briefly, cDNA was amplificated and PCR products were digested with the restriction enzyme PstI (Promega) for 2 h and then analyzed in a 1% agarose gel.

For qPCR analysis, BMDCs were treated with medium or stimulated with 100 μg/ml MEL, 100 μg/ml B16 lysate, 100 ng/ml LPS, 50 mg/ml HDM, or 1 μg/ml TM or DMSO ctrl for 8 h. Primers for Sec61 and XBP-1 were from Lee et al. (40), primers for Erp44, Bloc1s1, and Tapbp were from Osorio et al. (21). Other qPCR primers used in this study were from Roche Universal Probe Library: Bip forward (5′-ATGAGGCTGTAGCCTATG GTG-3′); Bip reverse (5′-GGGGACAAACATCAAGCAG-3); CHOP forward (5'-CCACCACACCTGAAAGCAG′-3′); CHOP reverse (5′-TCCTGCAGATCCTCATACCAG-3′); L27 forward (5′-GCCAAGCGATCCAAGATCAA-3′); L27 reverse (5′-GCT GGGTCCCTGAACACATC-3′).

Antigen Presentation Assays

$CD8^+$T cells were isolated from spleen of OT-I or Pmel-1 mice, while $CD4^+$ T cells were isolated from spleen and lymph nodes of Trp-1 mice. $CD8^+$ T Cells were isolated by negative selection using a lineage depletion cocktail of biotinylated antibodies and anti-biotin microbeads (Miltenyi Biotec) and labeled with 5 μM CFSE (eBioscience) when described. $CD4^+$ T cells were isolated by cell sorting gating on FSC/SSC/singlets/$CD3^+$/$CD4^+$ and labeled with 5 μM CellTrace™ Violet (CTV) (Thermofisher). BMDCs were treated with 50 μM 4μ8C or 60 μM STF-083010 or DMSO as vehicle control. One hour later, OVA (200 μg/ml) and/or MEL lysates (100 μg/ml) were added to the wells containing the inhibitors and cells were incubated for 5 additional hours. For MHC-I presentation of peptides, BMDCs were pulsed for the last 20 min of culture with the following peptides $OVA_{257-264}$ (300, 100, 50, or 10 pM); $hgp100_{25-33}$ (2.5 μM), $TRP\text{-}1_{106-130}$ (2.5 μM). For assays measuring early T cell activation, DCs were collected, washed with FACS buffer and fixed with PFA 1% for 10 min. Then cells were washed with 0.2 M glycine and were washed with media prior to coculture. 5×10^4 fixed DCs were cultured with 5×10^4 T cells (1:1 ratio) at 37°C for 16 h to analyse T cell activation by flow cytometry by means of CD69 expression. For proliferation assays, DCs were pulsed with inhibitors and antigens as described above with the exception that cells were not fixed at the end of the culture. 2×10^4 DCs were cultured with 5×10^4 CFSE o CTV labeled T cells for 3 days and proliferation was measured by flow cytometry.

Endogenous MHC-I Presentation Assay

BM from CD11c.DOG mice was used to generate FL-DCs as described. On day 8, DCs were centrifuged and incubated for at 4°C for 2 min with citric acid (Acid wash solution, pH = 3.94), 1% BSA to remove constitutive OVA peptides from surface MHC class I molecules (60). After the incubation, cells were washed 3 times with complete culture media. Then, DCs were allowed to recover in presence of 50 μM 4μ8C or DMSO control for 5 h in complete media at 37°C. Cells were then fixed in PFA and were cocultured with purified $CD8^+$ OT-I T cells in a 1:1 ratio. CD69 expression on $CD8^+$ T cells was measured by flow cytometry after 16 h of culture.

Phagocytic Uptake Assay

1×10^7 Mel2 cells were washed with un-supplemented RPMI (Corning) and stained with 2 μM PKH26 membrane linker solution (Sigma-Aldrich) following manufacturer's instructions. Cells lysates of PKH26 labeled cells were generated as previously described. For phagocytic uptake, FL-DCs were incubated with 50 μM 4μ8C or DMSO, in presence of PKH26 labeled MEL in a 2:1 Tumor cell: DC ratio for 0, 60, and 120 min at 37 or 4°C as control of phagocytosis. Internalization of PKH26 labeled material by DCs was assessed by flow cytometry, gating on the DC population.

H-2K^b-SIINFEKL Staining

2×10^5 FL-DCs per condition were incubated with 20 μM 4μ8C or DMSO for 5 h and then not treated or stimulated with 250 μg/ml OVA or 250 μg/ml OVA plus 100 μg/ml MEL for

16 h. Cells were collected and incubated at 4°C for 1 h with H-2K^b-SIINFEKL PE-Cy7 antibody (25.D1-16; Biolegend) in FACS Buffer. Then antibody cocktail plus Fc Block 2X was added and incubated at 4°C for 20 min in FACS Buffer. MHC-I/SIINFEKL complex were measured by flow cytometry.

Western Blot

BMDCs were spun at 400 g for 7 min, the supernatant was removed and the pellet resuspended in ice-cold PBS. After a next round of centrifugation (400 g, 7 min), the pellet was pipetted dry and resuspended in 30 or 50 μl of E1A buffer (1% NP40, 20 mM HEPES, pH 7.9, 250 mM NaCl, 1 mM EDTA) complemented with Complete-ULTRA (Roche) and PhosSTOP (Roche). Samples were incubated in buffer at 4°C for 15 min, vortexing every 5 min, then spun at 12,000 g to remove insoluble material and stored at −80°C until further use. Prior to SDS–PAGE, samples were resuspended in loading dye and heated at 95°C for 10 min. After wet transfer to polyvinyldifluoride membrane (Immobilon; Millipore), proteins were analyzed by immunoblotting and visualized by chemiluminescence (Luminata Forte Western HRP substrate; Millipore). Antibodies used recognize IRE1α (Rabbit 14C10; Cell Signaling; used 1/1,000), β-Actin (Mouse ab6276; Abcam; used 1/5,000); Secondary antibodies Anti-Rabbit (Cell signaling; used 1/4,000), Anti-Mouse (Cell signaling; used 1/4,000).

In vivo Proliferation Assay

For *in vivo* proliferation assay, 2×10^6 OT-I CD8 T cells (CD45.1$^+$) stained with CTV were intravenously transferred (i.v.) into CD45.2 congenic mice. Next day, mice were injected i.v. with 2.5×10^5 GMCSF BMDCs from IRE1^{trunc} or control DC mice stimulated for 16 h with 100 μg/ml B16- F10 lysates plus 200 μg/ml OVA. Four days later, the proliferation of transferred cells was measured in the spleen by flow cytometry.

Statistical Analysis

Differences between groups were analyzed by paired, two-tailed Student's *t*-tests or Mann–Whitney test. Results with a *P*-value of 0.05 or less were considered significant. Mean values, SEM and statistics were calculated using Graphpad Prism Software. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. No criteria of inclusion/exclusion of data were used in this study.

AUTHOR CONTRIBUTIONS

CC, BM, and DF conducted experiments. CP managed cell lines and generated cell lysates. AL, DS, and RP provided critical reagents. FS-O contributed with critical discussions and key reagents. TI provided ERAI reporter mice and *Ern1*$^{fl/fl}$ mice. CC, BM, DF, and FO designed the study, analyzed the data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Metabolic Control of Dendritic Cell Functions: Digesting Information

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Dendritic cells (DCs) control innate and adaptive immunity by patrolling tissues to gather antigens and danger signals derived from microbes and tissue. Subsequently, DCs integrate those environmental cues, orchestrate immunity or tolerance, and regulate tissue homeostasis. Recent advances in the field of immunometabolism highlight the notion that immune cells markedly alter cellular metabolic pathways during differentiation or upon activation, which has important implications on their functionality. Previous studies showed that active oxidative phosphorylation in mitochondria is associated with immature or tolerogenic DCs, while increased glycolysis upon pathogen sensing can promote immunogenic DC functions. However, new results in the last years suggest that regulation of DC metabolism in steady state, after immunogenic activation and during tolerance in different pathophysiological settings, may be more complex. Moreover, ontogenically distinct DC subsets show different functional specializations to control T cell responses. It is, thus, relevant how metabolism influences DC differentiation and plasticity, and what potential metabolic differences exist among DC subsets. Better understanding of the emerging connection between metabolic adaptions and functional DC specification will likely allow the development of therapeutic strategies to manipulate immune responses.

Keywords: dendritic cell, metabolism, mitochondria, glycolysis, mammalian target of rapamycin, hypoxia-inducible factor, AMP-activated protein kinase, DC subsets

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METABOLIC CONTROL OF DENDRITIC CELL DEVELOPMENT

Natural dendritic cells (DCs) present in steady state comprise type 1 conventional DCs (cDC1s), type 2 cDCs (cDC2s), double negative (CD8/CD103– CD11b–) DCs (DN-DCs), and plasmacytoid DCs (pDCs; **Table 1**). Natural DCs derive from myeloid progenitors in the bone marrow and require FMS-like tyrosine kinase 3 ligand (FLT3L) to differentiate via the common DC progenitor (CDP) and DC precursors (pre-DCs). In addition, other cells that are functionally similar to DCs, such as Langerhans cells (LCs), can derive from embryonic precursors. Moreover, during inflammatory settings, DCs can develop from blood monocytes (moDCs; **Table 1**).

Energy Metabolism During Dendritic Cell Generation

Differentiation of Dendritic Cells From Monocytes With GM-CSF

The importance of energy metabolism was first established in the development of human moDCs *in vitro*. Granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4-induced differentiation and survival of DCs from human monocytes rely on the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) activation via phosphoinositide 3-kinase (PI3K; **Figure 1**) and are abrogated by rapamycin, an mTOR/mTORC1 inhibitor [**Table 2** and (1, 2)]. The mTORC1 downstream target peroxisomal proliferator-activated receptor γ (PPARγ) is upregulated early in moDC differentiation, affecting cell maturation and function largely through control of lipid metabolism (3–6). Indeed, inhibition of cytosolic fatty acid synthesis (FAS) via blocking acetyl-CoA carboxylase (ACC) 1 reduces moDC differentiation (7). Moreover, PPARγ co-activator-1α (PGC1α) and mitochondrial transcription factor A (TFAM), fundamental inducers of mitochondrial biogenesis and also indirect mTORC1 targets (8, 9), are also elevated during moDC differentiation (10). In line, differentiated moDCs show a higher oxygen consumption rate (OCR), contain more mitochondria, and produce more adenosine triphosphate (ATP) compared to monocytes (10, 11). Importantly, blocking the electron transport chain (ETC) with the complex I (CI) inhibitor rotenone (**Figure 2**) partially prevents moDC differentiation, despite causing a notable increase in glycolysis/lactate production (10, 11). Hence, moDC differentiation depends on oxidative phosphorylation (OXPHOS) and a balanced fatty acid metabolism.

Likewise, the DC-like cells differentiated from mouse bone marrow cultured with GM-CSF *in vitro*, a culture system composed of a mixed population of DCs and macrophages [**Table 2**, GM-DCs and (12)], also show glucose uptake together with high mitochondrial membrane potential (ΔΨm) and oxygen consumption (13). Indeed, GM-DC differentiation under hypoxic conditions yields fewer total cells, and hypoxia-inducible factor (HIF)-1α deficiency further reduces the frequency of CD11c+ GM-DCs, linked to decreased ATP (14). As HIF1α is a key metabolic regulator and many of its target genes drive glycolysis (see the section Sustained Glycolysis: The Role of HIF1α), these observations point toward the importance of an active glucose metabolism involving oxidative and glycolytic pathways in GM-DCs. However, CD11c-Cre HIF1α$^{flox/-}$ mice display unaltered DC homeostasis in the steady state (15). Moreover, impairment of cytosolic FAS by blocking ACC1 with the inhibitor 5-(tetradecyloxy)-2-furoic acid (TOFA) in GM-DC cultures or by the administration of the fatty acid synthase (FASN) inhibitor methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid (C75; **Figure 2**) *in vivo* reduces the generation of DCs (7), further suggesting that balanced FA metabolism contributes to DC development. However, it is noteworthy that the inhibitor C75 can also cause mitochondrial dysfunction (16).

Natural Dendritic Cell Differentiation

Generally, the presence of CDPs, pre-DCs, cDCs, and pDCs is reduced in energy-restricted mice, while myeloid progenitors, blood monocytes, and spleen macrophages are increased. FLT3L administration is unable to rescue the effect (17), highlighting the intrinsic importance of uncompromised energy metabolism for *in vivo* DC differentiation compared to monocytes. In concert, natural mouse DC progenitors in the bone marrow (**Table 2**; FLT3L-DC cultures) are dependent on nutrient transporters and glucose uptake for proliferation upon FLT3L stimulation *in vitro* (18). Those FLT3L-stimulated bone marrow cultures allow for the separate evaluation of mouse CDP-derived DC subsets [**Table 2**; FLT3L-DCs and (19)]. Notably, the inhibition of fatty acid oxidation (FAO) with etomoxir (**Figure 2**), promoting mitochondrial fusion with M1 or blocking fission with Mdivi-1, does not affect pDCs but strongly skews cDC differentiation toward cDC2s, while reactive oxygen species (ROS) inhibition favors cDC1s (18). Of note, apart from inhibition of carnitine palmitoyltransferase 1 (Cpt1a), a crucial enzyme for long-chain FAO, etomoxir displays off-target effects and can independently block mitochondrial respiration or enhance the ΔΨm in T cells (20). Indeed, cDC1s generally display higher mitochondrial mass and ΔΨm than cDC2s *in vitro* and *in vivo* (18, 21, 22). The non-canonical Hippo pathway kinases mammalian sterile twenty-like (Mst) 1 and 2 are crucial for mitochondrial homeostasis, energy metabolism, and immunogenic function of cDC1s, but less for cDC2s, and are activated by FLT3L in cDC1s (21). In line, *in vivo* FLT3L administration to CD11c-Cre Mst1/2$^{flox/flox}$ mice yields reduced splenic cDC1 numbers compared to controls. Unexpectedly, CD11c-Cre Mst1/2$^{flox/flox}$ mice exhibit elevated frequencies of splenic cDC1s, unaltered pDCs, and reduced cDC2s in the steady state (21); hence, the precise role of (non-canonical) Hippo signaling in DC development needs further investigation.

Overall, these data highlight differential energy requirements for DC subset generation, where moDCs and spleen cDC1s appear more dependent on functional mitochondrial metabolism and OXPHOS than cDC2s or pDCs (**Tables 1**, **2**).

Nutrient-Sensing Pathways Affecting Dendritic Cell Development

Adaption to extra- and intracellular nutrient sensing via the mTOR network composed of mTORC1 and 2 complexes (**Figure 1**) is central for the development of DCs (23). This notion is supported by the fact that the DC differentiation-inducing factors GM-CSF and FLT3L directly induce mTOR activation (2, 24, 25).

Monocyte-Derived Dendritic Cells and Embryo-Derived Langerhans Cells

The generation and survival of the non-CDP-derived human moDCs and self-maintaining LCs depend on mTORC1 (**Tables 1**, **2**). As mentioned in the previous section, mTOR is constitutively active in cultured human moDCs, and the mTOR inhibitor rapamycin, which affects mTORC1 stronger than mTORC2, abrogates their differentiation, inducing apoptosis,

TABLE 1 | Dendritic cell subsets *in vivo*.

DC subset	Developmental origin	Presence *in vivo*	Main functional specialization	Selected surface markers	Metabolic requirements for development *in vivo* and involved signaling factors	Status iNOS expression
cDC1s	HSC → CDP → pre-cDC; depend on FLT3L	Lymphoid-resident, peripheral tissues, blood	Cross-presentation of exogenous antigens on MHCI. Th1 & CD8+ T cell immunity against intracellular pathogens and tumors	M: CD11c+ MHCII+ CD8α+(resident) CD103+(migratory) CD24+ XCR1+ DNGR1/Clec9A+ CD11b-/low	Reduced upon energy restriction; higher ECAR, OCR, mitochondrial mass & $\Delta\psi$m than cDC2; mTOR (mTORC1 & mTORC2), TSC1, PI3Kγ, AKT, PTEN, AMPK, L-Myc, Mst1/2	No (spleen)
				H: CD11c+ HLA-DR+ BDCA-3/CD141+ XCR1+ DNGR1/Clec9A+ DEC205+ CD1c-	Mst1/2	? (Some blood DCs can express iNOS)
cDC2s			Direct presentation of exogenous antigen on MHCII. Immunogenic CD4+ Th and regulatory T cell activation	M: CD11c+ MHCII+ CD11b+/hi SIRP1α+ CD8α- CD103-	Reduced upon energy restriction; mTOR (mTORC1 & mTORC2), TSC1	No (spleen)
				H: CD11c+ HLA-DR+ CD1c+ SIRP1α+ CD11b+ CD141- inducible CD14+	Not reported	? (Some blood DCs can express iNOS)
DN-DCs		Peripheral tissues, blood, spleen	Not well defined. CD8+ and CD4+ T cell priming upon uptake of cell-associated antigen suggested	M: CD11c+ MHCII+ XCR1- CD103- CD11b- (variation between tissues)	AMPK	Not reported
				H: CD11c+ HLA-DR+ CD141- sometimes CD1c+ CD206+	Not reported	
pDCs	HSC → CDP; depend on FLT3L	Lymphoid-resident, blood, lung (mouse), tonsil (human)	Type I interferon secretion	M: CD11c-low MHCII-low Ly6C+ B220+	mTORC1, TSC1	Not reported
				H: CD11c- HLA-DR-low CD123+ CD303+ CD304+	mTORC1, PI3K, PKB, PTEN (*in vitro*)	No (blood)
LCs	Yolk-sac macrophage, fetal liver and adult blood monocyte. Self-renew.	Epidermis and stratified epithelia, migrate to lymph node	Apoptotic cell clearance, antigen presentation to CD8+ T cells, Th17, regulatory and follicular T helper cells	M: CD11c+ MHCII+ Langerin+ CD11b+/low SIRP1α+ CD24+ EpCAM+ XCR1-	mTORC1/raptor, p14/LAMPTOR2	Yes
				H: CD11c+/low HLA-DR+ Langerin+ CD1a+ E-Cadherin+ EpCAM+	Not reported	
moDCs	Blood monocyte, depend on GM-CSF + M-CSF	Mainly induced upon inflammation in peripheral tissues	Context dependent: CD8+ T cell, Th1, Th2 and Th17-type immunity.	M: CD11c+ MHCII+ CD11b+ Ly6C+ CD64+ DC-SIGN+ F4/80+ CD14+ (depending on tissue)	Not reported	Tip-DCs, some i-moDCs express iNOS
				H: CD11c+ HLA-DR+ CD14+ CD141- often DC-SIGN+ CD16+ CD1c+ SIRP1α+ CD11b+	Not reported	Psoriatic Tip-DC-like cells express iNOS

Of note, iNOS is expressed by rat DCs in the thymus but not in the spleen or pseudo-afferent lymph. $\Delta\psi$m, mitochondrial membrane potential; cDC1s, conventional DC type 1; cDC2s, conventional DC type 2; CDP, common DC progenitor; DN-DCs, conventional double-negative DCs; FLT3L, FMS-like tyrosine kinase 3 ligand; GM-CSF, granulocyte–macrophage colony-stimulating factor; H, human; HSC, hematopoietic stem cell; i-moDCs, inflammatory moDC-like cells; iNOS, inducible nitric oxide synthase; LCs, Langerhans cell; M, mouse; M-CSF, macrophage colony-stimulating factor; MHC, major histocompatibility complex; moDCs, monocyte-derived DCs; pDCs, plasmacytoid DCs; Th, CD4+ T helper cell; Tip-DCs; TNF/iNOS-producing-DC subset that depends on CCR2.

in line with GM-CSF/IL-4 activating mTOR to sustain survival (1, 2). Mice deficient in the mTORC1 component Raptor in CD11c-expressing cells, but not the mTORC2 component Rictor (**Figure 1**), progressively lose epidermal LCs over time (26). In concert, LCs deficient in the Ragulator complex component p14 [a.k.a. lysosomal adaptor and mitogen-activated protein kinase and mTOR activator/regulator 2 (LAMPTOR2)], which display abrogated extracellular signaling-regulated kinase (ERK) and mTOR signaling, are increasingly mature and unable to self-renew due to reduced responsiveness to tumor growth factor (TGF)-β1 (27, 28), which is crucial for LC differentiation and maintenance (29).

Dendritic Cells Generated From Common Dendritic Cell Progenitors

Despite the Ras/PI3K/AKT/mTOR signaling axis (**Figure 1**) being activated by FLT3L (24, 25), the precise role of mTOR signaling is more ambiguous in FLT3L-dependent, CDP-derived DC subsets (**Tables 1**, **2**). There are conflicting observations depending on how mTOR signaling is targeted. A line of evidence

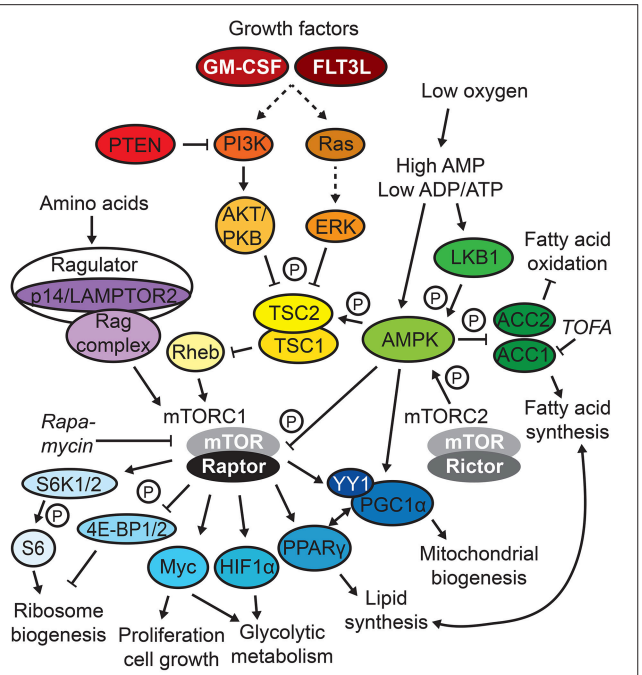

FIGURE 1 | mTOR/AMPK signaling. Selected signaling circuits of the complex mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) signaling network are depicted. Frequently used metabolic inhibitors are displayed in italics, and P indicates phosphorylation.

suggests that active mTOR signaling promotes generation of proper natural DC numbers and subset distribution. *In vitro*, generation of pDCs, cDC1s, and cDC2s in FLT3L-DCs is reduced by rapamycin and enhanced by loss of phosphatase and tensin homolog (PTEN), a negative regulator of PI3K/AKT/mTOR signaling (24) (**Figure 1**). Similarly, rapamycin administration to mice in the steady state decreases CDPs and pre-DCs in the bone marrow as well as total CD11c+ DCs, pDCs, and cDC2s in the spleen (25, 30). cDC1s and, to a lesser extent, cDC2s are profoundly reduced in the spleens and lungs of CD11c-Cre mTOR$^{flox/flox}$ mice, CD11c-Cre Raptor$^{flox/flox}$, Rictor$^{flox/flox}$ double-knockout mice and mice lacking functional PI3Kγ or AKT, upstream activators of mTOR (25, 31). In accordance, cDC1s are strongly expanded in lymphoid and peripheral organs in mice deficient for PTEN (CD11c-Cre PTEN$^{flox/flox}$ mice), a phenotype reversed by rapamycin administration (24). While pDC development is largely unaffected in PI3Kγ-deficient mice (25), human pDC differentiation *in vitro* is blocked by rapamycin, PI3K, and AKT/PKB inhibitors and facilitated by PTEN inhibition or enforced AKT activation (32).

In contrast, other reports suggest an inhibitory function of mTOR signaling for natural DC development. FLT3L-DCs show induction of AMP-activated protein kinase (AMPK) signaling, which antagonizes mTORC1 (**Figure 1**) (18, 33). AMPKα1 deficiency does not affect pDC or overall cDC differentiation but results in relative loss of cDC1s and DN-DCs (18, 33). Moreover, mTOR inhibition by rapamycin increases spleen cDC1 and cDC2 subsets and several DC subsets in peripheral organs upon FLT3L-mediated DC expansion *in vivo* (25). Loss of mTORC1 in DCs in CD11c-Cre Raptor$^{flox/flox}$ mice also expands CD11c+ DCs in the bone marrow, cDC1s in the spleen, and cDC2s in the small intestine (26, 34). Similarly, tuberous sclerosis 1 (TSC1) deficiency (**Figure 1**), using tamoxifen-inducible Rosa-Cre TSC1$^{flox/flox}$ mice, enhances mTOR activation and reduces pDCs, cDC1s, and cDC2s generated in FLT3L-DCs and *in vivo*, which is rescued by rapamycin (35). Conversely, CD11c-Cre TSC1$^{flox/flox}$ mice show no major alterations in DC development (24, 36). In humans, rapamycin treatment of kidney transplant patients does not affect cDC/pDC differentiation, while DCs appear more immunogenic (2).

In conclusion, a delicate balance of the complex system of nutrient sensing and mTOR (mTORC1) signaling is crucial to ensure appropriate development of DCs (23). Strikingly, loss of both mTOR complexes results in opposite effects on *in vivo* DC development compared with loss of mTORC1 alone, probably indicating differential inhibition of mTOR downstream targets and collaboration of mTOR complexes. Indeed, DC loss upon TSC1 deficiency is accompanied by increased DC apoptosis and enhanced metabolic activity due to TSC1-dependent inhibition of Myc, an effector downstream of mTOR (**Figure 1**), and reversed upon Myc loss (35). Of note, Myc itself regulates glucose and glutamine catabolism in activated T cells (37). Moreover, apart from controlling mTORC1 activity, AMPK is an important regulator of fatty acid metabolism limiting ACC1/2 activity (**Figure 1**), which is crucial for T cell activation (38). AMPK loss generally favors cytosolic FAS over mitochondrial FAO, which likely accounts for the decrease in differentiation in AMPKα1-deficient cDC1s, as this process was shown to be sensitive to FAO block (18) and, hence, could be independent from mTOR signaling.

Moreover, the context dependence of balanced mTOR signaling in DCs may be strongly influenced by FLT3L. First, rapamycin and Mst1/2 deficiency have different or even opposing effects on DC generation in the steady state compared with FLT3L-mediated DC expansion *in vivo* (21, 25, 30). Second, while FLT3L-mediated differentiation of DC subsets from mouse bone marrow *in vitro* clearly relies on appropriate mTOR activity (18, 24, 33), GM-CSF-induced DC development *in vitro* was not affected by mTOR deregulation. FLT3L and GM-CSF have both been shown to activate mTOR (2, 24, 25); however, this activation might serve different purposes. Third, deregulated mTOR signaling appears to have stronger effects on the generation of cDC1s than other natural DC subsets, in line with spleen cDC1s being more metabolically active and their reliance on functional mitochondrial respiration (18, 21). The notion that cDC1s appear to rely more on FLT3L than other subsets, especially in peripheral tissues (39), might provide a potential explanation. Indeed, cDC1s in the spleen have higher basal phosphorylation levels of S6 protein, a readout for mTORC1 activity (**Figure 1**), than other DC subsets and upregulate mTOR activation to a greater extent upon FLT3L administration *in vivo*. Moreover, the increase of cDC1s upon PTEN deficiency is specific to the FLT3L-responsive CX3CR1-negative subset (24).

TABLE 2 | Culture systems of dendritic cells.

DC culture	Origin	Culture conditions	Subset composition	Metabolic requirements of development *in vitro* and involved signaling factors	Status iNOS expression
GM-DCs	Mouse bone marrow (progenitors)	GM-CSF (+IL-4), 5-7 days	DC-like and macrophage- like cells	Glucose uptake, oxygen availability, and cytosolic FAS; HIF1α	Yes (inducible)
FLT3L-DCs		FLT3L (+GM-CSF), ca. 9 days	cDC1-like cells	Glucose uptake, FAO and mitochondrial fusion/fission; higher mitochondrial mass & $\Delta\psi$m than cDC2; mTORC1, TSC1, PTEN, AMPK	Not reported
			cDC2-like cells	Glucose uptake & ROS; mTORC1, TSC1, PTEN	
			pDC-like cells	Glucose uptake; mTORC1, TSC1, PTEN	
iCD103-DCs		FLT3L + GM-CSF, ca. 16 days	cDC1-like cells	Not reported	No (NO measured)
moDCs	Human blood monocytes	GM-CSF + IL-4, 6-7 days	moDCs	Cytosolic FAS, mitochondrial biogenesis, active OXPHOS; mTORC1/(PI3K), PPARγ	No (depending on differentiation)

Of note, iNOS is expressed by a mouse skin DC cell line. $\Delta\psi$m, mitochondrial membrane potential; FLT3L-DCs, mouse FLT3L (+GM-CSF)-induced DCs; GM-DCs, mouse GM-CSF-induced DCs; iCD103-DCs, mouse induced CD103+ DCs; iNOS, inducible nitric oxide synthase; moDCs, human GM-CSF+IL-4-induced monocyte-derived DCs.

Last, caution has to be taken when interpreting the effect of manipulating mTOR signaling in DCs. For instance, deletion of the positive mTORC1 regulator p14/LAMPTOR2 in CD11c-expressing cells increases pre-DCs in the bone marrow and amplifies DC subsets in spleen and lymph nodes (LNs) due to accumulation of FLT3 receptor on the DC surface, leading to activation of mTOR (40). Also, while the requirement of mTOR and its signaling components was assessed, the specific mechanisms or the direct role of this nutrient sensor in regulating metabolic pathways such as glycolysis, OXPHOS, or fatty acid metabolism during DC development largely remain to be defined and could account for some of the observed controversies.

METABOLIC REARRANGEMENTS UPON IMMUNOGENIC DENDRITIC CELL STIMULATION

Increasing efforts have been made over the past years to better understand metabolic changes that occur in DCs upon stimulation and how those affect DC functionalities. Resting DCs show a catabolic metabolism and continuously break down nutrients for energy generation and cell maintenance. This metabolic state manifests active OXPHOS, driven by the tricarboxylic acid (TCA) cycle fueled via FAO and glutaminolysis, and is largely regulated by AMPK (13, 41–45), as discussed in the section Metabolic Control of Dendritic Cell Development. Apart from glucose, steady-state DCs use intracellular glycogen to support basal glycolytic demands, which provides metabolic substrates for mitochondrial respiration (46). Upon immunogenic activation, DCs often adopt an anabolic metabolism for the generation of substrates for biosynthesis and cell growth. Activated DCs switch to glycolysis and lactic fermentation that provide energy and additionally reroute glycolytic intermediates into the pentose phosphate pathway (PPP). Moreover, production of nitric oxide (NO), which inhibits the ETC, is induced by some activated DC subsets (**Tables 1**, **2**). The TCA cycle is rewired, leading to accumulation of TCA intermediates that can serve as immunomodulatory signals and support FAS and production of ROS and NO upon DC activation (41–45) (**Figure 3**). Of note, most of the current knowledge on DC metabolism was obtained using DC-like cells differentiated with GM-CSF from mouse bone marrow *in vitro* (**Table 2**; GM-DCs), which also contain a significant proportion of macrophage-like cells (12). This DC culture model provides important insights on the basis of metabolic adaptions of DCs after activation but does not allow investigation of different DC subsets, which appear more and more relevant in light of the differential metabolic requirements for their development.

Increased Glycolytic Activity Determines Inflammatory Dendritic Cell Functions—A Consensus Among Activated DC Subsets?

An early elevation of glycolysis is a metabolic hallmark of activated DCs and occurs in different mouse DC cultures, human moDCs *in vitro*, and mouse/human DC subsets *in vivo*/*ex vivo* (**Figures 3**, **4**) shortly after pattern recognition receptor (PRR) stimulation with a wide range of pure pathogen-associated molecular patterns (PAMPs) or complex stimuli, such as lipopolysaccharides (LPSs) (13, 47–51), CpG oligodeoxynucleotides (13, 49), poly(I:C) (15, 49), R848/Resiquimod (49, 52), protamine–RNA complexes (pRNA) (53), zymosan (50), Pam_3CSK_4/Pam_2CSK_4 (49), *Aspergillus fumigatus* (54), *Chlamydia* (55), heat-killed *Propionibacterium acnes* (13), and influenza A virus or rhinovirus infection (52). Interestingly, stimulants such as LPS and zymosan strongly induce upregulation of costimulatory molecules and cytokines, whereas weak activators such as house dust mite (HDM) or zymosan lacking TLR ligands (ZymD) provoke a milder GM-DC maturation profile (56). Importantly, the potency of stimulants inducing GM-DC activation is directly correlated with enhanced degree and maintenance of glycolysis induction (56).

Requirement of Glycolysis for Functions of Activated Dendritic Cells

Interrupting the glucose-to-pyruvate pathway significantly impairs DC maturation, upregulation of co-stimulatory molecules, cytokine secretion, and T cell stimulatory capacity in

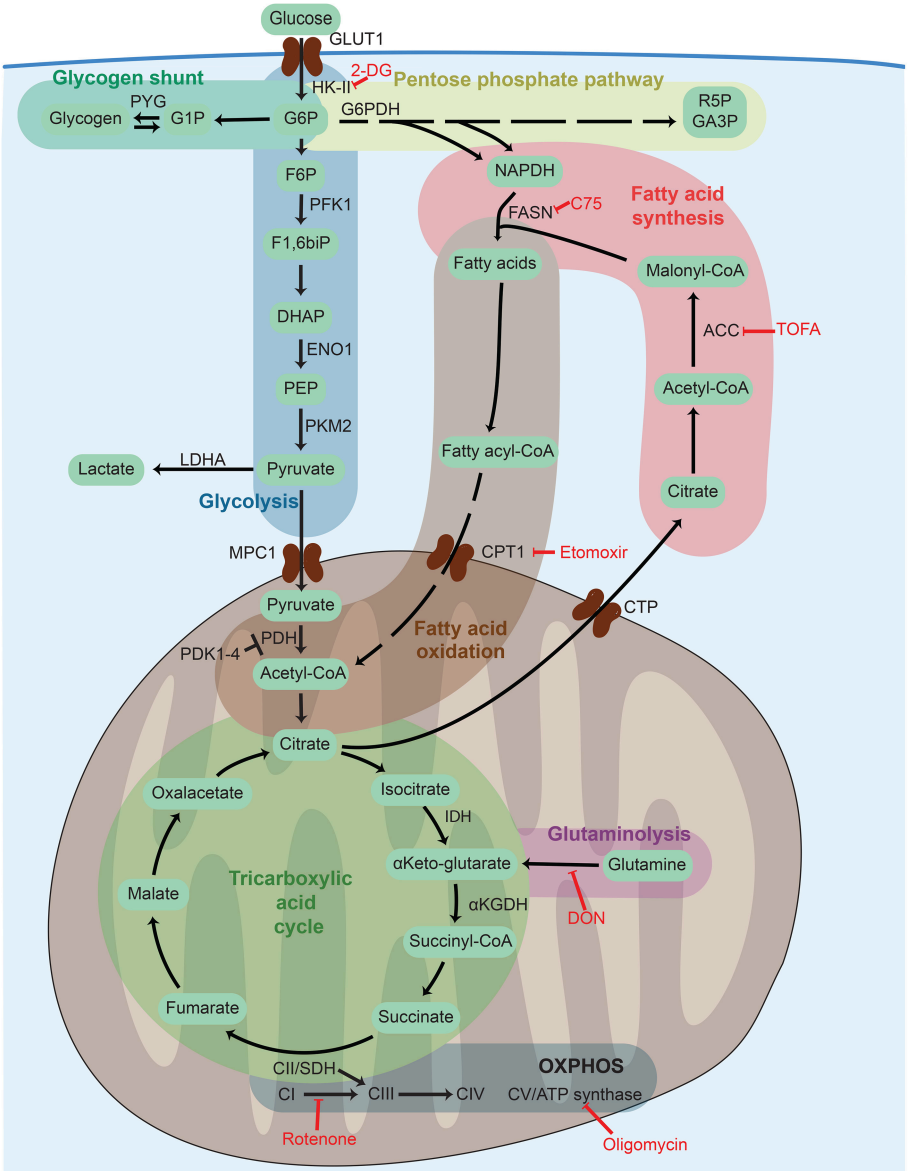

FIGURE 2 | Cellular metabolism networks. Glucose is imported from the extracellular environment and can generate glycogen stores, be used in the pentose phosphate pathway to generate reducing power, or be oxidized during glycolysis to obtain adenosine triphosphate (ATP). Pyruvate generated from glycolysis can either be partially oxidized to lactate to quickly regenerate the consumed nicotinamide adenine dinucleotide (NADH) or translocate into the mitochondria to be completely oxidized thought the tricarboxylic acid (TCA) cycle. The TCA cycle can also be fueled by fatty acids via fatty acid oxidation or glutamine via glutaminolysis. The electrons released by glycolysis and the TCA cycle enter into the electron transport chain composed of complex I–V (CI–CV) where ATP is generated by oxidative phosphorylation (OXPHOS). Frequently used metabolic inhibitors are indicated in red. 2-DG, 2-deoxy-D-glucose; ACC, acetyl-CoA carboxylase; αKGDH, α-ketoglutarate dehydrogenase; CoA, coenzyme A; CPT1, carnitine palmitoyltransferase 1, CTP, citrate transport protein; DHAP, dihydroxyacetone phosphate; DON, 6-Diazo-5-oxo-L-norleucine; ENO1, enolase 1; F1,6biP, fructose 1,6 biphosphate; F5P, fructose 5 phosphate; F6P, fructose 6 phosphate; FASN, fatty acid synthase; G1P, glucose 1 phosphate, G6P, glucose 6 phosphate; G6PDH, glucose 6 phosphate dehydrogenase; GA3P, glyceraldehyde 3 phosphate, GLUT1, glucose transporter 1; HK-II, hexokinase 2; IDH, isocitrate dehydrogenase; LDHA, lactate dehydrogenase A; MPC1, mitochondrial pyruvate carrier 1; NADPH, nicotinamide adenine dinucleotide phosphate; PDH, pyruvate dehydrogenase PDK1-4, pyruvate dehydrogenase kinase 1-4, PEP, phosphoenolpyruvate; PFK1, phosphofructokinase-1; PKM2, pyruvate kinase isozyme M2; PYG, glycogen phosphorylase; R5P, ribose 5-phosphate; SDH, succinate dehydrogenase; TOFA, 5-(Tetradecyloxy)-2-furoic acid.

the long term (**Figure 3**). For example, pharmacological blockade of glycolysis using 2-deoxyglucose (2-DG), genetic deficiency of glycolytic enzymes such as α-enolase (ENO1), or overexpression of lactate dehydrogenase A (LDHA) or pyruvate dehydrogenase kinase 1 (PDK1) (**Figure 2**) prevents GM-DC maturation and immunogenicity upon stimulation with LPS or *Chlamydia* (13, 47, 49, 57) and can skew GM-DCs toward inducing Th17 and regulatory T cells (Treg) rather than Th1 and Th2 responses

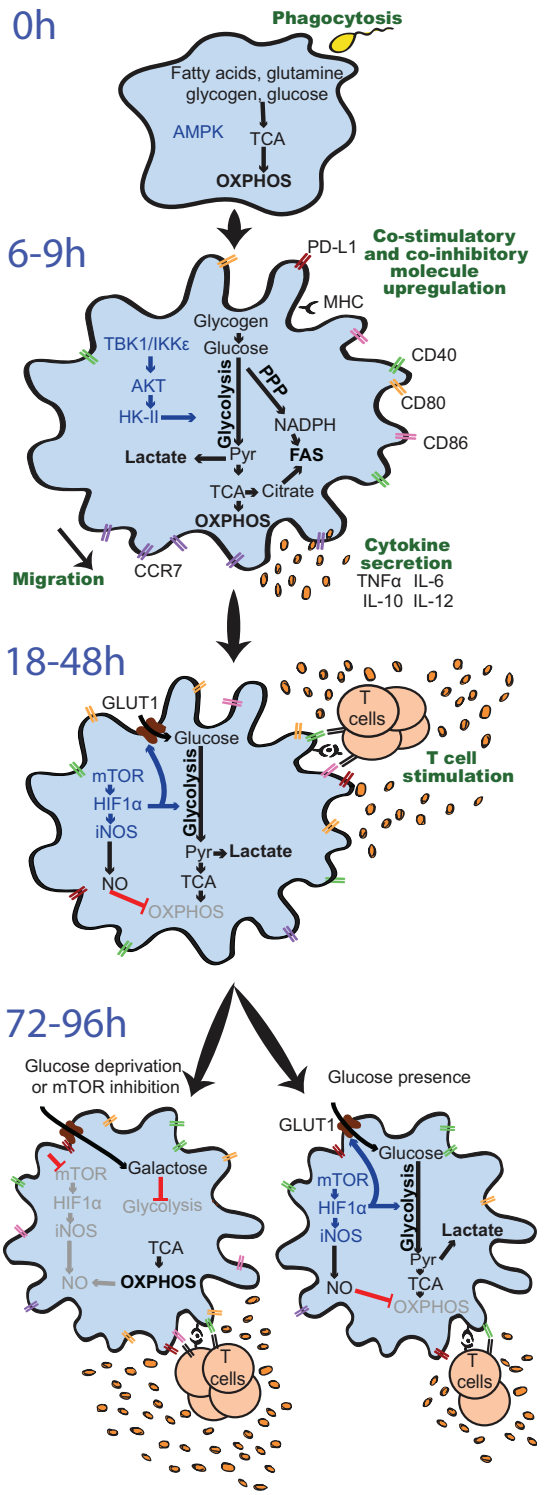

FIGURE 3 | Differential regulation and effects of glycolysis induction in GM-DCs upon stimulation over time. Resting GM-DCs (top) display a basal metabolism with active AMPK (AMP-activated protein kinase) and fatty acids, glutamine, glycogen, and glucose being fully oxidized to generate energy by oxidative phosphorylation (OXPHOS). Upon early stimulation after 6–9 h,

(Continued)

FIGURE 3 | GM-DCs are activated and exhibit transiently enhanced OXPHOS/mitochondrial membrane potential and an increased glycolytic metabolism mainly using glucose from intracellular glycogen stores. The induction of glycolysis is predominantly driven by a TBK1-IKKε/AKT/HK-II axis and largely devoted to fatty acid synthesis (FAS). Moreover, enhanced early glycolytic activity of GM-DCs is vital for their migration and upregulation of co-stimulatory/inhibitory molecules as well as cytokines. At later time points about 18–48 h after robust stimulation, a mTOR/HIF1α/iNOS axis is activated in GM-DCs, leading to enforced glycolysis via upregulation of glucose importers such as GLUT1 and inhibition of OXPHOS via nitric oxide (NO). This fostered glycolytic activity appears crucial for the interaction of GM-DCs with T cells. Nevertheless, the sustained inhibition of OXPHOS by NO and reliance on glycolysis for energy generation can reduce the ability of GM-DCs to stimulate T cells in the long term. Glucose deprivation or mTOR inhibition can preserve metabolic flexibility and functional OXPHOS in GM-DCs, sustaining their activity at least during 72–96 h and extending their life span. AKT, protein kinase B; CCR7, C-C chemokine receptor type 7; CD, cluster of differentiation; GLUT1, glucose transporter 1; GM-DC, GM-CSF, mouse GM-CSF-induced DCs; HIF1α, hypoxia-inducible factor 1-alpha; HK-II, hexokinase II; IKKε, IkB kinase; IL, interleukin; iNOS, inducible nitric oxide synthase; MHC, major histocompatibility complex; mTOR, mammalian target of rapamycin; NADPH, nicotinamide adenine dinucleotide phosphate; PD-L1, programmed death-ligand 1, Pyr, pyruvate; PPP, pentose phosphate pathway; TBK1, TANK-binding kinase 1; TCA, Tricarboxcylic acid cycle; TNFα, tumor necrosis factor α.

(49). In line, natural mouse cDC1s and cDC2s isolated from the spleen decrease expression of co-stimulatory molecules, IL-12 production, and activation of CD4+ and CD8+ T cells when activated by LPS in the presence of 2-DG (49). pRNA-stimulated human blood cDC2s require glycolytic activity for activation, evidenced by TNFα production, CD86, and programmed death ligand 1 (PD-L1) expression (53). Treatment of primary human pDCs with 2-DG upon influenza A virus stimulation also reduces co-stimulatory molecule and type I interferon (IFN-I) expression (52), while another study rather suggests induction of glutamine-fueled OXPHOS upon pRNA stimulation of human blood pDCs (53). However, the effects of inhibition of glycolysis by 2-DG in DCs have to be taken with caution, as 2-DG itself deregulates cytokine expression of human moDCs *in vitro* by activation of the endoplasmic reticulum (ER) stress response via the sensor inositol-requiring protein 1α (IRE1α) (50). In addition, 2-DG can impair the TCA cycle, OXPHOS, and ATP levels, as recently described in macrophages (58).

Other DC functions such as phagocytosis do not seem to be affected by inhibition of glycolysis during stimulation of human moDCs (50). However, reduced endocytic/phagocytic activity in aging mouse spleen cDC1s and DN-DCs [termed merocytic DCs (mcDCs)] and a resulting decline in antigen cross-presentation are linked to mitochondrial dysfunction with decreased basal OCR and $\Delta\Psi m$ as well as enhanced proton leakage and ROS. Importantly, inhibition of ATP synthase by oligomycin or the uncoupling agent carbonyl cyanide 4-(trifluromethoxy)phenyl-hydrazone (FCCP) corroborates the diminished phagocytosis of cDC1s and DN-DCs/mcDCs (22). Moreover, antigen uptake seems to decrease in GM-DCs in hypoxia, when glycolytic activity is increased by HIF1α stabilization, which is also observed in human moDCs after stimulation (47, 50).

In contrast, glucose and enhanced glycolytic activity are required for the ability of DCs to migrate (**Figure 3**).

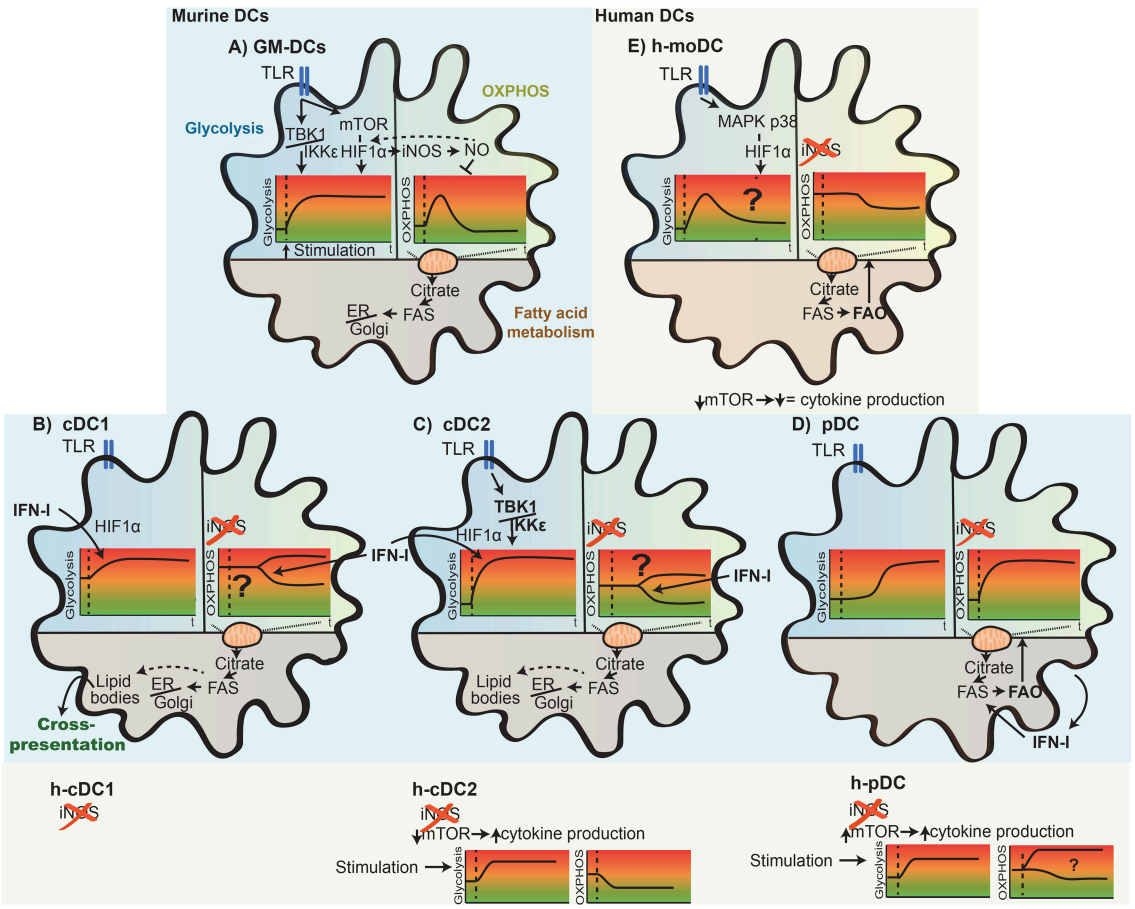

FIGURE 4 | Differential metabolic rearrangement in mouse and human DC subsets upon activation. Depicted here are key adaptions of the main metabolic pathways [glycolysis, OXPHOS (oxidative phosphorylation), and fatty acid metabolism] of DCs upon TLR stimulation. The glycolytic and OXPHOS state of the cells over time (t) is indicated as a schematic representation. In GM-DCs **(A)**, TLR stimulation leads first to induction of glycolysis, and later, mitochondrial OXPHOS is reduced (see also **Figure 3**). Whereas, this increase in glycolysis is consistently observed after stimulation, differences in the basal glycolytic state, promptness of the glycolytic induction, increased rate, and signaling factors driving these changes in distinct DC subsets are illustrated for naturally occurring mouse and human (h-) cDC1s **(B)**, cDC2s **(C)**, pDCs **(D)**, and human *in vitro*-generated moDCs (h-moDCs; **E**). The impact of TLR stimulation on OXPHOS metabolism among DC subsets likely differs due to the lack of iNOS expression in naturally occurring DCs and h-moDCs. In addition, OXPHOS rearrangements of activated cDCs are context dependent and appear to be down-modulated in splenic cDCs in an IFN-I-dependent manner but remain high in cultured FLT3L-cDCs **(B,C)**. An increase in fatty acid synthesis is generally ascribed to most DC subsets upon stimulation; however, differences in fatty acid use emerge, such as fuel of fatty acid oxidation to drive OXPHOS in h-moDCs and pDCs **(D,E)** or for organelle biosynthesis in GM-DCs, cDC1s, and cDC2s **(A–C)**. In line, fatty acids can accumulate within DCs and form lipid bodies that associate with enhanced cross-presentation potential of cDC1s. Lastly, the thus far reported role of TBK1/IKKε and mTOR/HIF1α regulating cDC, pDC, and h-moDC metabolism and function upon activation is displayed.

Independently of stimulation, glucose-deprived GM-DCs show reduced mobility, increased rounded morphology losing dendrites, and impaired oligomerization of CCR7, the chemokine receptor driving DC migration toward LNs. Subsequently, glucose limitation or 2-DG presence prevents migration of GM-DCs as well as splenic CD11c+ cDCs both *in vitro* and *in vivo* (49, 56). In line, HIF1α-deficient GM-DCs, which largely fail to induce glycolysis (see the section Sustained Glycolysis: The Role of HIF1α), display reduced CCR7 levels, and GM-DCs differentiated in hypoxic conditions exhibit elevated migratory potential *in vitro* and *in vivo* that is dependent on HIF1α (14).

Overall, early induction of glycolysis emerges as a general feature of immunogenic activation of most cultured DCs and primary DC subsets and appears necessary for several aspects of their maturation such as upregulation of co-stimulatory surface molecules and cytokine production, despite having no major effects on phagocytosis or antigen uptake. However, DC activation leads to cytoskeletal changes that support increased migratory capacity to migrate toward LNs and T cell zones, which is also affected by early induced glycolysis. Ultimately, in light of those findings, glycolytic increase in DCs upon stimulation is vital for adequate induction of adaptive T cell responses (59) and, hence, regulates immune homeostasis (**Figure 3**).

Mechanisms That Control Glycolytic Reprogramming in Activated Dendritic Cells

Fuels for Glycolytic Induction Upon Dendritic Cell Stimulation

Extracellular glucose consumption by DCs is required for some aspects of induction of glycolysis, functionality, and survival in activated DCs (13, 56). However, glucose uptake and its effects on DC activation emerge to be time and DC subset dependent. Expression of glycolytic enzymes is not increased in GM-DCs at 4 or 8 h after LPS, HDM, curdlan, or zymosan stimulation (56, 60), when cells already display an enhanced glycolytic activity (56), but is only detectable 18–24 h after stimulation. Moreover, switching GM-DCs from a glucose-containing to a galactose-containing medium, which only supports a low glycolytic rate, 8 h after LPS stimulation actually enhances co-stimulatory molecule expression, IL-12 production, and their potential to activate CD8+ T cells, which is ascribed to deregulation of the mTORC1/HIF1α network (60) (**Figure 3**). Indeed, increased glycolysis may be preferentially supported by glycogenolysis of intracellular glycogen reserves during the first 6 h post-stimulation of DCs, rather than extracellular glucose (46). GM-DCs activated with LPS or IL-4 during differentiation accumulate intracellular glycogen, which correlates with their enhanced T cell stimulation potential (61). At later stages after GM-DC stimulation, extracellular glucose uptake is enhanced via the upregulation of glucose transporters such as the glucose transporter 1 (GLUT1) (13, 46, 56), and GLUT1 inhibition 24 h after LPS stimulation reduces CD40 and CD86 expression (46). Of note, expression levels of GLUT1 might be a suboptimal readout for its induction or activity. In fact, GLUT1 is translocated from intracellular vesicles to the cell membrane for glucose uptake upon LPS stimulation in macrophages, which does not entirely correlate with mRNA expression levels (62). Moreover, a significant amount of glucose imported from the extracellular environment by activated DCs still appears to be metabolized to glycogen first before entering glycolysis (glycogen shunt; **Figure 2**) (46). Additionally, upon 6 h pRNA stimulation of primary human blood cDC2s, glycolytic metabolism appears to rely on BCL2 interacting protein 3 (BNIP3)-dependent mitophagy, despite reported 2-DG-sensitive glucose uptake and ENO2 upregulation (53).

Early Glycolytic Induction: The TBK1/IKKε/AKT/HK-II Axis

Glycolytic reprogramming upon activation of DCs appears to be largely driven by TANK-binding kinase-1 (TBK1)/IκB kinase-ε (IKKε)/AKT/hexokinase (HK)-II activation in the short term and regulated by AMPK loss and induction of mTOR and/or HIF1α in the long term (**Figure 3**). TBK1 and IKKε, both non-canonical IκB kinase homologs downstream of TLRs, are activated in GM-DCs within minutes after LPS stimulation, leading to PI3K-independent AKT phosphorylation and association of the rate-limiting glycolytic enzyme HK-II with mitochondria. These events promote glycolytic flux and support early induction of glycolysis in LPS-stimulated GM-DCs as well as in primary mouse spleen cDC2s *ex vivo* (49) (**Figure 4**). Indeed, early induction of TBK1, AKT, and mTORC1 occurs upon stimulation with potent and weak stimuli, correlating with early increase in glycolytic activity (56). LPS-stimulated human moDCs *in vitro* also enhance HK-II expression and activity in concert with enhanced glycolysis and cytokine production in the long term; however, HK-II induction and glycolysis in this setting appear to rely on HIF1α activity mediated by p38/mitogen-activated protein kinase (MAPK; **Figure 4**). Nevertheless, this p38/MAPK/HIF1α axis does not seem to be involved in enhanced glycolysis by human moDCs after TLR2/6-mediated activation but relies on TBK1 (51). Notably, HK-II itself can act as a PRR and cause inflammasome activation (63).

Glycolytic Reprogramming: AMPK vs. the PI3K/AKT/mTOR Pathway

Loss of AMPK and induction of the PI3K/AKT/mTOR pathway (**Figure 1**) at longer time points after LPS stimulation of GM-DCs (18–24 h) ultimately lead to upregulation of glycolytic enzymes such as LDHA, pyruvate kinase 2 (PKM2), or phosphofructokinase (PFK), as well as glucose transporters like GLUT1 (13, 56), which depend on glucose availability (60) (**Figure 3**). Mechanistically, inactivation of AMPK occurs upon LPS stimulation, alleviating mTORC1 inhibition (13, 60). In line, activation of AKT, mTORC1, and mTORC2 declines 18 h after weak stimulation of GM-DCs hand in hand with loss of increased glycolysis activity (56). Enforced AMPK activation or inhibition/loss can prevent or foster GM-DC maturation, respectively (13, 33), associating active AMPK with diminishing proinflammatory DC functions (59). Human pRNA-activated cDC2s downregulate AMPKα1 levels, which appears to be dependent on mitophagy in this system (53). A reduction in glycolysis and activation of GM-DCs upon early inhibition of glycogenolysis also associate with a rapid drop in intracellular ATP and AMPK activation (46). Inhibition of mTOR/mTORC1 blunts glucose consumption, lactate production, upregulation of glycolytic enzymes/glucose transporters, and increased extracellular acidification rate (ECAR) in GM-DCs 20 h or longer after LPS stimulation (60, 64). Hence, mTOR activation appears to control DC activation, especially maintaining it for prolonged periods of time (43) (**Figure 3**). Indeed, ectopic AKT/PKB activation, which sustains mTOR activation, enhances co-stimulatory molecule expression and cytokine secretion in human pDCs (32). Also, mTOR signaling is essential for induction of IFN-I responses of (primary) mouse and human pDCs (65). In concert, rapamycin treatment of anti-CD40-stimulated GM-DCs *in vitro* or IL-4-treated spleen CD11c+ DCs *in vivo* downregulates co-stimulatory molecules/cytokines and promotes activation of Tregs, but not allogeneic CD4+ T cells (30, 66).

Nevertheless, sustained mTOR signaling may also be detrimental for proinflammatory DC functions (23, 41, 59, 67). For example, knockdown or pharmacological inhibition of mTOR enhances life span, prolongs the expression of co-stimulatory molecules, cytokine production, and promotes T cell stimulatory activity of LPS-stimulated GM-DCs (64, 68, 69) (**Figure 3**). Indeed, mTOR promotes NO production by activated

GM-DCs, which limits their mitochondrial energy metabolism, while mTOR inhibition restores the metabolic flexibility of those cells in the long term (68) (**Figures 3**, **4**). However, loss of the negative mTORC1 regulator TSC1 in mouse DCs causes impaired cytokine production and antigen presentation upon TLR4 stimulation (36). mTORC1 inhibition in human CD1c+ cDC2s enhances proinflammatory cytokine production upon stimulation with various agents but has the opposite effect on LPS-stimulated human moDCs. Those contrasting effects are ascribed to differential activation of NFκB upon mTORC1 blockade, which increases in LPS-stimulated CD1c+ cDC2s but remains unchanged in moDCs (2). A spatiotemporal model to integrate the ambiguous roles of mTOR regulating DC functions has been proposed (23).

Sustained Glycolysis: The Role of HIF1α

HIF1α stabilization is also involved in enhanced glycolytic activity of GM-DCs and human moDCs upon stimulation *in vitro* and of natural mouse cDCs *in vivo* (15, 47, 51) (**Figures 3**, **4**). Many glycolytic genes are HIF1α targets and are downregulated in DCs upon HIF1α loss, such as GLUT1 and LDHA (47, 50, 54, 60). Moreover, only potent GM-DC stimulation that leads to long-term induction of glycolysis causes HIF1α stabilization and induction of its target genes, while weak activation fails to do so (56). In line, GM-DCs in the steady state express higher MHCII and co-stimulatory molecule levels in hypoxic conditions (14). LPS stimulation of GM-DCs in hypoxia compared with normoxia further elevates HIF1α activation, glucose consumption, glycolytic enzyme expression, and lactate and ATP production, enhancing GM-DC activation (47). Similar effects are also observed upon *in vitro Aspergillus fumigatus* stimulation of human moDCs in hypoxia *in vitro* (54). Inhibition or loss of HIF1α in GM-DCs or human moDCs prevents the increase in glycolytic rate and upregulation of glycolytic genes upon LPS or *Aspergillus fumigatus* stimulation and reduces co-stimulatory molecule expression, proinflammatory cytokine production (including IL-12), and CD4+ T cell stimulatory capacity in the long term (47, 51, 54). However, LPS-stimulated HIF1α-deficient GM-DCs show enhanced IL-12 expression and CD8+ T cell activation (60). Hence, further efforts will be necessary to clarify the exact role of HIF1α on DC functions. Nevertheless, spleen CD11c+ MHCII+ cDCs of mice lacking HIF1α in CD11c-expressing cells also fail to induce higher glycolysis and display reduced immunogenicity 14–18 h after poly(I:C) stimulation. However, some of those effects might be ascribed to elevated death of HIF1α-deficient spleen cDCs (15). Importantly, HIF1α can be induced or stabilized by many other mechanisms apart from mTORC1 or hypoxia, such as glucose withdrawal (60), which might differentially influence the effects on immunogenic DC activation. Moreover, HIF1α can be activated by intracellular pyruvate or lactate produced by glycolysis (70, 71). Indeed, the timing of HIF1α stabilization occurring in human moDCs 4 h after LPS or zymosan stimulation trails the immediate increase in glycolysis (50). Notably, weakly stimulated GM-DCs do not accumulate HIF1α while still inducing early glycolysis, in contrast to strongly activated GM-DCs that stabilize HIF1α and maintain high glycolytic activity at later stages (56). Taken together, HIF1α is implicated in the maintenance rather than in the early induction of glycolysis after DC stimulation (50) (**Figures 3**, **4**) and appears to partially depend on glucose availability (60).

Extracellular Cues Influencing Glycolytic Metabolism of Activated Dendritic Cells

Signals in the microenvironment can strongly influence DC function via modulating their glucose metabolism. For example, the anti-inflammatory cytokine IL-10 inhibits the LPS-mediated increase in glycolysis and GM-DC maturation likely via maintaining active AMPK (13), and IL-10-deficient GM-DCs display higher levels of the glycolytic enzyme ENO1 (57). Similarly, IL-10 loss in macrophages causes enhanced glycolytic reprogramming upon LPS stimulation, which is ascribed to mTORC1 inhibition by autocrine IL-10 via signal transducer and activator of transcription 3 (STAT3) and DNA damage inducible transcript 4 (DDIT4). Notably, they also accumulate dysfunctional mitochondria due to reduced autophagy independent of NO (62).

Metabolic reprogramming of mouse spleen cDCs may rely on type I IFNs in concert with PRR signaling, as IFNα/β receptor (IFNAR)-deficient cDCs fail to elevate glycolytic activity after poly(I:C) stimulation *in vivo* while maintaining active OXPHOS (15), and mouse pDCs from FLT3L-DC cultures increase their OCR and ECAR upon 24 h exposure to IFNα (72) (**Figure 4**). However, IFNα treatment or IFNAR inhibition in primary human blood pDCs *ex vivo* does not affect induction of glycolysis after stimulation with influenza A virus (52).

Last, exogenous metabolites such as fatty acids or lactate are sensed by DCs, leading to an adaption of their metabolism and functions [reviewed in Pearce and Everts (41)], such as lactate-mediated effects on HIF1α (70, 71, 73). For example, the short-chain fatty acid butyrate can prevent maturation and glycolytic reprogramming of human moDCs upon LPS stimulation, driving them to induce Tregs (74).

Fatty Acid Synthesis and ER Stress During Dendritic Cell Activation

Generation of TCA cycle intermediates regulates function and *de novo* FAS upon DC stimulation. Indeed, while glycolysis-derived ATP appears to be dispensable for early GM-DC activation, incorporation of pyruvate into the mitochondrial TCA cycle is essential, as knockdown of the mitochondrial pyruvate carrier MPC-1 (**Figure 2**) limits GM-DC maturation and cytokine production (49). Accumulation of TCA intermediates such as citrate, succinate, and fumarate in stimulated DCs contributes to the regulation of inflammatory responses as well as cytokine production (45, 75). Additionally, citrate escaping the mitochondria serves as an important substrate for protein acetylation, nicotinamide adenine dinucleotide phosphate (NADPH) production, and, importantly, cytosolic FAS in activated DCs (49, 75) (**Figures 2**, **3**). In addition, knockdown of the PPP enzyme glucose-6-phosphate dehydrogenase (G6PDH) reduces LPS-induced maturation of GM-DCs (49). The PPP produces ribose 5-phosphate (R5P), a precursor for biosynthesis

of nucleotides, and NADPH, which is needed for production of ROS and NO as well as for cytosolic FAS (**Figure 2**).

De novo FAS and accumulation of phospholipids increase upon GM-DC stimulation with LPS (49) and after activation of *in vitro* bone marrow-derived cDC1-like cells (iCD103; **Table 2**) with LPS, CpG, and *Mycobacterium bovis* Bacille Calmette Guerin (BCG) (76) (**Figure 4**). Indeed, accumulation of intracellular fat in LPS- or IL-4-stimulated GM-DCs correlates with enhanced T cell activation capacity (61). FAS also leads to increased lipid storage in lipid bodies (LBs) in GM-DCs (49), organelles composed of a core of neutral lipids such as cholesteryl esters or triglycerides (TAG) surrounded by a single layer of phospholipids (77). Notably, intracellular LB formation associates with induction of cross-presentation potential in GM-DCs, FLT3L-DCs, and mouse spleen cDCs that is at least partially dependent on inflammasome activation or IFNγ-induced protein immunity-related GTPase family member m3 (Irgm3) (78, 79). Accordingly, the specialized cross-presenting CD8+ cDC1 subset (**Table 1**) in the spleen harbors more LBs than CD8– cDCs (79). Human and mouse liver DCs with high lipid content are more potent activators of NK, CD4+, and CD8+ T cells, which is reduced by inhibition of FAS (80). In line, FAS blockade in GM-DCs by knockdown of the mitochondria–cytosol citrate shuttle citrate transport protein (CTP) or by the FASN or ACC inhibitors C75 and TOFA (**Figure 2**) prevents LPS-induced activation and proinflammatory functions of GM-DCs (49). However, non-activated GM-DCs or human moDCs differentiated in the presence of TOFA show high levels of ER stress, ERK and AKT signaling, and PPARγ expression, linked to enhanced DC immunogenicity and T cell priming (7). In the iCD103 culture system that rather represents cDC1-like DCs (**Table 2**), deficiency in ACC1 or 2 or their inhibition by TOFA does not affect co-stimulatory surface marker expression and their inflammatory cytokine profile upon CpG or *Mycobacterium bovis* BCG stimulation. T cell priming capacity or *in vivo* mycobacterial control of iCD103 DCs also remains unaffected by interference with FAS (76). Of note, FAS impairment in iCD103s also results in enhanced uptake of extracellular fatty acids, which might represent a compensatory mechanism for fatty acid generation. Nevertheless, the actual role and subsequent usage of fatty acids produced by DCs appear to be dependent on the context and DC subsets (**Figure 4**). For example, *de novo* synthesized fatty acids provide building blocks for expansion of the Golgi apparatus and the ER in LPS-stimulated GM-DCs and are ultimately required for activated DCs to produce and secrete large amounts of cytokines, which can lead to ER stress and the unfolded protein response (41, 49). Liver DCs containing high amounts of lipids have an increased ER stress, and its blockade reduces their ability to induce immune responses (80). Indeed, ER stress can enhance IL-23 production in zymosan-stimulated human moDCs via IRE1α and X-box binding protein 1 (XBP1) (50). In contrast, in mouse pDCs sorted from FLT3L-DC cultures, an increase in ECAR late after CpG or IFNα stimulation associates with enhanced FAS, which, in this setting, serves as a source of fatty acids for FAO to maintain high OXPHOS levels (72) (**Figure 4**).

Overall, regulation of ER stress and lipid metabolism in activated DCs can notably influence their function to release cytokines and to present antigen (41, 81), and further efforts will be needed to understand the precise functions in different settings. In that regard, the importance of *de novo* FAS and lipid accumulation in tolerogenic or dysfunctional DCs in cancer is discussed in the section Lipid Accumulation and Dendritic Cell Dysfunction in Cancer.

Mitochondrial Energy Generation Regulating Dendritic Cell Activation—Specific to Dendritic Cell Subsets and the Context

Mouse GM-CSF Dendritic Cell Cultures

Development of natural DCs largely relies on FAO to fuel OXPHOS (see the section Metabolic Control of Dendritic Cell Development). However, in cultured GM-DCs, mitochondrial energy metabolism is dramatically reduced upon immunogenic stimulation in the long term (13) (**Figures 3**, **4**). Indeed, the FAO inhibitor etomoxir, the glutaminolysis inhibitor 6-diazo-5-oxo-L-norleucine (DON), or glutamine deprivation has no effect on GM-DC maturation upon LPS stimulation (46, 49). Furthermore, GM-DCs display irresponsiveness to ETC inhibitors and exhibit decreased OCR and $\Delta\Psi m$ 18 or 24 h post-LPS stimulation, which is independent of PI3K/AKT signaling (13, 48). The production of NO via the enzyme inducible NO synthase (iNOS) is central to the collapse of OXPHOS of activated GM-DCs in the long term and their functions (48) and was recently reviewed (82). In brief, NO is induced in GM-DCs within 6 h after LPS stimulation, and their enhanced glycolytic rate becomes NO dependent about 9 h after stimulation, when OXPHOS declines (49). Stabilized HIF1α enhances NO generation by increasing the expression of iNOS, which, in turn, leads to the inhibition of prolyl hydroxilases (PHDs) that label HIF1α for degradation. This positive loop causes NO accumulation, which leads to nitrosilation of some ETC complexes and inhibits their functionality (48, 60, 82) (**Figures 3**, **4**). A small proportion of mouse moDCs induced by *Listeria monocytogenes* infection also display a comparable NO-mediated inhibition of OCR late after stimulation that is compensated by enhanced glycolysis (48). Based on those and other studies in tolerogenic DCs (see the section DC Metabolism in Tolerance), anabolic metabolism, and glycolysis are generally associated with immunogenicity of DCs, while catabolic metabolism and active mitochondrial respiration, regulated via AMPK/PGC1α, are related to tolerogenicity of DCs (41, 43, 83).

However, several pieces of evidence point toward a potential role of mitochondrial energy metabolism and functional OXPHOS in immunogenic, activated DCs. Indeed, $\Delta\Psi m$ and OCR are actually increased in GM-DCs in the short term up to 6 h after LPS stimulation before iNOS becomes expressed (**Figure 4**), which is prevented by 2-DG (48, 49), and weak stimuli like HDM or ZymD do not reduce mitochondrial respiration 18 h post-activation (56). Moreover, decreased mitochondrial abundance is usually not associated with NO-mediated OXPHOS inhibition upon GM-DC activation (13), and 24 h LPS-activated GM-DCs or mouse moDCs fully restore their mitochondrial respiratory profile when NO production is diminished (48). Also,

ENO1 loss causes a profound dysregulation of mitochondrial morphology in short-term (2 h) *Chlamydia*-stimulated GM-DCs associated with a drop of intracellular pyruvate levels and enhanced cell death (57). Additionally, antiviral responses of DCs promoted by cytoplasmic RNA sensor RIG-I-like receptor (RLR) signaling depend on the mitochondrial localization of the antiviral signaling protein (MAVS), which requires active $\Delta\Psi m$ (41).

These observations suggest that mitochondrial energy generation contributes to DC activation in certain settings. Indeed, deficiency or inhibition of iNOS in LPS-activated GM-DCs maintains active OXPHOS and even enhances aspects of DC activation, such as CD8+ T cell stimulation and CD86 and MHC molecule expression in the long term (48) (**Figure 3**). The presence of the mTORC1 inhibitor rapamycin attenuates NO production and ameliorates the decrease in mitochondrial-dependent OCR in activated GM-DCs (60, 68). The maintenance of functional OXPHOS permits the cells to use FAO and glutaminolysis for energy generation (68). Also, the culture of LPS-activated GM-DCs in galactose enhances OCR, while ECAR levels plummet (60). Indeed, in the long term, rapamycin-treated or galactose-cultured activated GM-DCs display a prolonged life span together with extended co-stimulatory molecule and IL-12 expression that leads to more potent activation of CD8+ T cells, which is at least partially dependent on suppression of HIF1α/iNOS signaling (60, 64, 68) (**Figure 3**).

Natural Mouse and Human Dendritic Cell Subsets

Crucially, contrary to cultured GM-DCs, most DC subsets present in lymphoid organs do not express detectable levels of iNOS, foremost naturally occurring cDC1s and cDC2s, as well as cultured human moDCs (82) (**Tables 1**, **2** and **Figure 4**). In line, mitochondrial energy metabolism and OXPHOS remain intact in *in vitro* LPS- or zymosan-stimulated human moDCs (50). Also, splenic mouse cDC1 and cDC2 increase their ECAR shortly after *in vivo* LPS stimulation (49); however, notably, they do not display any differences in the ECAR/OCR ratio 24 h after *ex vivo* LPS stimulation (48) (**Figure 4**). Uptake of dead cell material and cross-presentation potential of unstimulated natural mouse spleen cDC1s and DN-DCs/mcDCs (**Table 1**) are diminished upon abrogated mitochondrial function caused by aging or ETC inhibition (22). Conversely, 14 h *in vivo* poly(I:C) stimulation reduces $\Delta\Psi m$ and OCR of total spleen cDCs, which is prevented by IFNAR deletion (15) (**Figure 4**), suggesting an additional context-mediated mechanism. In the same study, maintenance of mitochondrial energy metabolism and reduction in ECAR upon poly(I:C) stimulation by HIF1α loss in spleen cDCs reduce their T cell activation potential. However, this effect is ascribed to unbalancing cellular metabolism leading to enhanced ROS production, lower ATP levels, and increased cell death (15). Nevertheless, in 6 h-stimulated human blood cDC2s, mitochondrial morphology and dynamics are altered, the OCR is strongly reduced, and BNIP3-dependent mitophagy is triggered, which appears necessary for glycolytic activity and activation (53).

Notably, pDCs appear to show distinctive rewiring of their mitochondrial energy metabolism in different settings (**Figure 4**). While human pDCs mildly decrease their OCR after 24 h *ex vivo* influenza or rhinovirus infection (52), they elevate OXPHOS 6 h post-pRNA stimulation, which appears to be mediated by autophagy-induced glutaminolysis (53). Importantly, the induction of mitochondrial energy metabolism in human pDCs is required for the production of IFNα, CD80, and PD-L1 expression (53). Mouse pDCs sorted from FLT3L-DC cultures enhance mitochondrial pyruvate import and FAO that fuel elevated OXPHOS 24 h post-CpC stimulation. This effect is due to IFN-I induction, with IFNα itself promoting FAO via PPARα (72), in contrast to mouse spleen cDCs where IFNAR deficiency maintains high OCR (15) (**Figure 4**).

Hence, no general conclusion can be reached as to the importance and function of mitochondrial energy metabolism, OCR, and OXPHOS in activated DCs, and it appears context and DC subset dependent (**Figure 4**). Metabolic flexibility of activated DCs to switch their carbon source for ATP generation from glucose to galactose, glutamine or fatty acids would be of benefit in DC function and indeed, prevention of OXPHOS collapse and metabolic plasticity enhance DC survival and activation upon glucose deprivation and mTOR or iNOS inhibition (48, 60, 68). In the future, it will be interesting to determine the influence of the microenvironment in which DCs are activated. Not only nutrient or oxygen availability but also other environmental factors can strongly influence DC metabolism, such as extracellular lactate, fatty acids (41), the TCA intermediates citrate, succinate, and fumarate (45, 75), as well as IL-10 (13, 57) or IFN-I (15, 52, 72), as discussed in the section Extracellular Cues Influencing Glycolytic Metabolism of Activated Dendritic Cells. Moreover, NO produced by neighboring cells can cause HIF1α stabilization and trigger a cellular loop in DCs, leading to a glycolytic switch (60, 68).

Development and maintenance of different DC subsets display differential metabolic requirements (discussed in the section Metabolic Control of Dendritic Cell Development), which will likely reflect on their metabolic reprogramming upon activation. Considering that different DC subsets specialize on distinct functions (**Table 1**), their metabolic requirements to exert those tasks might differ, as suggested in a recent study (53). Moreover, a fine regulation of OXPHOS activity, such as reported in the case of supercomplex assembly in macrophages (84, 85), may also have a functional effect on DCs.

DENDRITIC CELL METABOLISM IN TOLERANCE

DCs contribute to the maintenance of immunological tolerance in order to prevent hyperactivation of the immune system and subsequent autoimmune diseases. Generally, such tolerogenic DCs arise in the steady state during uptake of (self-) antigen in the absence of danger signals, upon sensing of anti-inflammatory cytokines/factors, and during various pathological states, including cancer, due to tolerizing signals (86, 87). Tolerogenic or semimature DCs can be identified by upregulation of regulatory surface molecules or receptors such as PD-L1 and tolerogenic cytokines IL-10, IL-27, and TGFβ,

leading to induction of Treg activation at the expense of effector T cells (83, 86). Much of the functionality of tolerogenic DCs is intertwined with metabolic activity, such as lipid accumulation or catabolism of amino acids [tryptophan (Trp) and arginine (Arg)].

Metabolic State(s) and Their Regulatory Cellular Pathways in Tolerogenic Dendritic Cells

Metabolic Adaptions of Tolerized Dendritic Cells

Our understanding of energy metabolism of tolerogenic DCs is largely based on observations in human moDC cultures treated with vitamin D3 or D2 (VitD3 or VitD2), dexamethasone (DEX), and/or resveratrol (83, 88–91). Resveratrol is a plant-derived polyphenol that induces regulatory properties in mouse and human DCs, preventing their maturation and immunogenic activation (92, 93). Glucocorticoid receptor engagement by DEX modulates many aspects of DC maturation, including antigen presentation and cytokine production, leading to a tolerant phenotype (83, 94, 95). VitD3 skews DC functionality toward an inhibitory phenotype inducing Tregs and enhancing expression of inhibitory receptors (96, 97).

Tolerogenic human moDCs, generated either by treatment with DEX+VitD3 for 48 h or 1,25-dihydroxyvitamin D3 [1,25(OH)2-VitD3, the active form of VitD3] for 24 h, exhibit enhanced catabolism and metabolic plasticity, increased expression of genes involved in OXPHOS, glycolysis/glucose metabolism, and FAO in concert with higher mitochondrial respiration (OCR) and glycolytic activity (ECAR) than untreated moDCs (88, 89). Intriguingly, LPS stimulation of DEX+VitD3-tolerized moDCs slightly decreases their OXPHOS capacity; however, their glycolytic capacity drops to levels of immunogenic LPS-stimulated DCs, which are, in this study, lower than those of untreated moDCs (88). Functionally, while MHCII expression of LPS-stimulated immunogenic moDCs is sensitive to glycolysis inhibition, LPS-stimulated DEX+VitD3-tolerogenic moDCs remain unaffected. DEX+VitD3-tolerogenic moDCs increase their MHCII levels upon inhibition of FAO instead. In line, FAO inhibition rescues the ability of DEX+VitD3-tolerogenic moDCs to induce expression of activation markers on CD4+ T cells upon LPS stimulation (88). Moreover, in the context of melanoma, a Wnt5a/β-catenin and PPARγ pathway induces FAO and a tolerogenic indoleamine 2,3-dioxygenase (IDO)-producing and Treg-activating phenotype in DCs (98). In contrast, in moDCs tolerized by DEX+VitD2, immunogenic stimulation induces even higher glycolysis/cellular LDH activity than in activated moDCs (91). Nevertheless, the maintenance of tolerogenic features of both 1,25(OH)2-VitD3-treated and (re-stimulated) DEX+VitD2-treated moDCs relies on glycolysis, and their tolerogenic phenotype is abrogated by 2-DG treatment (89, 91). Notably, levels of FAO are unaltered in 1,25(OH)2-VitD3-treated vs. control moDCs, and FAO inhibition by etomoxir does not affect their tolerogenic hallmarks (89). Accumulation of pyruvate during glycolysis may partially cause the concomitant increase in OXPHOS in those tolerogenic moDCs in concert with elevated OXPHOS-related gene expression (89). Those results indicate a metabolic plasticity and responsiveness of tolerogenic moDCs, which display a very active metabolism, despite showing differential dependencies on glycolysis vs. FAO/OXPHOS. Those controversies may be due to the different experimental settings, presence or absence of immunogenic stimulation, and the fact that 1,25(OH)2-VitD3 has stronger effects on OXPHOS, lipid, and glucose metabolism of tolerogenic moDCs than DEX (99). Nevertheless, tolerogenic DCs appear to rely less on glycolysis than LPS-activated immunogenic DCs for their functionality and, as they largely upregulate functional OXPHOS, might be able to adapt their metabolism depending on the context. However, those conclusions are solely based on cultured human moDCs, and the metabolism of other tolerized DC subsets in complex *in vivo* settings largely remains to be investigated.

AMPK and mTOR Pathways Influence Tolerogenicity of Dendritic Cells

The tolerogenic status of DCs is also influenced by a balance of the nutrient-sensing pathways AMPK and mTOR, which appear to be equally context dependent as for immunogenic stimulation of DCs. Inflammatory activation of DCs involves enhanced glycolytic activity and anabolic metabolism compared to immature DCs that largely appear to be controlled by mTOR signaling (see the section Mechanisms That Control Glycolytic Reprogramming in Activated Dendritic Cells), and, despite controversial findings (64, 68), mTOR inhibition by rapamycin can cause DC tolerization (30, 66). Accordingly, DEX or resveratrol treatment of macrophages can block iNOS expression and NO generation (100, 101), whose upregulation associates with LPS-activated GM-DCs (82), while VitD3 had varying effects (102). Indeed, an axis involving AMPK, PGC1α, and PPARγ is suggested to control tolerogenicity of DCs, largely by preventing biosynthetic metabolic adaptions or pathways driving immunogenic DC activation such as mTOR (41, 43, 59, 83). This concept is founded on the observations that tolerogenic DCs show enhanced mitochondrial respiration and that AMPK activation favors catabolic metabolism, FAO, and OXPHOS, largely via PPARγ and the mitochondrial biogenesis inducer PGC1α (41, 43, 59, 83). Indeed, DEX+VitD3- and 1,25(OH)2-VitD3-treated human moDCs upregulate AMPK activity and signaling (88, 89), human cDC2s (53) and GM-DCs reduce AMPK activation upon pRNA or LPS exposure, and the inhibitory effect of IL-10 on LPS-mediated maturation of mouse GM-DCs appears to be AMPK dependent (13). Further, AMPKα1-deficient LPS-stimulated GM-DCs show augmented proinflammatory features such as enhanced co-stimulatory molecule expression and CD40 signaling, increased IL-6 and TNFα, but decreased IL-10 production and skewing of CD4+ T cell activation toward a Th1 and Th17 phenotype (33). The AMPK inducer 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) is equally potent in blocking glucose consumption by LPS-stimulated GM-DCs as 2-DG (13) and AMPK activation after uptake of dead cells induces autophagy, tolerogenic properties, and reduced anti-tumor immune responses (103). Intriguingly, several studies also implicate VitD3, resveratrol, and DEX in enhancing AMPK activation in various other settings and cell types (104–110). Moreover, resveratrol treatment promotes

OXPHOS and mitochondrial biosynthesis in mice and humans via mechanisms similar to AMPK, such as activating the histone deacetylase Sirtuin 1 and augmenting PGC1α expression (90, 111). Loss of the PGC1α targets PPARγ or nuclear factor erythroid 2-related factor 2 (NRF2) enhances DC maturity and proimmunogenic functionality (41).

However, the precise role of balanced mTOR/AMPK signaling in tolerogenic DCs remains controversial. Indeed, the PI3K/AKT/mTOR axis is reported to be vital for tolerogenic features of moDCs, independent from AMPK (89, 91). Human restimulated DEX+VitD2-tolerized moDCs strongly upregulate mTOR phosphorylation and signaling compared to non-tolerized controls (91). PI3K or mTOR inhibition (by LY294002 or rapamycin, respectively) enhances MHC and co-stimulatory molecule expression and reduces co-inhibitory molecules as well as the IL-10/IL-12p70 expression ratio by 1,25(OH)2-VitD3-treated and DEX+VitD2-treated moDCs without or after immunogenic activation. Induction of CD4+ and CD8+ T cell proliferation and IFNγ production is also enhanced by mTOR inhibition in both tolerogenic human moDC cultures (89, 91). Importantly, in this setting, AMPK activation by AICAR is ineffective in altering the tolerogenic phenotype of 1,25(OH)2-VitD3-treated moDCs (89). Moreover, the context dependence of cellular metabolism associated with active mTOR signaling is highlighted by a recent study of allergic airway inflammation in mice harboring mTOR-deficient CD11c-expressing cells (31). There, HDM exposure induces the generation of lung CD11c+ MHCII+ CD11b+ DCs that depend on macrophage CSF (M-CSF) and, hence, likely represent moDCs (**Table 1**). Upon loss of mTOR, those induced CD11b+ DCs show enhanced expression of CD80 and CD86 co-stimulatory molecules and skew the HDM-mediated Th2-polarized allergy toward a neutrophilic Th17-mediated lung inflammation. Moreover, mTOR-deficient CD11b+ DCs accumulate fatty acid metabolites, and FAO inhibition by etomoxir diminishes their activated phenotype (31). Those observations suggest anti-inflammatory/tolerizing effects of mTOR associated with inhibition of FAO that, in turn, appears functionally important for an activated state and Th17 polarization capacity of lung CD11b+ inflammatory DCs in allergic airway inflammation.

In summary, research on primary DC subsets in settings of immune tolerance, additional to tolerized DC cultures, will be needed to advance our knowledge on tolerogenic DC metabolism.

Lipid Accumulation and Dendritic Cell Dysfunction in Cancer

The role of lipid metabolism for immunogenic and tolerogenic DC function is ambiguous. Although lipid accumulation in DCs seems to support immunogenic immune responses and cross-presentation (78, 79) (see the section Fatty Acid Synthesis and ER Stress During Dendritic Cell Activation), it also associates with DC dysfunction in tumor settings. Tumor-associated DCs accumulate high amounts of cytosolic lipids in both mice and humans. Lipid-laden DCs isolated from tumor-bearing mice exhibit defective T cell stimulation ability due to altered antigen processing and presentation (112). The aberrant lipid accumulation in DCs is fostered by yet-unknown factors secreted by tumor cells and mediated by macrophage scavenger receptor 1 (Msr1) on DCs (112), a receptor that binds primarily modified lipoproteins (113). Inhibition of Msr1 or blockade of FAS with TOFA restores lipid content and DC immunogenicity, indicating that enhanced lipid uptake, FAS, or a combination impairs DC-mediated antitumor immunity. Interestingly, this effect is observed in cDC1s and cDC2s but not in pDCs (112), which might be a reflection of the different functions and/or metabolic pathway usage among DC subsets *in vivo* (**Table 1** and **Figure 4**). Indeed, CD103+ cDC1s from draining LNs (dLNs) of tumor-bearing mice accumulate more LBs compared to the CD103– DC counterparts, which substantially reduces their ability to cross-present antigens (114). Cross-presentation plays a central role in the generation of efficacious anticancer CD8+ cytotoxic T cell responses (115), and these data provide a metabolic explanation for the impaired ability of tumor-infiltrating DCs to induce potent antitumor adaptive responses.

The differential effect of lipid accumulation in DCs seen in tumor settings may be due to accumulation and/or signaling by modified lipid species. For instance, tumor-derived factors act on DCs activating liver X receptor (LXR)-α signaling, whose natural ligands are oxidized cholesterol (oxysterols), and reduce the expression of CCR7, inhibiting their migration to the dLNs (116). Consistently, LXR-α/LXR-β-deficient GM-DCs show impaired migration in response to the CCR7 ligands CCL19 and CCL21, and this response is partially dependent on the LXR target CD38, a molecule that is linked to leukocyte trafficking (117). Oxidized lipids contained in tumor-associated DCs also affect cross-presentation (118). Accumulation of oxidized polyunsaturated fatty acids, cholesterol esters, and TAG impairs cross-presentation without affecting the presentation of endogenous antigens. Notably, the accumulation of non-oxidized lipids does not alter cross-presentation, supporting the idea that it is not the mere storage of lipids but the accumulation of modified lipids that alters DC function (114, 118). Consistent with these observations, tumor-derived factors trigger lipid peroxidation in tumor-associated DCs, which activates the ER stress response mediated by IRE-1α and its target XBP1. XBP1 activation, in turn, induces a lipid biosynthetic program that results in the accumulation of LBs and blunted antigen presentation, leading to a reduced ability to control tumor growth (119). Regarding the mechanisms by which LBs and modified lipids could impair cross-presentation, oxidatively truncated TAG accumulate on the surface of LBs and bind the heat shock-induced chaperone heat shock protein 70 (HSP70). As a result of this interaction, peptide–MHCI complexes do not traffic to the cell surface and rather accumulate in lysosomal/late endosomal compartments (114), although the mechanism by which HSP70 controls antigen cross-presentation remains to be elucidated.

Taken together, these data illustrate mechanisms by which capabilities of DCs are suppressed in tumors through modification of their lipid metabolism, either by secreted factors or indirectly by an altered tumor microenvironment. Tumor-associated DCs exert their functions in a tissue where glucose is scarce due to the high glycolytic rates of tumor cells (120), and

the inability to adopt a glycolytic metabolism can impair DC effector functions (see the section Metabolic Rearrangements Upon Immunogenic Dendritic Cell Stimulation). Alternatively, tumor-derived factors can enforce FAO and OXPHOS in DCs and promote accumulation of lipids, which can, in turn, inhibit secretion of proinflammatory cytokines and antigen cross-presentation, respectively (98, 112, 119). Nonetheless, it remains unanswered why and how tumor-associated DCs accumulate high amounts of lipids. Some reports indicate that lipid accumulation is due to activation of a lipogenic program (119), while others suggest increased lipid uptake (112). Moreover, tumor cells also secrete other factors to the local milieu that act on tumor-infiltrating DCs and support the acquisition of a tolerogenic phenotype such as adenosine (121) or lactate (70, 71, 73). Thus, the metabolic reprogramming of tumor-associated DCs can contribute to tumor progression.

Amino Acid Metabolism and Tolerizing Dendritic Cell Functions

Catabolism of the essential amino acid Trp is critical in balancing inflammation and tolerance. Trp is metabolized by the enzyme IDO1, generating kynurenine (Kyn) in a process that consumes oxygen (122). This enzyme is highly expressed by tumor cells and exploited as a mechanism for immune evasion (123). IDO1-mediated Trp catabolism promotes local immunosuppression by two means: (1) Trp starvation limits T cell proliferation by impairing the T cell cycle machinery (124, 125), and (2) Kyn products induce T cell apoptosis (126), inhibit T cell cytotoxicity via downregulation of T cell receptor (TCR) CD3 ζ-chain (127), and induce differentiation of Tregs (127, 128). Notably, a subset of tumor-associated pDCs that accumulate in tumor-draining LNs (tdLNs) express IDO and mediate antigen-specific T cell anergy, contributing to tumor progression (129, 130). Cytokines such as IFNγ and TGFβ (131–134) and immunosuppressive drugs such as DEX (131) induce IDO in pDCs. Remarkably, cytotoxic T-lymphocyte-associated protein (CTLA)-4-expressing Tregs bind B7 family receptors on pDCs also triggering IDO1 expression (132, 133). This bidirectional conditioning also happens upon glucocorticoid-inducible TNF receptor-related protein (GITR) ligand (GITRL) engagement by GITR, expressed by Tregs and pDCs, respectively, inducing IDO1 expression via activation of the IKB–IKKα non-canonical NFkB pathway in pDCs in an IFNα-dependent manner (131). This crosstalk would establish a positive feedback loop to favor long-term immunosuppression. DEX induces this tolerogenic pathway by concomitant upregulation of GITR on CD4+ T cells and GITRL on pDCs (131). DEX treatment is a frequently used treatment to tolerize human moDCs *in vitro* (see the section Metabolic Adaptions of Tolerized Dendritic Cells), which often display high FAO and OXPHOS rates (88, 89). Therefore, one could hypothesize that FAO and IDO1 activities collaborate in establishing a tolerogenic program in DCs. Indeed, an oxidative metabolic profile adopted by tolerogenic DCs supports IDO1 function, providing a direct link between FAO and tolerogenic DC responses *in vivo* (98).

Arg is another amino acid that has a central immunomodulatory role. In immune cells, Arg is metabolized by iNOS under inflammatory conditions to generate L-citruline and NO (135), the latter being associated with activated GM-DCs (82). Alternatively, Arg can be metabolized by arginases 1 and 2 (Arg1 and 2) to produce ornithine, a precursor for polyamines that can support tumor cell proliferation (135, 136). Notably, tumor-infiltrating DCs act as Arg sinks, contributing to local Arg depletion and indirectly inhibiting T cell antitumor responses (137). Additionally, Arg1-dependent production of the polyamine spermidine by DCs induces both IDO1 enzymatic and signaling activities, allowing the establishment of a tolerogenic phenotype in response to TGFβ (138). Interestingly, myeloid-derived suppressor cells also release polyamines that condition DCs to express IDO1 and, therefore, amplify the immune suppression exerted through joint modulation of amino acid catabolism in cancer (138).

Enhanced Trp and Arg catabolism causes amino acid depletion in the local microenvironment, which is sensed by T cells via the Ser/Thr kinase general control non-derepressible 2 kinase (GCN2) and results in limited protein synthesis and proliferative arrest (139–141). Intriguingly, GCN2 activation in response to amino acid scarcity improves antigen presentation by human moDCs *in vitro* in response to yellow fever vaccine YF-17D by enhancing autophagy (142). Indeed, human CD8+ T cell responses after YF-17D vaccination correlate with increased expression of GCN2 and autophagy-related genes, and mice deficient for GCN2 or autophagy related-proteins 5 or 7 in the CD11c compartment show impaired antiviral T cell responses (142). Hence, active Trp and Arg amino acid metabolism by DCs influences the microenvironment and T cell responses and is involved in immune suppression.

CONCLUDING REMARKS

DCs are functionally defined by their ability to prime immunity and tolerance, but how their cellular metabolism (**Figure 2**) is affected by sensing of environmental cues and how this metabolic rewiring affects, in turn, DC function is an emerging fascinating field. The diversity of DCs (**Tables 1**, **2**) and the fact that a great body of literature has been generated using DC-like cells from mouse bone marrow cultures with GM-CSF (12) limit our ability to predict what are the regulation and consequences of metabolic rearrangements in natural DCs *in vivo*.

Moreover, the use of inhibitors or genetic deletion of metabolic regulators to interrogate modulation of metabolic pathways is debated. Metabolic inhibitors have the advantage of immediate action on otherwise unaltered DCs and universal application on primary mouse and human DCs *ex vivo*. However, their applicability for DC-specific *in vivo* studies is limited, and they can have off-target effects, such as reported for C75, etomoxir, and 2-DG (16, 20, 58). On the other hand, genetic deletion of metabolic regulators in DCs using Cre-expressing mouse lines or other genetic approaches such as shRNA or CRISPR/Cas9 largely circumvents side effects and allows investigation of DCs with metabolic impairment

in vivo. Nevertheless, genetic deficiency of important metabolic regulators can cause a deregulation of DC development (see the section Metabolic Control of Dendritic Cell Development) that challenges investigation of their functions, and unrelated compensating mechanisms that are difficult to control. While there is probably no consensus on the ideal strategy, studying consequences of manipulation of DC metabolism *in vivo*, rather than *in vitro*, may be of high relevance, as the microenvironment is crucial for cellular metabolism. Additionally, future studies of DC metabolism employing combined approaches of pharmacological inhibition and genetic deficiency will be most convincing.

Nevertheless, some patterns are starting to emerge showing that moDC and cDC1 generation is more dependent on functional mitochondrial metabolism and OXPHOS than cDC2s or pDCs (**Tables 1**, **2**). Early induction of glycolysis characterizes and is required for immunogenic activation of cultured DCs and primary DC subsets, while long-term glycolytic reprogramming is finely regulated and may have suboptimal consequences (**Figure 3**). Indeed, important differences of metabolic/glycolytic adaptions of DCs early or late after stimulation are emerging, such as the different signaling pathways regulating early (49) and, likely, rather late glycolytic reprogramming (50, 56) or the time-dependent substrate use for glycolysis (46). Moreover, while weak and potent stimulants induce early glycolytic activity in GM-DCs, only strong activation achieves maintenance of increased glycolysis for 18 h or longer (56), further supporting the action of different mechanisms. Notably, metabolic flexibility for energy generation of long-term activated GM-DCs (3 days or more) seems to benefit their immunogenic functions (60, 64, 68) (**Figure 3**).

In contrast, tolerogenic DCs appear to generally rely more on OXPHOS than glycolysis, based on cultured human moDCs tolerized with specific stimuli. However, we only understand fragments of the cellular energy metabolism of tolerogenic DCs and the signaling pathways controlling their induction and maintenance of their functions.

Importantly, different DC subsets (**Table 1**) emerge to display pronounced variations in their adaption of mitochondrial energy metabolism upon immunogenic activation (**Figure 4**), reaching from strong induction of OXPHOS in pDCs, context-dependent alterations in cDCs, to a long-term reduction in cultured GM-DCs or human moDCs. Additionally, while enhanced glycolysis and FAS appear as general features of activated DCs, the further application of fatty acids as building blocks for the ER/Golgi or substrate for FAO also largely varies among DC subsets (**Figure 4**). Further efforts in primary DC subsets in different settings will likely contribute to a better understanding of context dependence and regulation of immunogenic and tolerogenic DC subset metabolism, as highlighted for lung inflammatory DCs (31).

Overall, integration of nutrient sensing and adequate adaption of mTOR/AMPK signaling (**Figure 1**) are crucial for metabolic adjustments by DCs. However, the complexity of metabolic reprogramming of DCs (upon stimulation) is highlighted by the fact that the signaling mechanisms involved in inducing glycolytic activity show context dependency and even contradictory effects with regard to regulating DC function. This controversy might be explained by differential routes of activation and additional functions and nutrient-dependent regulations of those important cellular signaling networks in DCs, apart from controlling glycolytic metabolism, that remain to be defined. For example, mTOR signaling is often linked with immunogenic DC activation due to increasing glycolytic and anabolic metabolism (41, 43, 59). However, tolerized moDCs also exhibit increased glycolysis compared to control moDCs in the steady state or after additional stimulation (88, 89), which was, indeed, also dependent on mTOR and reduced by rapamycin (91). Those observations indicate that the general association of a metabolic state, anabolic glycolysis vs. catabolic FAO/mitochondrial respiration (**Figure 2**), and concomitantly pathways controlling metabolic adaption to nutrients, mTOR vs. AMPK activation, cannot be generally ascribed to immunogenic vs. tolerogenic DCs.

Indeed, the influence of the particular immunogenic or tolerogenic context, ontogenic constraints of distinct DC subsets, and additional (environmental) factors on the balance of nutrient-sensing pathways and metabolic adaptions of DCs will have to be carefully assessed in the future.

AUTHOR CONTRIBUTIONS

SKW prepared tables and figures and conceptualized and wrote the manuscript. SCK conceptualized and wrote part of the manuscript. EP and IH-M helped conceptualize the manuscript and prepared the figures. DS contributed to funding acquisition and supervised, conceptualized, and wrote the manuscript. All authors declare no conflict of interest, contributed to manuscript revision, and read and approved the final version.

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Human Dendritic Cell Subsets Undergo Distinct Metabolic Reprogramming for Immune Response

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Toll-like receptor (TLR) agonists induce metabolic reprogramming, which is required for immune activation. We have investigated mechanisms that regulate metabolic adaptation upon TLR-stimulation in human blood DC subsets, CD1c$^+$ myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). We show that TLR-stimulation changes expression of genes regulating oxidative phosphorylation (OXPHOS) and glutamine metabolism in pDC. TLR-stimulation increases mitochondrial content and intracellular glutamine in an autophagy-dependent manner in pDC. TLR-induced glutaminolysis fuels OXPHOS in pDCs. Notably, inhibition of glutaminolysis and OXPHOS prevents pDC activation. Conversely, TLR-stimulation reduces mitochondrial content, OXPHOS activity and induces glycolysis in CD1c$^+$ mDC. Inhibition of mitochondrial fragmentation or promotion of mitochondrial fusion impairs TLR-stimulation induced glycolysis and activation of CD1c$^+$ mDCs. TLR-stimulation triggers BNIP3-dependent mitophagy, which regulates transcriptional activity of *AMPKα1*. BNIP3-dependent mitophagy is required for induction of glycolysis and activation of CD1c$^+$ mDCs. Our findings reveal that TLR stimulation differentially regulates mitochondrial dynamics in distinct human DC subsets, which contributes to their activation.

Keywords: CD1c$^+$ mDC, pDC, glutaminolysis, mitophagy, mitochondrial dynamics, OXPHOS, glycolysis

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INTRODUCTION

Dendritic cells (DCs) regulate the immune homeostasis and development of adaptive immune responses. In human peripheral blood, there are two main subsets of naturally circulating DCs, namely CD1c$^+$ myeloid dendritic cells (CD1c$^+$ mDCs) and plasmacytoid dendritic cells (pDC) (1, 2). These subsets differ in function, localization, and phenotype. CD1c$^+$ mDCs are primarily localized in the marginal zone of the lymph nodes and confer immunity against bacteria and fungi (3, 4) by inducing Th1 responses *via* the production of IL-12 (5, 6). Conversely, pDCs localize to the T-cell areas in lymph nodes and are proficient in viral antigen recognition (7). Mature pDCs abundantly produce type I IFNs upon activation and induce T cell responses (2, 8).

Under non-inflammatory conditions, DCs are poorly immunogenic. However, inflammatory stimuli or pathogen-derived products trigger a group of pattern recognition receptors, including Toll-like receptors (TLRs), which results in a process of cellular activation, termed DC maturation, hence making them highly immunogenic (9). DC maturation is a tightly coordinated response, which involves various signaling pathways, molecular trafficking, cytokine production and cytoskeletal remodeling (10–12). These processes require metabolic adaptations, which are essential for DC survival, migration and eventually the development of immunity. DC activation upon TLR stimulation is associated with metabolic reprogramming and expression of genes encoding cytokines and chemokines, which promote immune response (13, 14). Effector functions requires a glycolytic switch in mouse bone-marrow DCs cultured in GM-CSF (14, 15), while lipid metabolism and OXPHOS are indispensable for murine pDC immune function (16).

Mitochondrial dynamics and bioenergetics are reciprocally coupled to adjust bioenergetic adaptation to metabolic needs of the cell (17). Mitochondrial dynamics are controlled by a group of dynamin-related GTPases, i.e., mitofusin 1 and 2 (Mfn1/2) and optic atrophy 1 (Opa1) for fusion and dynamin related protein 1 (Drp1) for fission (18). Mfn1 plays a crucial role in mitochondrial fusion, while Mfn2 is central to mitochondrial metabolism, by regulating mitochondrial membrane potential and the OXPHOS system (17). The balanced mitochondrial dynamics is critical for normal mitochondrial function, bioenergetics and quality control *via* mitophagy (19–21). Mitophagy is a process by which a cell removes damaged mitochondria to use them as additional fuels during stress (22, 23). Upon stress or damage, mitochondria exhibit compromised metabolism, ATP production and reduction in membrane potential, which are characteristics of mitochondrial dysfunction and the initial trigger for mitophagy (24).

Understanding of metabolic changes underpinning human DC-subsets immune function are less known and insights into these changes can help develop new strategies for controlling immunogenicity. Given the distinct ontogeny and functional specializations of $CD1c^+$ mDC and pDC, we aimed at identifying metabolic adaptations engaged by human DC-subsets for effector function. We here demonstrate that TLR-stimulation in $CD1c^+$ mDC and pDC results in differential mitochondrial rewiring and metabolic adaptations. TLR stimulation results in increased glutaminolysis and OXPHOS in pDC, while it promotes mitophagy and glycolysis in $CD1c^+$ mDC. Notably, these metabolic adaptations are indispensable for activation of $CD1c^+$ mDC and pDC. Our data provides novel insights into subset-specific regulation of mitochondrial metabolism, which impacts DC function.

Abbreviations: PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; BNIP3, BCL2 interacting protein 3; Mfn1/2, mitofusin 1/2; Drp1, dynamin-related protein; ENO2, enolase; BPTES, bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide; 3-MA, 3-methyladenine; ROT, rotenone; AA, antimycin A; OXPHOS, oxidative phosphorylation; ETC, electron transport chain; pDC, plasmacytoid dendritic cell; $CD1c^+$ mDC, $CD1c^+$ myeloid dendritic cells; 2-NBDG, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose.

MATERIALS AND METHODS

Chemicals

Mdivi-1 (#M0199), Niclosamide (#N3510), 6-Diazo-5-oxo-L-norleucine (#D2141), 2-Deoxy-D-glucose (#D8375), BPTES (#SML0601), Chloroquine (#C6628), 3-Methyladenine (#M9281), Poly-D-lysine hydrobromide (#P7280), Antimycin A (#A8674), Oligomycin A (#O4876) and Rotenone (#R8875) were obtained from Sigma-Aldrich. Olomoucine (#10010240) was obtained from Caymanchem. Piericidin A (#ALX-380-235-M002) was obtained from Enzo Life Sciences. MitoTracker™ Green FM (#M7514), MitoTracker™ Red CMXRos (#M7512) and 2-NBDG (#N13195) were obtained from Thermo Fisher Scientific. EnzyChrom™ Glutamine Assay Kit (#EGLN-100) was purchased from BioAssay Systems. 15-oxospiramilactone (S3) was kindly provided by Prof. Xiaojiang Hao (The State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650204, China). SF2312 was kindly provided by Dr. Florian Muller (The University of Texas MD Anderson Cancer Center, USA).

Cytokine detection–Supernatant was taken from each sample after overnight incubation and analyzed with standard sandwich ELISAs to detect TNF-α using human TNF-α ELISA Kit (#88-7346-22) from Thermo Fisher Scientific and IFN-α (#BMS216INSTCE) from Bender Medsystems, Vienna.

DC Isolation and Culture

For functional assays, DCs were isolated from buffy coats of healthy volunteers (Sanquin, Nijmegen, The Netherlands). Written informed consent per the Declaration of Helsinki and according to institutional guidelines, were obtained from healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated by using Ficoll density centrifugation (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway). CD1c isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) was used to isolate $CD1c^+$ mDCs, as per manufacturer's instructions. Next, monocytes were depleted by either plastic adhesion, or by the use of CD14 microbeads (Miltenyi Biotec). Consequently, pDCs were purified by positive selection using anti–BDCA-4–conjugated magnetic microbeads (Miltenyi Biotec). DCs were cultured in X-VIVO-15 medium (Lonza, Basel, Switzerland) supplemented with 2% human serum (Sanquin). DCs were stimulated with: pRNA (15 μg/ml) freshly prepared 5–10 min before adding to the cell culture. pDCs were cultured with IL-3 (10 ng/mL) (Cellgenix, Freiburg, Germany) as a survival factor in addition to the stimuli.

Flow Cytometry

The phenotype of pDC and $CD1c^+$ mDC populations was determined by flow cytometry. DC purity was assessed by double staining $CD11c^+/CD1c^+$ for $CD1c^+$ mDCs (above 95%) and BDCA2/CD123 for pDCs (above 95%; all Miltenyi Biotec) (25). The following primary monoclonal antibodies (mAbs) were used to determine the maturation state of the DCs: anti–CD80-APC, anti–PD-L1-APC (all BD Bioscience, San Jose, CA). Anti-BNIP-3 Antibody (#sc-56167 FITC) was purchased from Santa Cruz Biotechnology. Anti-Mfn2 (#M6444) and Anti-Drp1 (#ABT155)

were purchased from Sigma-Aldrich Anti-Porin (#529536) was purchased from Calbiochem. Anti-NDUFA10 (#ab174829) was purchased from abcam. Autophagosomes were detected using Autophagy detection kit (Enzo Life Sciences # ENZ 51031-0500) according to the manufacturer's instructions. Briefly, cells were incubated with CYTO-ID Green autophagy detection dye (1:2,000) for 30 min at 37°C. Subsequently, cells were washed and analyzed by flow cytometry. Cell viability was determined using Fixable Viability Dye eFluor™ 780 (Invitrogen # 65-0865-14) according to manufacturer's instructions. Briefly, cells were incubated with Fixable Viability Dye eFluor™ 780 (1:2000) at 4°C for 20 min. Subsequently, cells were washed and analyzed by flow cytometry. Measurements were performed on FACSVerse flowcytometers (BD).

Metabolism Assay

An XF-96 Extracellular Flux Analyzer (Seahorse Bioscience) was used for Extracellular flux analyses of $CD1c^+$ mDC and pDCs (50,000 cells/well) (26). For mitochondrial fitness tests, OCR was measured sequentially at basal, and following the addition of 1 μM oligomycin, 3 μM FCCP (fluorocarbonyl cyanide phenylhydrazone), 1 μM ROT + 1 μM AA. Intracellular concentrations of glutamine were determined using a quantitative colorimetric enzyme assay kit (#EGLN-100; BioAssay Systems, Hayward, CA). Samples were diluted (1:2) with distilled water. All materials and chemicals were provided by the manufacturer, and manufacturer's instruction were followed.

Protamine-RNA Complexes

pRNA complexes were made freshly before adding to the cells. Protamine (protaminehydrochloride MPH 5000 IE/ml; Meda Pharma BV Amstelveen, The Netherlands) was diluted to 0.5 mg/ml in RNase free water and mixed with 2-kbp-long single-stranded mRNA (coding for gp100). It was extensively mixed and incubated for 5–10 min at room temperature, before added to the cells.

Quantitative Real-Time PCR (qPCR)

qPCR was carried out in 25-μl reaction mixture containing 2 μl of cDNA, 12.5 μl of SYBR Green master mix (Applied Biosystems #A25742, Austin, USA) and 250 nmol of forward and reverse primer. The reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. For qPCR following primer sequences were used; AMPK1α forward, 5′-TGCGTGTACGAAGGAAGAATCC-3′ and reverse, 5′-TGTGACTTCCAGGTCTTGGAGTT-3′; β-Actin forward, 5′-TGACAGGATCGAGAAGGAGA-3′ and reverse 5′-CGCTCAGGAGGAGCAATG-3′.

RNA Sequencing

Total RNA was isolated from $CD1c^+$ mDCs and pDCs using Trizol (Invitrogen, MA, USA). RNA sequencing and read alignment were performed by BGI TECH SOLUTIONS (Hong Kong). Reads were aligned to human genome version 19. RNA sequencing data is deposited at the Gene Expression Omnibus (GEO; accession number: GSE89442). Data was analyzed using the R platform package "edgeR," version 3.12, to analyze whole transcriptome principal coordinates analysis (using the "plotMDS" command), differential expression analysis, and GO term analysis. Differential expression was determined by fitting a generalized linear model using the "glmFit" command, and significance was determined using the likelihood ratio test provided by the "glmLRT" command (27).

RESULTS

Mitochondrial Dynamics Is Differentially Regulated in $CD1c^+$ mDC and pDC Upon TLR7/8 Stimulation

To investigate changes in metabolism, human $CD1c^+$ mDC and pDC were stimulated with a complex of protamine and mRNA (pRNA) that acts as a TLR7/8 ligand. pRNA has been shown to activate $CD1c^+$ mDCs and pDCs and induces them to release IL-12 and IFN-α, respectively (28). Previously, we analyzed the whole-transcriptome of human $CD1c^+$ mDC and pDC upon TLR7/8 stimulation (27). Our data demonstrated that pRNA upregulated cytokines and migration-related genes in $CD1c^+$ mDCs as well as type I and III interferons (IFN-α and IFN-λ) related genes in pDC. Moreover, we demonstrated that pRNA stimulation increased expression of maturation markers (i.e., CD80, PD-L1 & CD40) in both $CD1c^+$ mDC and pDC, in addition to increase in immunostimulatory cytokines i.e., TNFα and INFα for $CD1c^+$ mDC and pDC, respectively (27). To investigate whether changes in metabolism are required for human DC-subsets immune response, we analyzed expression of OXPHOS related genes in human $CD1c^+$ mDC and pDC. OXPHOS related genes were significantly downregulated in $CD1c^+$ mDCs upon pRNA-stimulation (**Figure 1A**). Conversely pRNA-stimulation increased expression of *NDUFAF1, NDUFA9*, *COX7A2*, *ATP5H*, and *ATP6V1F* in pDC (**Figure 1B**) suggesting up-regulation of OXPHOS in pDC.

To explore the question whether TLR-stimulation modulates OXPHOS, we next examined the effect of pRNA on NDUFA10 protein, which is an accessory subunit of the mitochondrial respiratory chain complex I (29). Importantly, pRNA stimulation reduced NDUFA10 in $CD1c^+$ mDC, in comparison to increase of NDUFA10 in pDC (**Figure 1C**). Given, the crucial role of Mfn2 and Drp1 in regulating OXPHOS system and metabolism (17, 30–32), we analyzed the effect of TLR-stimulation on Mfn2 and Drp1 protein levels. Intriguingly, analysis of protein expression revealed that pRNA-stimulation increased levels of Drp1 in $CD1c^+$ mDC whereas Mfn2 levels remained unchanged (**Figure 1C**). Conversely, in pDC, pRNA-stimulation increased Mfn2 protein levels, whereas Drp1 protein levels remained unchanged (**Figure 1C**). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) controls mitochondrial biogenesis, oxidative phosphorylation (33, 34) and mitochondrial dynamics (35, 36). TLR7/8-stimulation increased PGC-1α expression in pDC, whereas it had no effect on PGC-1α expression in $CD1c^+$ mDC (**Figure 1C**). The Voltage-Dependent Anion Channel (VDAC or porin) is an outer membrane mitochondrial protein, which is implicated in alteration of

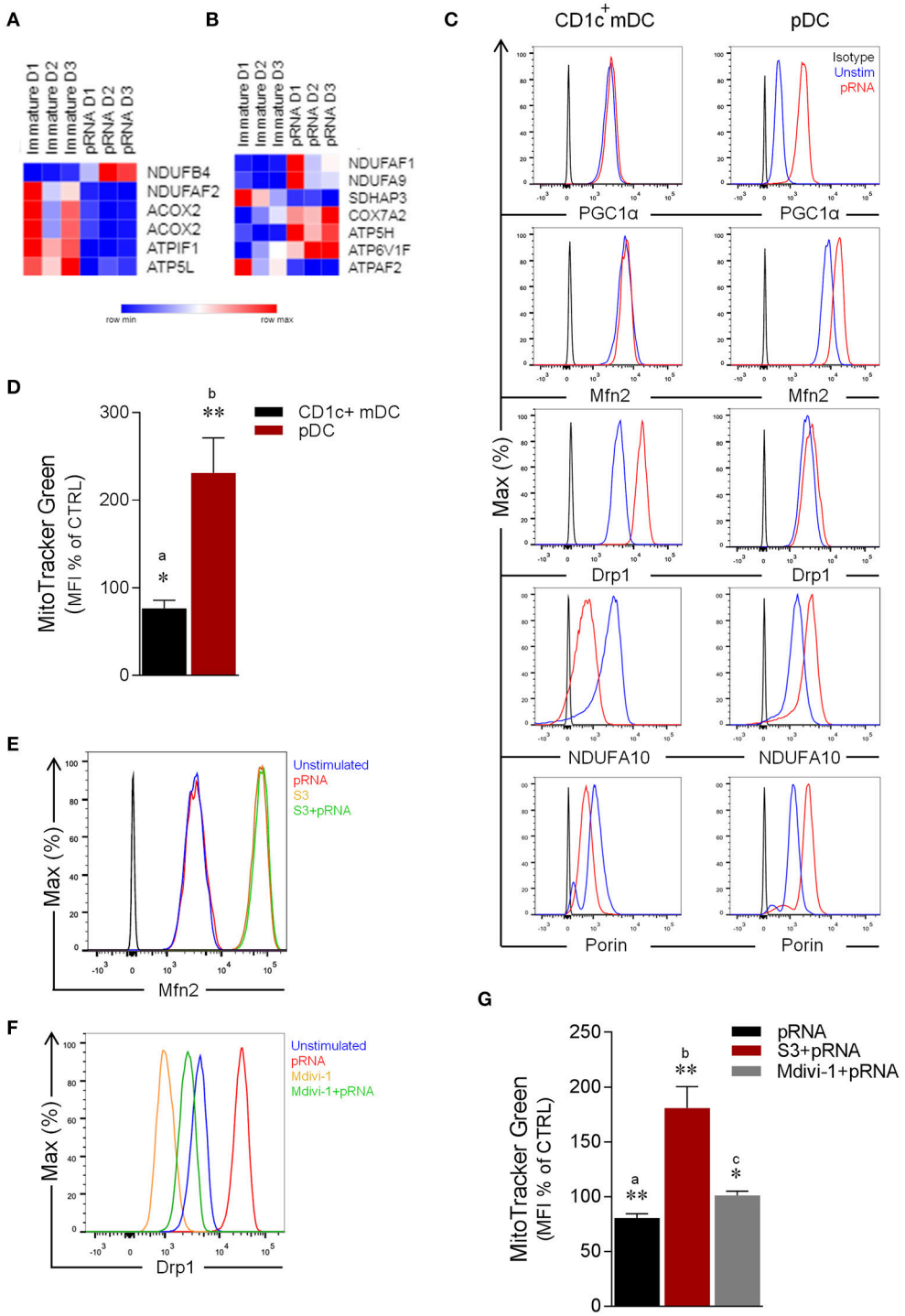

FIGURE 1 | Effect of pRNA on mitochondrial dynamics in CD1c$^+$ mDC and pDC. **(A)** Heatmap showing expression of significantly changed genes which regulate OXPHOS in CD1c$^+$ mDC upon pRNA-stimulation. Red color indicates increased expression while blue color shows decreased expression. **(B)** Heatmap showing expression of significantly changed genes which regulate OXPHOS in pDC upon pRNA-stimulation. Red color indicates increased expression while blue color shows decreased expression. **(C)** Flow cytometry histograms of PGC1α, Mfn2, NDUFA10, Porin and Drp1 in Drp1 in CD1c$^+$ mDC and pDC. Black represents isotype control, blue represents unstimulated control and red represents pRNA stimulated cells for 6 h. **(D)** Percentage mean fluorescence intensity of cells stained with MitoTracker Green FM and stimulated with pRNA for 6 h. Data represents mean ± SEM of four independent experiments $*p < 0.05$; $**p < 0.01$ (Student's *t*-test). **(E)** Flow cytometry histograms of Mfn2 in CD1c$^+$ mDC. Blue represents unstimulated control, red represents pRNA stimulated cells for 6 h, brown represents S3 and green represents S3+pRNA. **(F)** Flow cytometry histograms of Drp1 in CD1c$^+$ mDC. Blue represents unstimulated control, red represents pRNA stimulated cells, brown represents Mdivi-1 and green represents Mdivi-1+pRNA. **(G)** Percentage mean fluorescence intensity of cells stained with MitoTracker Green FM and stimulated with pRNA for 6 h in the presence or absence of 5 μM S3 or 1 μM Mdivi-1. Data represents mean ± SEM of four independent experiments. $*p < 0.05$; $**p < 0.01$ (Student's *t*-test).

mitochondrial morphology (37). Importantly, pRNA-stimulation reduced porin levels in $CD1c^+$ mDC and increased porin levels in pDC (**Figure 1C**). Of note, pRNA-stimulation did not affect viability of $CD1c^+$ mDC and pDC (**Supplementary Figures 4**, **6**).

Based on these findings, we hypothesized that TLR7/8-stimulation alters mitochondrial content in $CD1c^+$ mDC. To test this, $CD1c^+$ mDC were stained with MitoTracker™ Green FM, a fluorescent dye that localizes to mitochondria in a mitochondrial membrane potential independent manner. Indeed, TLR7/8-stimulation significantly decreased mitochondrial content in $CD1c^+$ mDC (**Figure 1D**). By comparison, staining of pDC with MitoTracker™ Green FM showed a significant increase in mitochondrial content upon TLR7/8-stimulation (**Figure 1D**) consistent with increased Mfn2 and PGC1α levels. To confirm the involvement of mitochondrial dynamics in regulating mitochondrial mass, we stimulated $CD1c^+$ mDC with pRNA in the presence or absence of a fusion promoter (15-oxospiramilactone, S3) (38) or a fission inhibitor (Mdivi-1) (39). Interestingly, S3 increased Mfn2 expression and Mdivi-1 reduced both endogenous and pRNA-induced Drp1 levels in $CD1c^+$ mDC (**Figures 1E,F**). Of note, S3 and Mdivi-1 significantly prevented loss of mitochondrial content in $CD1c^+$ mDC upon TLR7/8-stimulation (**Figure 1G**). Collectively, these data indicate that TLR7/8-stimulation results in mitochondrial fragmentation and reduced mitochondrial content in $CD1c^+$ mDCs and increased mitochondrial biogenesis, fusion and content in pDCs.

pDC Stimulated via TLR7/8 have Increased Glutaminolysis and OXPHOS Which Are Crucial For Activation

We next asked whether increased mitochondrial fusion and content along with upregulation of NDUFA10 and OXPHOS related genes in TLR7/8-stimulated pDCs was associated with metabolic changes. OXPHOS is driven by NADH and $FADH_2$, produced by the tricarboxylic acid (TCA) cycle (40, 41) and the amino acid glutamine is among the key metabolites that support the TCA cycle. Glutaminolysis is a metabolic pathway, which requires deamination of glutamine by glutaminase (GLS), generating glutamate, which in turn is converted to α-KG, a TCA cycle intermediate (42, 43). To determine whether glutaminolysis contributes to increased OXPHOS upon TLR7/8-stimulation in pDC, we examined expression of genes related to amino acid metabolism. pRNA-stimulation significantly increased expression of *GLS* and *SLC1A3* in pDC (**Figure 2A**). *GLS* catalyzes the conversion of glutamine to glutamate (44) while *SLC1A3* is a glutamate transporter (45, 46). Upregulation of these genes suggests increased glutaminolysis in pDCs upon TLR-stimulation. To test this, we measured intracellular glutamine levels in pDC. pRNA-stimulation significantly increased intracellular glutamine in pDC, which could be inhibited by 6-Diazo-5-oxo-L-norleucine (DON) (**Figure 2B**), a glutamine antagonist, which inhibits glutamine utilizing enzymes by irreversible alkylation of L-cysteinyl residues (47).

Notably, extracellular flux analysis (EFA) revealed increased basal oxygen consumption rate (OCR), maximal OCR (**Figure 2C**; **Supplementary Figures 1B,C**), ATP-linked OCR, mitochondrial OCR and spare respiratory capacity (SRC) in pRNA-stimulated pDC compared to unstimulated pDC (**Figures 2D–F**). To explore whether increased OXPHOS activity in pRNA-stimulated pDC is due to increased glutaminolysis, we pharmacologically attenuated Glutaminase, an enzyme responsible for conversion of glutamine into glutamate. pDC were stimulated with pRNA in the presence or absence of BPTES, a chemical inhibitor of GLS. BPTES inhibited in pDC the pRNA-induced increase in basal OCR (**Figure 2C**; **Supplementary Figures 1A–C**), ATP-linked OCR (**Figure 2D**), maximal OCR (**Supplementary Figure 1C**) mitochondrial OCR (**Figure 2E**) and SRC (**Figure 2F**). These results indicate that pRNA stimulation of pDC results in increased OXPHOS due to increased glutaminolysis. Intriguingly, we did not observe an increase in ECAR (**Supplementary Figure 1D**) and 2-NBDG uptake (**Figure 2G**) upon pRNA-stimulation.

We next asked whether these metabolic changes are required for pDC activation. Activation of these cells was assessed by measuring secretion of immunostimulatory cytokine IFNα and membrane expression of co-stimulatory molecule CD80 and co-inhibitory molecule PD-L1. A reduced secretion of IFNα by pRNA-stimulated in pDC was observed when Rotenone (ROT), Antimycin A (AA), BPTES and DON were added to the culture medium (**Figure 2H**). Addition of these factors also significantly reduced the pRNA-mediated upregulation of CD80 and PD-L1 on pDC (**Figure 2I**). By comparison, we observed no effect of ROT, AA, BPTES and DON on pRNA-stimulated TNFα (**Supplementary Figure 3B**) and CD80 and PD-L1 in $CD1c^+$ mDC (**Supplementary Figure 3C**).

Of note, TLR stimulation triggers autophagy in pDC, which is required to produce type I IFN (48–52). Consistently, we observed significant increase in autophagosomes upon pRNA-stimulation in pDC (**Figure 3A**). Intriguingly, autophagy has been reported to supply metabolic substrates to preserve mitochondrial function (53–57). We hypothesized that increased glutamine and glutaminolysis in TLR7/8-stimulated pDCs is provided by autophagy. To investigate this, autophagy inhibitor 3-MA was added during the pRNA stimulation of pDC. 3-MA significantly reduced the pRNA-induced increase in glutamine levels in pDC (**Figure 3B**). Consistently, 3-MA significantly reduced pRNA-induced increase in basal OCR (**Figure 3C**; **Supplementary Figure 1E**), maximal OCR (**Supplementary Figure 1F**), ATP-linked OCR (**Supplementary Figure 1G**), SRC (**Supplementary Figure 1H**) and mitochondrial OCR (**Figure 3D**) indicating the requirement of autophagy for optimal induction of OXPHOS upon TLR-stimulation of pDC. Notably, 3-MA significantly reduced both IFNα secretion (**Figure 3E**) as well as expression of CD80 and PD-L1 upon pRNA-stimulation of pDCs (**Figure 3F**). Since, TLR7/8 stimulated pDC activation was prevented by pharmacological attenuation of OXPHOS, glutaminolysis and autophagy, we next asked whether the observed reduction was due to effect on cell viability. Analysis of cell viability revealed that BPTES, DON, 3-MA, ROT and AA did not affect viability of pDC

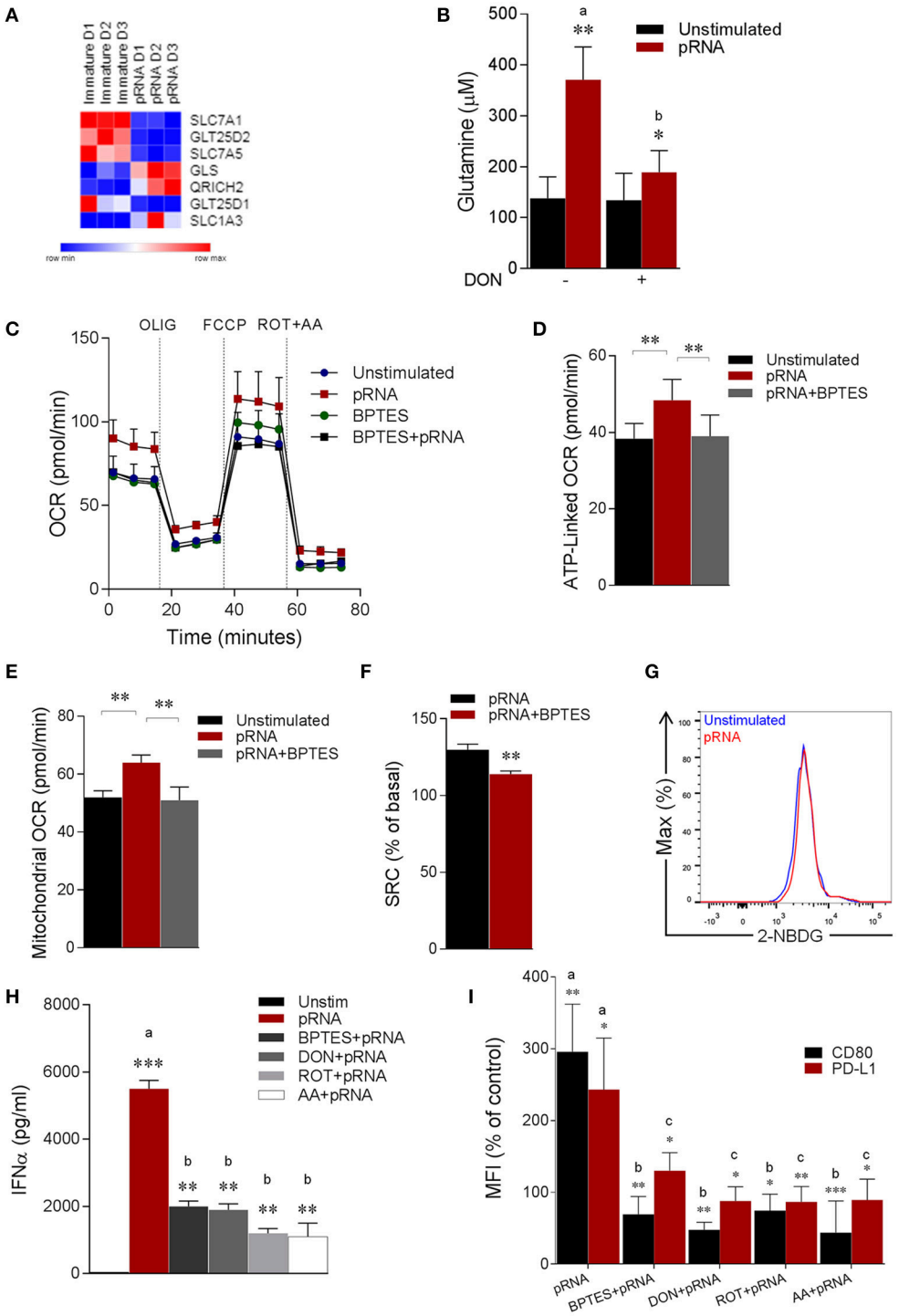

FIGURE 2 | pDC stimulated with pRNA have increased glutaminolysis and OXPHOS which are required for activation. **(A)** Heatmap showing expression of significantly changed genes which regulate amino acid metabolism in pDCs upon pRNA-stimulation for 6 h. Red color indicates increased expression while blue color shows decreased expression. **(B)** Glutamine concentration measured by a coupled glutaminase, glutamate dehydrogenase assay with correction for glutamate concentration. Data represents mean ± SEM of experiments from six donors. $^{*}p < 0.05$; $^{**}p < 0.01$ (Student's *t*-test). **(C)** Mitochondrial fitness test of pDCs stimulated with pRNA for 6 h in the presence or absence of 5 μM BPTES. Data represents mean ± SEM of three independent experiments. **(D–F)** Data was collected within same experiments as C, but is shown separately for better understanding. Data represents mean ± SEM of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ (Student's *t*-test). **(G)** Flow cytometry histograms of 2-NBDG stained pDCs. Blue represents unstimulated control and red represents pRNA-stimulated cells pDC for 6 h. **(H)** IFN-α levels on protein level were measured in the supernatant of the pDCs stimulated for 6 h. Data represents mean ± SEM of three independent experiments $^{**}p < 0.01$; $^{***}p < 0.001$ (Student's *t*-test). **(I)** Percentage mean flouresence intensity of maturation markers (CD80 and PD-L1) in pDCs stimulated for 6 h. Data represents mean ± SEM of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (Student's *t*-test).

alone or in combination with pRNA (**Supplementary Figures 7**, **8**). Together, these data show that TLR7/8-stimulated pDC activation requires autophagy-supplemented glutaminolysis to fuel OXPHOS.

TLR7/8 Stimulated Alterations in Mitochondrial Dynamics Triggers Glycolysis Which Is Required For CD1c$^+$ mDC Activation

Our data show that TLR7/8-stimulation reduces expression of OXPHOS related genes and mitochondrial content in CD1c$^+$ mDCs, which is associated with metabolic changes with a shift toward glycolysis (58) to compensate for the reduced activity of the respiratory chain to generate ATP (17). In this sense, we wondered whether mitochondrial alterations induced by TLR7/8-stimulation led to a metabolic shift in CD1c$^+$ mDC. To this end, analysis of glycolysis related genes showed significant upregulation of *ENO2* (**Figure 4A**). *ENO2* encodes a dimeric enzyme, Enolase, which catalyzes the second last step in glycolysis i.e., interconverting 2-phosphoglycerate (2-PGA) and phosphoenolpyruvate (PEP) (59). Next, we monitored EFA in pRNA-stimulated CD1c$^+$ mDC. We found that TLR7/8-stimulation significantly reduced OCR (**Figure 4B**; **Supplementary Figure 2C**). To test our hypothesis that mitochondrial fragmentation leads to induction of glycolysis in CD1c$^+$ mDC upon TLR7/8-stimulation, we monitored EFA in the presence of S3 and Mdivi-1. Interestingly, S3 and Mdivi-1 significantly prevented the pRNA-induced decrease in OCR (**Figure 4B**; **Supplementary Figures 2A–C**), SRC (**Figure 4C**), mitochondrial OCR (**Figure 4D**) ATP-linked OCR (**Figure 4E**) and maximal OCR (**Supplementary Figure 2D**) in CD1c$^+$ mDCs.

To investigate the induction of glycolysis, we monitored pRNA-induced ECAR in CD1c$^+$ mDC. Importantly, pRNA stimulation significantly increased ECAR in CD1c$^+$ mDC (**Figure 4F**). Of note, S3 and Mdivi-1 significantly reduced the pRNA-induced increase in ECAR (**Figure 4F**), indicating that indeed mitochondrial fragmentation induced by TLR7/8-stimulation leads to a shift toward glycolysis in CD1c$^+$ mDC.

To further investigate the induction of glycolysis, we determined glucose uptake in CD1c$^+$ mDCs upon TLR7/8-stimulation using 2-NBDG. Consistent with the increase in ECAR, pRNA-stimulation significantly increased the uptake of 2-NBDG in CD1c$^+$ mDC, which could be prevented by glycolysis inhibitor, 2-DG (**Figure 4G**). Additionally, given the significant upregulation of *ENO2* upon pRNA-stimulation in CD1c$^+$ mDC, we determined 2-NBDG uptake in the presence of a specific Enolase inhibitor, SF2312 (59). Consistently, the pRNA-induced 2-NBDG uptake in CD1c$^+$ mDCs was significantly reduced in the presence of SF2312 (**Figure 4H**; **Supplementary Figure 3**). Similarly, S3 and Mdivi-1 treatment significantly reduced pRNA-induced 2-NBDG uptake (**Figure 4H**; **Supplementary Figure 3A**). Taken together, these data indicate that mitochondrial fragmentation induced by TLR7/8-stimulation leads to a shift toward glycolysis in CD1c$^+$ mDC.

Next, we asked whether TLR7/8-stimulation induced alteration in mitochondrial dynamics are required for CD1c$^+$ mDC activation. Importantly, pRNA stimulation significantly increased TNFα production, which was attenuated by S3 and Mdivi-1 (**Figure 4I**). Similarly, pRNA stimulation significantly upregulated maturation markers i.e., CD80 and PD-L1 on CD1c$^+$ mDC, which were significantly inhibited by S3 and Mdivi-1 (**Figure 4J**). By comparison, we observed no effect of S3 and Mdivi-1 on pRNA-stimulated IFNα (**Supplementary Figure 3E**) and CD80 and PD-L1 in pDC (**Supplementary Figure 3D**). Collectively, these data indicate that TLR7/8-induced mitochondrial fragmentation is required for induction of glycolysis and immune response of CD1c$^+$ mDC.

TLR7/8-Stimulation Triggers BNIP3-Dependent Mitophagy in CD1c$^+$ mDC

Mitophagy is a highly regulated autophagy process during which damaged mitochondria are degraded and removed from the cell (23, 60–62). Given the alteration in mitochondrial dynamics in CD1c$^+$ mDC upon TLR7/8-stimulation, we hypothesize that mitophagy is induced in CD1c$^+$ mDC. To this end, analysis of autophagy-related genes revealed that pRNA-stimulation significantly increased expression of *EPG5, MAP1LC3A, DRAM1* & *AMBRA1* (**Figure 5A**), indicating involvement of autophagy. Consistent with increased expression of autophagy-related genes, pRNA significantly increased autophagosomes in CD1c$^+$ mDC (**Figure 5B**). Damaged mitochondria exhibit dissipated membrane potential, which is the initial trigger for mitophagy (22, 63). To test whether pRNA-stimulation affects mitochondrial membrane potential ($\Delta\psi$) in CD1c$^+$ mDC, we measured $\Delta\psi$ using MitoTracker Red CMXRos, a red-fluorescent dye which stains mitochondria in a membrane potential dependent manner (64). Importantly, pRNA-stimulation significantly induced $\Delta\psi$ depolarization in CD1c$^+$ mDC (**Figure 5C**). Two distinct mitophagy pathways have been described. One engages ubiquitination of OMM proteins *via* the PINK1/Parkin-mediated pathway. Consequently, ubiquitinated proteins recruit autophagosomal membrane via specific receptors, which can recognize ubiquitin chains on mitochondrial proteins and LC3 at autophagosomal membrane (65). The other mitophagy pathway involves BNIP3, a Bcl-2 family member that regulates mitophagy by associating itself on the outer mitochondrial membrane (OMM) through C-terminal transmembrane domain and interacts with LC3 through its LC3-interacting region (LIR) domain located at N-terminal part (66–68). To determine which mitophagy pathway is involved upon TLR7/8-stimulation of CD1c$^+$ mDC, the gene expression data were examined. Interestingly, PINK1 did not significantly change upon pRNA stimulation, whereas BNIP3 was significantly increased in CD1c$^+$ mDC upon pRNA-stimulation (**Supplementary Figure 2E**).

To specify the involvement of BNIP3, the effect of olomoucine, a transcriptional inhibitor of BNIP3 (69) on TLR7/8-induced mitophagy in CD1c$^+$ mDC was examined.

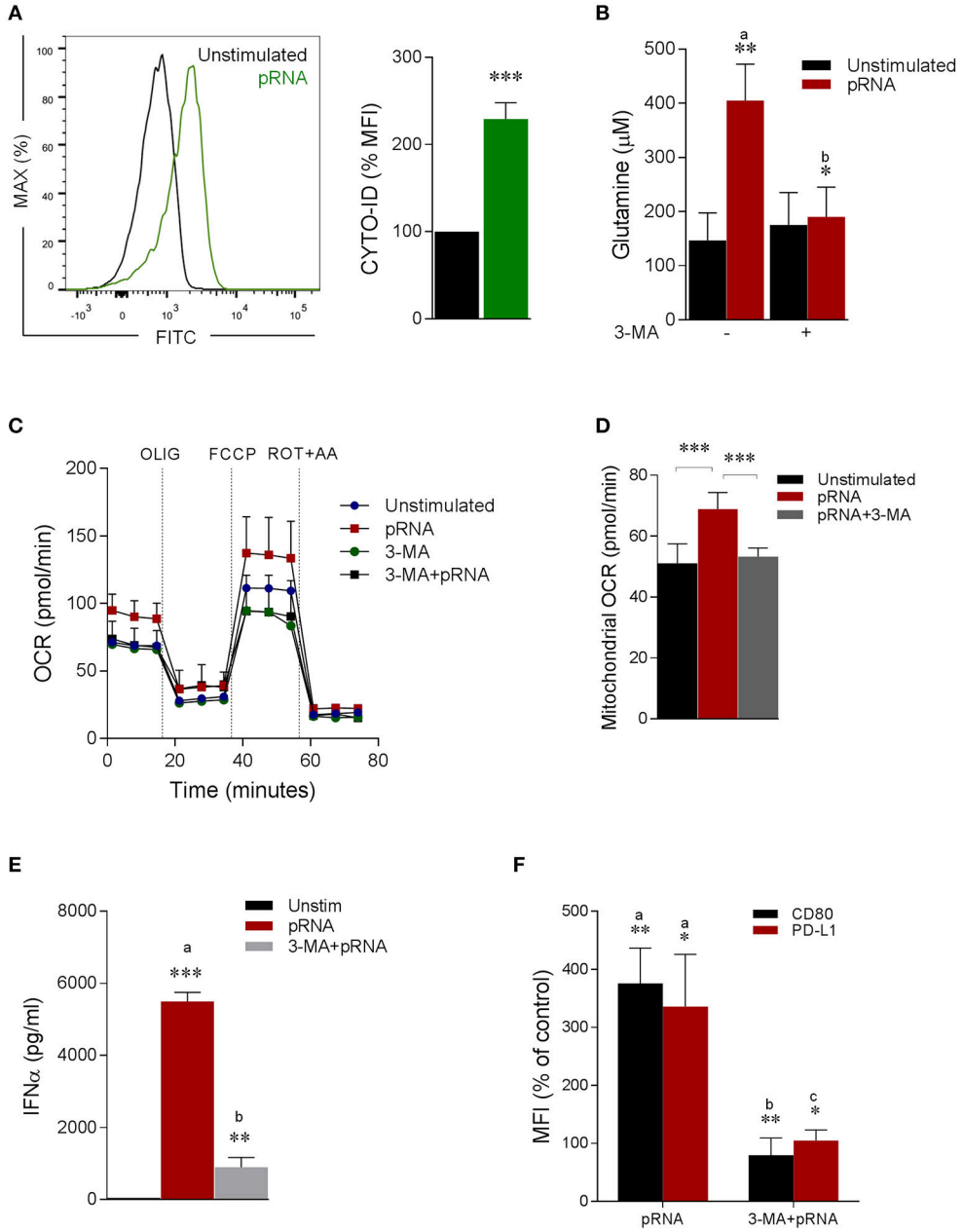

FIGURE 3 | Autophagy provides glutamine for pDC activation. **(A)** Fluorescence intensity of autophagosomal marker CYTO-ID in pDC stimulated with pRNA for 6 h ***$p < 0.001$ (Student's *t*-test). **(B)** Glutamine concentration measured by a coupled glutaminase, glutamate dehydrogenase assay with correction for glutamate concentration. Data represents mean ± SEM of experiments from six donors. *$p < 0.05$; **$p < 0.01$ (Student's *t*-test). **(C)** Mitochondrial fitness test of pDCs stimulated with pRNA for 6 h in the presence or absence of 25 μM 3-MA. Data represents mean ± SEM of three independent experiments. **(D)** Data was collected within same experiments as **(C)** but is shown separately for better understanding. Data represents mean ± SEM of three independent experiments. **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test). **(E)** IFN-α levels on protein level were measured in the supernatant of the pDCs stimulated for 6 h. Data represents mean ± SEM of three independent experiments. **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test). **(F)** Percentage mean flouresence intensity of maturation markers (CD80 and PD-L1) in pDCs stimulated for 6 h. Data represents mean ± SEM of three independent experiments. *$p < 0.05$; **$p < 0.01$ (Student's *t*-test).

Olomoucine significantly reduced steady state BNIP3 (**Figure 5D**) and the pRNA-induced increase of BNIP3 in CD1c$^+$ mDC (**Figure 5D**). Niclosamide is a transcriptional inhibitor of S100A4 (70), which is transcriptional repressor of of BNIP3 (71). Niclosamide increased BNIP3 expression in CD1c$^+$ mDC (**Figure 5D**). To quantitatively asses mitophagy in CD1c$^+$ mDC cells, we employed flow cytometry based method (72). This approach is suitable to robustly assess

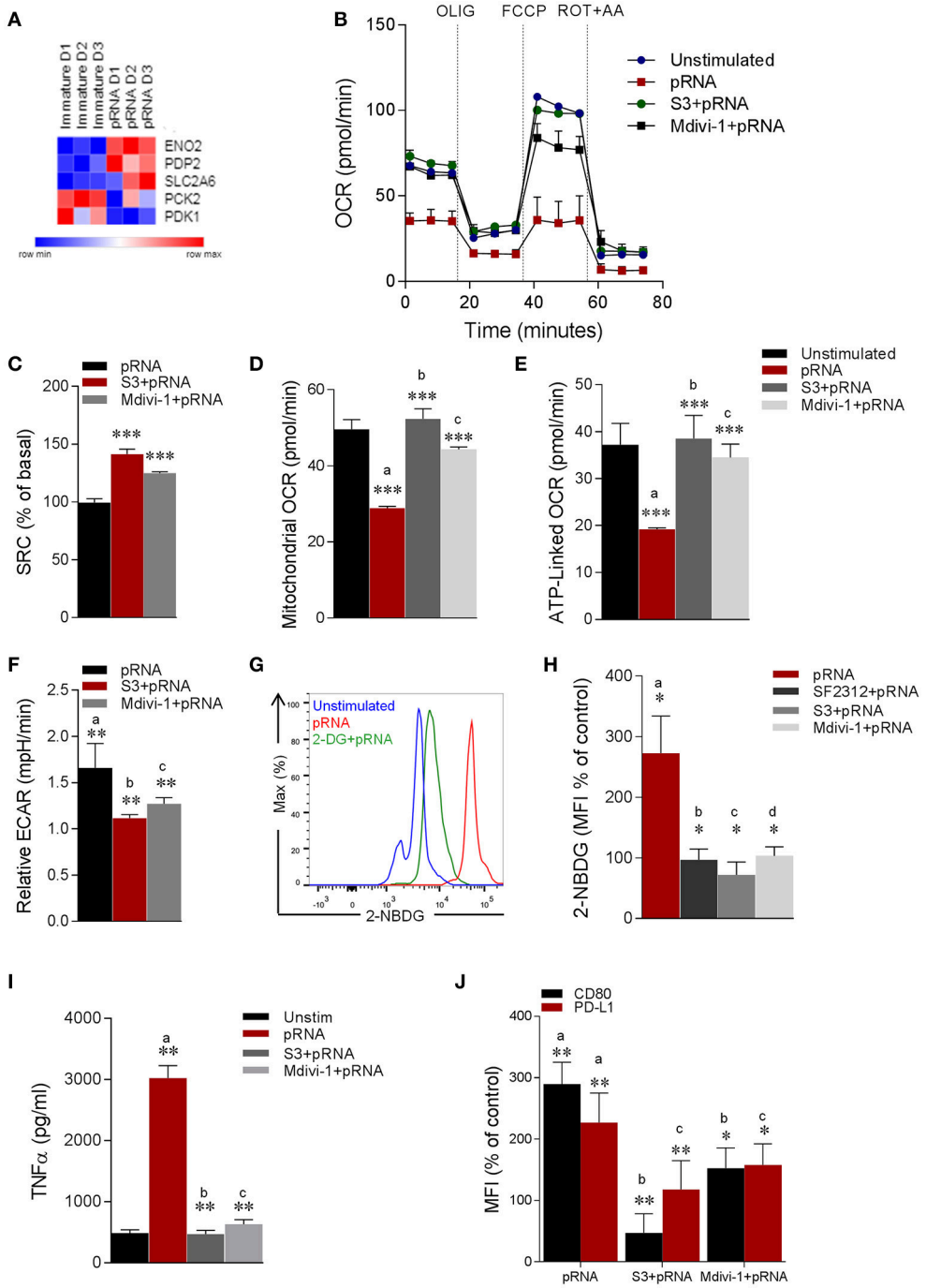

FIGURE 4 | pRNA-stimulation alters mitochondrial morphology in CD1c$^+$ mDC to induce glycolysis. **(A)** Heatmap showing expression of significantly changed genes which regulate glycolysis in CD1c$^+$ mDC upon pRNA-stimulation for 6 h. Red color indicates increased expression while blue color shows decreased expression. **(B)** Mitochondrial fitness test of CD1c$^+$ mDC stimulated with pRNA for 6 h in the presence or absence of 5 μM S3 or 1 μM Mdivi-1. Data represents mean ± SEM of three independent experiments. **(C–F)** Data was collected within same experiments as **(B)**, but is shown separately for better understanding. Data represents mean ± SEM of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test). **(G)** Flow cytometry histograms of 2-NBDG stained CD1c$^+$ mDC cells. **(H)** Percentage mean fluorescence intensity of cells stained with 2-NBDG. Data represents mean ± SEM of four independent experiments *$p < 0.05$ (Student's *t*-test). **(I)** TNF-α levels on protein level were measured in the supernatant of the stimulated CD1c$^+$ mDC cells stimulated for 6 h in the presence or absence of 5 μM S3 or 1 μM Mdivi-1. Data represents mean ± SEM of three independent experiments. **$p < 0.01$ (Student's *t*-test). **(J)** Percentage mean fluorescence intensity of maturation markers (CD80 and PD-L1) in CD1c$^+$ mDC cells stimulated for 6 h in the presence or absence of 5 μM S3 or 1 μM Mdivi-1. Data represents mean ± SEM of three independent experiments. *$p < 0.05$; **$p < 0.01$ (Student's *t*-test).

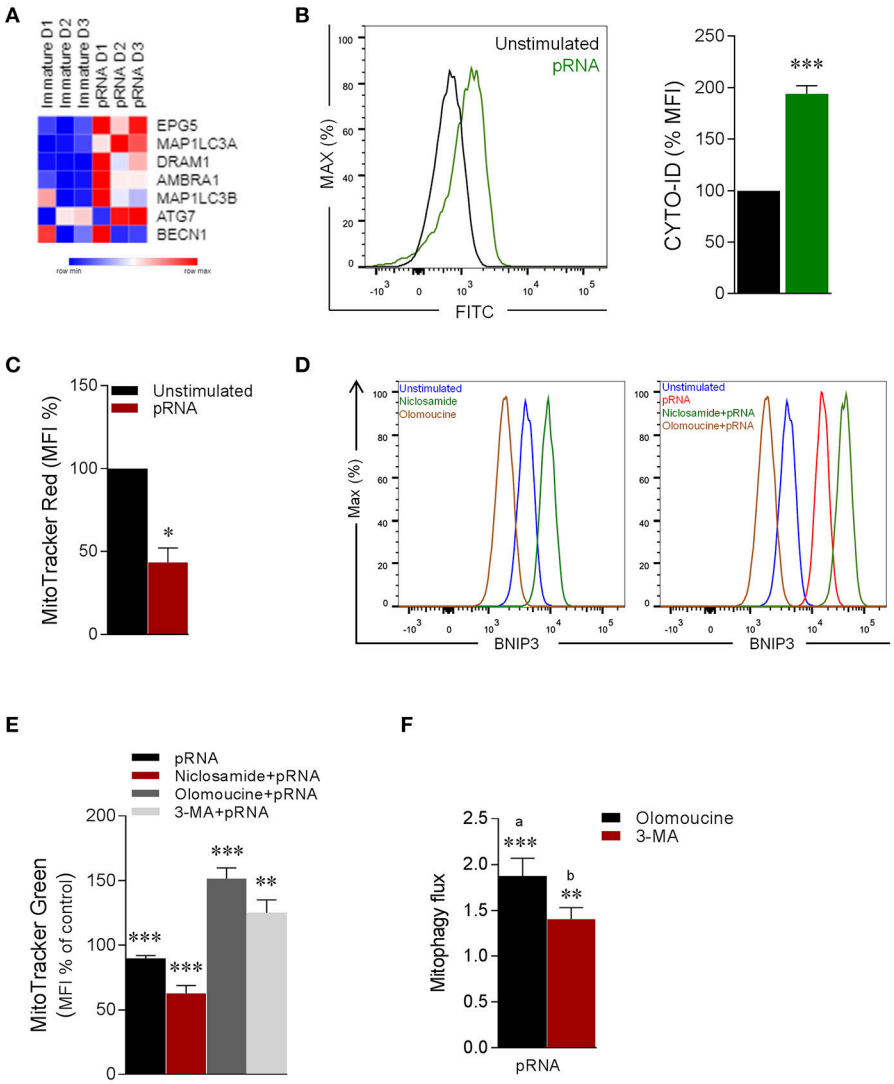

FIGURE 5 | pRNA-stimulation triggers BNIP3-dependent mitophagy in CD1c$^+$ mDC. **(A)** Heatmap showing expression of significantly changed genes which regulate autophagy in CD1c$^+$ mDC upon pRNA-stimulation for 6 h. Red color indicates increased expression while blue color shows decreased expression. **(B)** Fluorescence intensity of autophagosomal marker CYTO-ID in pDC stimulated with pRNA for 6 h ***$p < 0.001$ (Student's *t*-test). **(C)** Percentage mean flouresence intensity of CD1c$^+$ mDC cells stained with MitoTracker Red stimulated with pRNA for 6 h. Data represents mean ± SEM of three independent experiments. *$p < 0.05$ (Student's *t*-test). **(D)** Flow cytometry histograms of BNIP3 in CD1c$^+$ mDC cells in the presence or absence of 2 μM niclosamide or 10 μM olomoucine for 6 h. **(E)** Percentage mean fluorescence intensity of CD1c$^+$ mDC cells stained with MitoTracker Green stimulated with pRNA for 6 h in the presence or absence of 2 μM niclosamide or 10 μM olomoucine or 25 μM 3-MA. Data represents mean ± SEM of three independent experiments **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test). **(F)** Mitophagy flux in CD1c$^+$ mDC stimulated with pRNA for 6 h. Data represents mean ± SEM of three independent experiments **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test).

mitophagy without need to perform traditional fluorescence microscopy of mitochondrial-autophagosome colocalization in BNIP3 transfected cells, in order to avoid transfection and prolonged culture-induced cell death in rare human CD1c$^+$ mDC cells. The reversal in alteration in MitoTracker upon mitophagy inhibitors (i.e., olomoucine and 3-MA) indicates induction of mitophagy and can be used to calculate mitophagic flux (72). Of note, loss of pRNA-induced mitochondrial content in CD1c$^+$ mDC cells was significantly potentiated by niclosamide, which augments BNIP3 expression (**Figure 5E**). On other hand, loss of pRNA-induced mitochondrial content in CD1c$^+$ mDC cells was significantly reversed by olomoucine and 3-MA (**Figure 5E**) indiacating induction of mitophagy. Furthermore, analysis of mitophagic flux, revealed that pRNA stimulation significantly increased mitophagic flux in CD1c$^+$ mDC (**Figure 5F**). This data indicates that TLR7/8-stimulation triggers BNIP3-dependent mitophagy in CD1c$^+$ mDC cells.

TLR7/8-Stimulated BNIP3-Dependent Mitophagy Is Indispensable For Induction of Glycolysis and Activation of CD1c$^+$ mDC

Notably, mitophagy has been reported to be required for glycolytic switch in tumor cells (73). Given, the metabolic reprogramming toward glycolysis in CD1c$^+$ mDC upon TLR7/8 stimulation, we next asked whether BNIP3-dependnet mitophagy is required for induction of glycolysis in CD1c$^+$ mDC. To investigate this, we monitored EFA in the presence or absence of olomoucine and 3-MA in CD1c$^+$ mDC. Intriguingly, olomoucine and 3-MA significantly prevented the pRNA-induced decrease in OCR (**Figure 6A**; **Supplementary Figure 4A**), mitochondrial OCR (**Figure 6B**), ATP-linked OCR (**Supplementary Figure 4B**), maximal OCR (**Supplementary Figure 4C**) and SRC (**Supplementary Figure 4D**) in CD1c$^+$ mDCs. Moreover, olomoucine and 3-MA prevented pRNA-stimulated uptake of 2-NBDG (**Figure 6C**). These experiments indicate that BNIP3-dependent mitophagy is indispensible for induction of glycolysis in CD1c+ mDC upon TLR7/8 stimulation. To elucidate the mechanism underlying BNIP3 regulation of glycolysis, we examined the involvement of AMPK, which is key regulator of metabolic homeostasis (74). pRNA stimulation significantly reduced *AMPK1α* mRNA levels in CD1c$^+$ mDC, which were significantly rescued by olomoucine and 3-MA (**Figure 6D**). Interestingly, mitophagy inhibition attenuated TLR7/8-stimulated immune response in CD1c$^+$ mDC, as olomoucine and 3-MA significantly reduced pRNA-stimulated TNFα levels (**Figure 6E**). Moreover, the pRNA-induced increase in maturation markers CD80 and PD-L1 was significantly decreased in the presence of olomoucine and 3-MA (**Figure 6F**). By comparison, olomoucine had no effect on pRNA stimulated IFNα (**Supplementary Figure 3E**) and CD80 and PD-L1 in pDC (**Supplementary Figure 3D**). Of note, 2-DG, SF2313, Mdivi-1, S3, 3-MA, olomoucine and niclosamide did not affect viability of CD1c$^+$ mDC alone or in combination with pRNA (**Supplementary Figures 5**, **6**). Collectively, these data suggest that TLR7/8-stimulated BNIP3-dependent mitophagy is crucial for induction of glycolysis, which contributes to CD1c$^+$ mDC activation.

DISCUSSION

Changes in metabolism following TLR stimulation are indispensable for DC activation. However, the metabolic signature generated in naturally occurring human DCs in response to TLR-stimulation is not known in detail. Herein, we investigated TLR-induced metabolic changes in two human blood DC-subsets, CD1c$^+$ mDC and pDC. Our data show that TLR stimulation results in a differential mitochondrial rewiring in pDC and CD1c$^+$ mDC. We have focused on mitochondria as metabolic hubs critical for signals downstream of innate receptors in myeloid cells (75). Promotion of mitochondrial fusion results in increased OXPHOS activity *via* formation of supercomplexes (76). Supercomplex reorganization in macrophages is also driven by innate sensing of microbes, regulating macrophage cytokine production (77). Conversely, mitochondrial fission results in decreased OXPHOS activity and induction of glycolysis (21). Interestingly, mitochondrial dynamics play an important role in differentiation and migration of immature DC (78). Mitochondrial fusion proteins are upregulated during differentiation of bone marrow progenitors to immature DC. Mitochondrial fusion-related proteins i.e., Mfn2 and Opa1 have been shown to be required for migration of immature DC (78).

Here, we investigated the role of mitochondrial dynamics in regulating immune function of human DC subsets. We find that stimulation of pDCs with TLR7/8 agonist increases expression of PGC1α and Mfn2, which suggests increase in mitochondrial mass. Indeed, we observed that TLR7/8-stimulation resulted in increased mitochondrial mass in pDC, as demonstrated by MitoTracker Green and Porin levels. Moreover, PGC-1α positively regulates mitochondrial fusion by stimulating Mfn2 expression *via* targeting the Mfn2 promoter in an ERRα-binding element-dependent manner (79). Importantly, increased Mfn2 expression results in increased glucose oxidation and expression of OXPHOS complex I, IV and V (80). Consistently, we observed increased expression of OXPHOS related genes and protein levels of NDUFA10 upon TLR7/8-stimulation in pDC, indicating upregulation of OXPHOS. Taken together, these data indicate that TLR7/8 stimulation increases mitochondrial fusion, mass and increased OXPHOS activity in pDC. Conversely, pRNA stimulation of CD1c$^+$ mDCs results in increased expression of Drp1, which contributes to mitochondrial fission (81, 82), which lead to decrease in mitochondrial mass as shown by decreased levels of MitoTracker Green and Porin. Mitochondrial fission promotes a shift to aerobic glycolysis (58, 83, 84). Our data shows that TLR-stimulation leads to increased glycolysis in CD1c$^+$ mDC. Increased expression of Drp1 together with decreased expression of NDUFA10 and mitochondrial mass, in CD1c$^+$ mDC indicates induction of mitochondrial fission, which is linked to glycolysis (21, 85, 86). Intriguingly, Drp1 has been demonstrated to be required for the activation of bone marrow-derived DCs upon LPS-stimulation (87). It has been reported that TLR-stimulated metabolic reprogramming is required to meet the energy demand for the activation process in DC (14, 16, 88). Of note, our data show that mitochondrial dynamics modulate expression of inflammatory mediators (i.e., TNFα, CD80, and PD-L1) in human DC-subsets. Our data highlights the importance of mitochondrial remodeling in innate sensing.

Both fission and fusion proteins also play a key role in mitophagy regulation. Upon stress, Drp1 specifically splits a mitochondrion into a healthy fraction and a damaged fraction, to promote degradation of damaged fraction *via* mitophagy (23). To this end, our data show that TLR-stimulation induces BNIP3-dependent mitophagy in CD1c$^+$ mDC. Additionally, we demonstrate that TLR-stimulated mitophagy and glycolysis are essential for CD1c$^+$ mDC activation. We further demonstrate induction of *Enolase*-dependent glycolysis in CD1c$^+$ mDC upon TLR-stimulation. Consistently, ENO2 inhibition impairs CD1c$^+$ mDC maturation and activation.

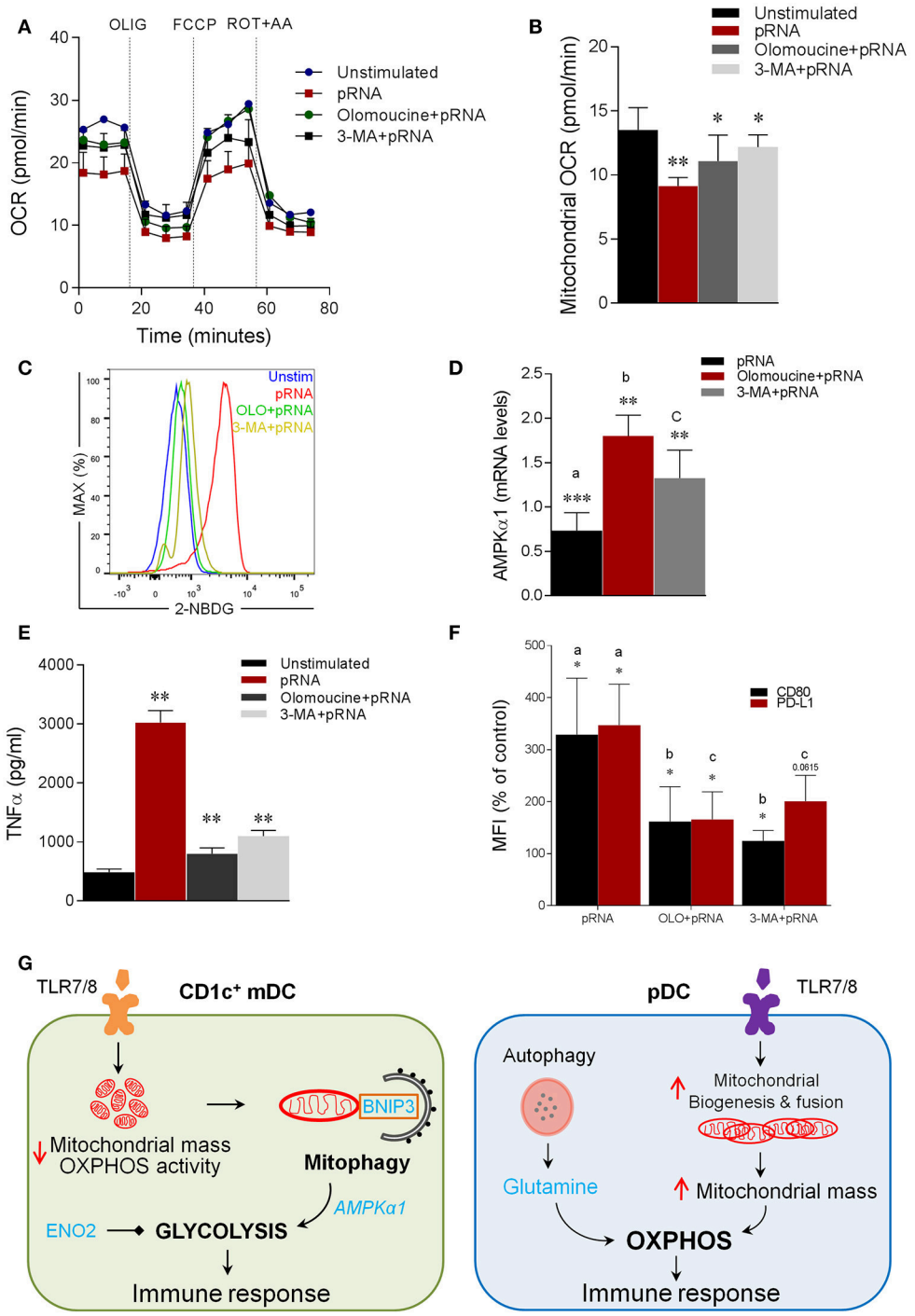

FIGURE 6 | Mitophagy is indispensable for induction of glycolysis and activation of CD1c$^+$ mDC **(A)** Mitochondrial fitness test of CD1c$^+$ mDC stimulated with pRNA for 6 h in the presence or absence of 10 μM olomoucine or 25 μM 3-MA. Data represents mean ± SEM of three independent experiments. **(B)** Data was collected within same experiments as **(A)** but is shown separately for better understanding. Data represents mean ± SEM of three independent experiments. *$p < 0.05$; **$p < 0.01$ (Student's *t*-test). **(C)** Flow cytometry histograms of 2-NBDG stained CD1c$^+$ mDCs stimulated with pRNA pDC for 6 h. **(D)** *AMPKα1* mRNA levels were analyzed after 6 h of pRNA stimulation by (qPCR) and normalized to β-actin expression by using the $2^{\Delta\Delta CT}$ method. Data represents Mean±SEM of three independent experiments **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test). **(E)** TNF-α levels on protein level were measured in the supernatant of the CD1c$^+$ mDC stimulated for 6 h. Data represents mean ± SEM of three independent experiments **$p < 0.01$ (Student's *t*-test). **(F)** Percentage mean flouresence intensity of maturation markers (CD80 and PD-L1) in CD1c$^+$ mDC cells stimulated for 6 h in the presence or absence of 10 μM olomoucine or 25 μM 3-MA. Data represents mean ± SEM of three independent experiments *$p < 0.05$ (Student's *t*-test). **(G)** Proposed model of human DC-subsets activation via TLR7/8 agonist (CD1c$^+$ mDC) TLR-stimulation reduces mitochondrial content, OXPHOS activity and induces glycolysis in CD1c$^+$ mDC. TLR-stimulation in CD1c$^+$ mDCs results in depolarized mitochondrial membrane potential ($\Delta\psi$) and triggers BNIP3-dependent mitophagy which is required for induction of glycolysis and activation of CD1c$^+$ mDC (pDC) TLR-stimulation increases OXPHOS and mitochondrial content as result of increased protein levels of Mfn2 and PGC1α in pDC. Moreover, TLR-stimulation in pDC increases intracellular glutamine in an autophagy-dependent manner. TLR-induced glutaminolysis fuels increases OXPHOS in pDCs which are indispensable for pDC activation.

These results implicate increased glycolysis for proficient antigen processing and presentation by $CD1c^+$ mDC to induce a robust immune response. Previously, *Chlamydia* infection was shown to increase mitochondrial permeability in parallel with mitochondrial remodeling in Enolase1 (ENO1)-dependent manner in mouse bone marrow-derived DCs (89). Intriguingly, BNIP3-dependent mitophagy contributes to mitochondrial elimination during polarization toward pro-inflammatory and glycolytic macrophages (90).

Of note, metabolic reprogramming toward glycolysis is regulated by mitophagy, as mitophagy inhibition reduced expression of glycolysis regulators e.g., *PFKFB3, HK2, GAPDH*, and *PKM2* (90). Therefore, it is conceivable that BNIP3-dependent mitophagy similarly controls glycolysis regulators in $CD1c^+$ mDC. We found that BNIP3 regulates transcriptional activity of *AMPKα1*. AMPK is a negative regulator of aerobic glycolysis (91). Intriguingly, AMPK activation has been reported to antagonize glycolytic switch in DCs (14). Our data shows that TLR7/8-stimulation decreases *AMPKα1* which can be restored upon BNIP3 inhibition. In contrast, loss of BNIP3 has been reported to reduce AMPK activity in liver (92). However, recent studies have demonstrated that AMPK activation can also be regulated *via* reactive oxygen species (ROS) (93). Of note, mitophagy regulates ROS (19), which in turn can act as transcription factor to control gene expression (94). Therefore, it is possible that BNIP3 inhibition reduces mitophagy, which in turn suppresses ROS levels to modulate *AMPKα1* in $CD1c^+$ mDC. Glycolysis is also required for canonical activation of the inflammasome in macrophages (95, 96). Interestingly, TLR-stimulation has been shown to induce inflammasome activation in $CD1c^+$ mDC (97). Intriguingly, autophagy negatively regulates NLRP3 inflammasome activation in macrophages and bone marrow derived DC (98, 99). Moreover, mitophagy prevents hyper-inflammation triggered by NLRP3 inflammasome activation in macrophages (100). Our data show that mitophagy is indispensable for $CD1c^+$ mDC activation. Collectively, our data suggest a scenario in which TLR-stimulation results in mitochondrial fission leading to induction of mitophagy, which in turn regulates glycolysis via *AMPKα1* to activate $CD1c^+$ mDC.

It has been demonstrated that autophagy is required for production of type I IFNs in pDC following TLR7 signaling *in vitro* and *in vivo* (48–52). To this end, TLR7-stimulated autophagy deficient pDCs are unable to produce IFNα, in comparison to their autophagy proficient counterparts (48, 49). We here demonstrate that autophagy serves to provide glutamine to fuel OXPHOS in pDC upon TLR-stimulation, similar to mechanisms previously shown in tumor cells (54–56). Our data show that TLR-stimulation in pDCs increases cellular glutamine levels in an autophagy dependent-manner. Additionally, autophagy inhibition abrogates glutamine fueled OXPHOS in pDCs upon TLR stimulation. Autophagy is involved in regulating several DC functions e.g., DC maturation, antigen presentation, cytokine production, DC migration and T-cell activation (101). Herein, we provide novel insight into pDC innate sensing mechanism by providing link between autophagy and type I IFN production by demonstrating that autophagy serves to provide glutamine, which is required for IFNα production. Conversely, selective autophagy i.e., mitophagy is required for induction of glycolysis *via* AMPKα1 regulation. Thus, our data provides novel mechanistic insight in differential role of autophagy in human DC subsets that can lead to immunostimulatory phenotype.

TLR stimulation triggers a shift in metabolism toward aerobic glycolysis, in human mDCs and mouse bone-marrow derived DCs (BMDCs), which is indispensable for the immune effector function and survival of DCs (14, 15, 102, 103). This shift toward glycolysis is required to support the metabolic requirements coupled with increased protein synthesis, which contributes to DC immunogenicity. This TLR-induced surge in glycolysis initiates *de novo* fatty acid synthesis through glucose-dependent citrate metabolism, which sustains the synthesis and secretion of inflammatory cytokines (103, 104). Furthermore, disrupting the glucose-to-citrate pathway reduces DC maturation, cytokine secretion and in turn T cell stimulatory capacity. Influenza virus (flu), Rhinovirus (RV) and a TLR7 agonist induce early glycolysis in human pDC, which is required for type I IFN production and upregulation of HLA-DR, CD80, CD86 (105). However, the generated type I IFN can in turn signal through IFNAR in a paracrine way to trigger FAO and OXPHOS in pDC (16). We find increased glutamine levels after TLR-stimulation in pDC. Of note, glutaminase inhibition in pDCs attenuated OXPHOS, suggesting that glutaminolysis drives OXPHOS induction in response to TLR stimulation in pDC. The requirement of glutamine for various immune effector functions has been demonstrated, e.g., LPS-driven inflammatory response in succinate-dependent anaplerosis (106, 107). However, these reports show that activity of glutamine depends on glycolysis. In contrast, it has also been reported that glutamine drives glucose-independent TCA cycle (108). Additionally, glutamine has been demonstrated to be required for trained immunity in monocytes (109), for activated T cells to fuel metabolism (110) and cytokine production by lymphocytes and macrophages (111). Tumor associated M2-like macrophages utilize glutamine for TCA cycle activity, which is required for M2 polarization (112). Moreover, tumor associated macrophages in glioblastoma show increased glutamate transport and metabolism (113). Intriguingly, glutaminolysis has been reported to be dispensable for mouse bone marrow-derived DCs cultured in the presence of GM-CSF for activation upon TLR-stimulation (114). Moreover, it is possible that type I IFN paracrine signaling in TLR-stimulated pDC contributes to the induction of fatty acid oxidation, as shown for CpG stimulated murine pDC (16).

Our study provides several novel insights into TLR-stimulated metabolic adaptations in human DC subsets. Our data demonstrate that different DC-subsets engage distinct metabolic adaptations in a mitochondrial dynamics-dependent manner following TLR stimulation. Furthermore, our study provides novel mechanistic insights in human DC-subset metabolism by demonstrating the involvement of mitophagy dependent-glycolysis in $CD1c^+$ mDC and autophagy supplemented

glutaminolysis for OXPHOS in pDC (**Figure 6G**). As metabolic manipulation results in modulation of DC activation, our results may have important implications in development of DC-based therapies.

AUTHOR CONTRIBUTIONS

FB and TM performed the experiments. FB analyzed the data. FB, DS, and IdV wrote the manuscript. IdV supervised the research.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02489/full#supplementary-material

Supplementary Figure 1 | (A–D) Data were collected within the same experiments as **Figure 2C** but are shown separately for clarity. Data represents mean ± SEM of three independent experiments. ***$p < 0.001$ (Student's *t*-test). **(E–H)** Data were collected within the same experiments as **Figure 3C** but are shown separately for clarity. Data represents mean ± SEM of three independent experiments. **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test).

Supplementary Figure 2 | (A–D) Data were collected within the same experiments as **Figure 4B** but are shown separately for clarity. Data represent mean ± SEM of three independent experiments. ***$p < 0.001$ (Student's *t*-test). **(E)** Relative gene expression of mitophagy related genes in CD1c$^+$ mDC. Data represents mean ± SEM of three independent experiments. *$p < 0.05$; **$p < 0.01$ (Student's *t*-test).

Supplementary Figure 3 | (A) Depicted is the mean fluorescence intensity of cells stained with 2-NBDG as percentage of the mean fluorescence intensity of control cells ± SEM of four independent experiments *$p < 0.05$ (Student's *t*-test). **(B)** TNF-α levels on protein level were measured in the supernatant of the CD1c$^+$ mDC stimulated for 6 h in the presence or absence of 10 nM rotenone or 10 nM antimycin A. Data represents mean ± SEM of three independent experiments **$p < 0.01$ (Student's *t*-test). **(C)** Percentage mean fluorescence intensity of maturation markers (CD80 and PD-L1) in CD1c$^+$ mDCs stimulated for 6 h in the presence or absence of 10 nM rotenone or 10 nM antimycin A. Data represents mean ± SEM of three independent experiments. **$p < 0.01$ (Student's *t*-test). **(D)** IFN-α levels on protein level were measured in the supernatant of the pDC stimulated for 6 h in the presence or absence of either 5 μM S3 or 1 μM Mdivi-1 or 10 μM olomycine. Data represents mean ± SEM of three independent experiments ***$p < 0.001$ (Student's *t*-test). **(E)** Percentage mean fluorescence intensity of maturation markers (CD80 and PD-L1) in pDC stimulated for 6 h in the presence or absence of either 5 μM S3 or 1 μM Mdivi-1 or 10 μM olomycine. Data represents mean ± SEM of three independent experiments. **$p < 0.01$; *$p < 0.05$ (Student's *t*-test).

Supplementary Figure 4 | (A–D) Data was collected within same experiments as 6A, but is shown separately for better understanding. Data represents mean ± SEM of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test).

Supplementary Figure 5 | CD1c$^+$ mDC were stimulated with pRNA for 12 h in the presence or absence of 5 mM 2-DG or 500 nM SF2312 or 1 μM Mdivi-1. CD1c$^+$ mDCs were stained with Fixable Viability Dye eFluor™ 780.

Supplementary Figure 6 | CD1c$^+$ mDC were stimulated with pRNA for 12 h in the presence or absence of 5 μM S3 or 25 μM 3-MA or 10 μM olomoucine or 2 μM niclosamide. CD1c$^+$ mDCs were stained with Fixable Viability Dye eFluor™ 780.

Supplementary Figure 7 | PDC were stimulated with pRNA for 12 h in the presence or absence of 5 μM BPTES or 10 μM DON or 25 μM 3-MA. pDCs were stained with Fixable Viability Dye eFluor™ 780.

Supplementary Figure 8 | PDC were stimulated with pRNA for 12 h in the presence or absence of 10 nM rotenone or 10 nM antimycin A. pDCs were stained with Fixable Viability Dye eFluor™ 780.

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REVIEW
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Regulation of Dendritic Cell Immune Function and Metabolism by Cellular Nutrient Sensor Mammalian Target of Rapamycin (mTOR)

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Dendritic cell (DC) activation is characterized by an acute increase in glucose metabolic flux that is required to fuel the high anabolic rates associated with DC activation. Inhibition of glycolysis significantly attenuates most aspects of DC immune effector function including antigen presentation, inflammatory cytokine production, and T cell stimulatory capacity. The cellular nutrient sensor mammalian/mechanistic Target of Rapamycin (mTOR) is an important upstream regulator of glycolytic metabolism and plays a central role in coordinating DC metabolic changes and immune responses. Because mTOR signaling can be activated by a variety of immunological stimuli, including signaling through the Toll-like Receptor (TLR) family of receptors, mTOR is involved in orchestrating many aspects of the DC metabolic response to microbial stimuli. It has become increasingly clear that mTOR's role in promoting or attenuating inflammatory processes in DCs is highly context-dependent and varies according to specific cellular subsets and the immunological conditions being studied. This review will address key aspects of the complex role of mTOR in regulating DC metabolism and effector function.

Keywords: dendritic cell (DC), mTOR, immune metabolism, glycolysis, metabolism regulation

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INTRODUCTION

As the quintessential professional antigen presenting cells of the immune system, dendritic cells (DCs) play a central role in coordinating both innate and adaptive immune responses through efficient recognition and uptake of extracellular material and the potent ability to provide both presented antigen and costimulatory signals required for proper T lymphocyte activation (1). DC activation is typically initiated by Pattern Recognition Receptor (PRR) interactions with microbe-associated ligands, as has been exhaustively characterized for the Toll-like receptor (TLR) family of innate immune receptors (2–4). Signaling downstream of these receptors induces important transcription and translation programs in DCs that are essential for the induction of their immune effector function. While DCs share many of the innate immune features of other myeloid cells of the mononuclear phagocyte lineage such as the expression of PRRs, efficient endocytic, and phagocytic clearance of extracellular matter, and robust induction of cytokine-driven inflammatory response upon activation, DCs undergo a distinct cellular program of maturation that is affiliated with their important contributions to T cell activation. These latter functions include the processing and presentation of antigens on MHC molecules, the upregulation

of co-stimulatory molecule expression, and the induction of chemokine receptor expression that drives DC migration to secondary lymphoid organs where DCs encounter and activate T lymphocytes through cognate antigen interactions (1, 5, 6).

While historically the field of immunology has focused on immune cell regulation at the transcriptional and translational levels, the recent emergence of the field of "immunometabolism" has provided the scientific community with a new framework for thinking about immune cell activation; specifically, how nutrient availability and usage controls the cellular effector functions important for immunological protection. One of the cornerstone findings of recent advances in the field is the observation that immune cell activation, in both the lymphoid and myeloid lineages, is broadly characterized by an increased flux of glucose metabolism, often termed in the literature as "aerobic glycolysis" as both a historical nod to the analogous "Warburg metabolism" described in cancer cells [reviewed in Potter et al. (7)] and to emphasize that the increase in glucose metabolism is not systemically induced by hypoxic conditions (8–18). Analogous to lymphocyte dependence on glucose metabolism for activation [reviewed in (17, 19)], TLR stimulation of DCs induces significant upregulation of aerobic glycolysis that is required for the survival and immune effector function of both human and mouse DCs (10–12, 15, 20, 21). As a cellular nutrient sensor and important upstream regulator of glycolytic metabolism, Mammalian Target of Rapamycin (mTOR) plays a central role in coordinating DC metabolic changes and immune responses. mTOR's role in cell biology is far-reaching and highly complex, regulating a diverse network of cellular responses including cell metabolism, energy homeostasis, protein translation, cellular differentiation, and proliferation, autophagy, and cell survival. Despite this complexity, the existence of highly selective, non-toxic, and FDA-approved mTOR inhibitors such as rapamycin has allowed the research community to broadly interrogate the role of mTOR function in DC biology at both the cellular and organism/patient level. As the role of mTOR in immune cell development and autophagy regulation have been covered comprehensively by previous reviews [reviewed in (22–24)], these aspects of mTOR biology will not be covered in depth. Instead, the focus of this review will be to highlight and discuss the current understanding of mTOR-dependent metabolic regulation of DC function.

THE ROLE OF mTOR IN CELLULAR METABOLISM

The hierarchical regulation of cellular metabolism and energy homeostasis can be functionally partitioned into two opposing "programs," anabolism and catabolism, each governed by a distinct central upstream regulator. Energetic anabolism, generically characterized by reduced metabolic activity coupled to energy conservation and production, is controlled by AMP-activated protein kinase (AMPK) in response to low cellular ATP levels or nutrient starvation [reviewed in (25)]. Catabolism, contrastingly comprised by high rates of energy expenditure for nutrient breakdown and molecular biosynthesis, is controlled by mTOR complex activity [reviewed in (26)]. Not surprisingly, these two processes cross-regulate each other, most notably by AMPK inhibition of mTOR activation. While AMPK has been implicated in important aspects of DC biology (10, 27, 28), it is a notably understudied aspect of DC metabolic biology and this review will focus primarily on mTOR-mediated metabolic regulation of DCs.

The mTOR protein itself functions as a required component of two major signaling complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). While mTORC1 is primarily responsible for cellular energy expenditure and protein translation, mTORC2 serves an important role as a positive regulator of mTORC1. For the purposes of this review, "mTOR activity" will refer to mTORC1 functions unless otherwise noted. It is notable that while mTOR promotes sustained catabolism of carbohydrates, it concurrently supports the *de novo* synthesis of lipids, proteins, and amino acids, serving as an important checkpoint in converting increased cellular fuel consumption into processes such as cell division and protein production that have obvious implications for broad physiological responses, including those carried out by immune cells (29). As a downstream target of the PI3K/Akt signaling axis, mTOR activation in DCs can be initiated by a number of immunologically relevant factors, including cytokine signaling, growth factor signaling, and PRR signaling. In light of this, mTOR is positioned as a critical molecule integrating immunological stimuli into changes in cellular metabolism that regulate protein translation events required for the immunological function of these cells. The role of mTOR in governing immune cell homeostasis and the use of mTOR inhibitors as viable immunoregulatory strategies continue to be of intense interest to the field (23).

DC COMMITMENT TO AEROBIC GLYCOLYSIS

Activation of DCs via TLRs promotes significant upregulation of aerobic glycolysis, which regulates the immune function of both human and mouse DCs (10–12, 18, 20, 21, 30, 31). To date, ligands for both MyD88 -dependent and -independent TLR members have been shown to result in an acute upregulation of glycolysis (20), as well as ligands for the C-type Lectin Receptors Dectin-1/2 (21), suggesting that this metabolic reprogramming may be a broadly conserved feature of PRR signaling. A wide variety of approaches, including inhibition of glycolysis through culture with 2-deoxy-glucose (2DG), pharmacological inhibition of glycolysis-regulating signaling pathways, and genetic silencing of rate-limiting glycolysis enzymes, have demonstrated that loss of glycolytic capability significantly impairs DC effector functions, including antigen presentation, co-stimulatory molecule expression, chemotaxis, cytokine secretion, and T lymphocyte stimulatory capacity (10–12, 18, 20, 21, 30, 31). The prevailing consensus has emerged that acute, and in some cases sustained, metabolic commitment to elevated rates of glucose catabolism are an essential metabolic requirement for proper DC activation. We have previously

argued that DC metabolic reprogramming can be functionally partitioned into two temporal phases governed by distinct signaling events (32): (1) an acute induction of glycolysis occurring within minutes of TLR activation that supports the high biosynthetic demand associated with early DC maturation for several hours (20); (2) a long-term commitment to glycolysis in subsets of nitric oxide (NO) -producing DCs that is required for their metabolic adaptation to NO-mediated mitochondrial toxicity (12, 15).

Acute Glycolytic Reprogramming in DCs Is mTOR-Independent

Rapid induction of glycolysis in DCs, occurring within minutes of TLR stimulation, is controlled by a PI3K/TBK1/IKKε/Akt signaling axis that promotes the rapid translocation of Hexokinase 2 (HK2) to the mitochondria which supports the rapid flux of glucose catabolism associated with DC maturation (20). Glucose is rapidly consumed by activated DCs and glucose-derived carbons are primarily invested in pentose phosphate pathway (PPP) metabolism, lactate production, and citrate synthesis via the mitochondrial citrate shuttle, the latter presumably supporting fatty acid synthesis associated with endoplasmic reticulum, and Golgi body -dependent translation and secretory pathways that control inflammatory cytokine production (16, 20). The source of glucose that fuels this early activation comes from both the import of extracellular glucose, and the catabolism of intracellular glycogen pools that these cells possess in the resting state (18). The acute induction of glycolysis mediated by the PI3K/TBK1/IKKε/Akt signaling axis, conserved in multiple DC subsets in both mouse and human systems (20, 21, 31), is transient (lasting approximately 6–8 h) after which glycolysis levels gradually wane close to their pre-activation levels (20). The inability of mTOR inhibitors to attenuate this early wave of glycolysis indicates that mTOR activation is positioned downstream of early glycolysis commitment in DCs (12, 15, 20).

Sustained Glycolytic Reprogramming in DCs Is mTOR-Dependent

While a number of studies have concluded that that mTOR, and one of its downstream transcription factors HIF1α, are required for DC glycolytic reprogramming (9, 30, 33–35), we and others have shown that this is primarily the case for the long-term commitment to glycolysis observed in NO-producing DCs that express inducible nitric oxide synthase (iNOS) and is independent of the acute glycolytic reprogramming events described above (12, 15). In iNOS-expressing DCs, largely restricted to inflammatory monocyte-derived DCs in the mouse and minor subsets of human DCs [previously reviewed in (32)], mTOR–dependent HIF1α activity promotes iNOS expression in TLR-activated DCs (30, 36). iNOS protein expression becomes detectable just as the acute induction of glycolysis begins to wane (12, 15), followed by NO-mediated suppression of DC mitochondrial activity (12, 15) through reversible inhibition of mitochondrial cytochrome c oxidase function (37, 38). Through its regulation of iNOS expression, mTOR regulates the long-term commitment of these cells to glycolytic metabolism in a NO-dependent manner (12, 15). Notably, mTOR inhibition decreases NO production and restores mitochondrial function in iNOS-expressing DCs which leads to increased metabolic flexibility and enhanced inflammatory activity in these cells (11). While the multifaceted and highly complex NO-independent impacts of mTOR on DC function are discussed in more detail below, it is impossible to ignore the important role of mTOR in regulating DC iNOS expression and the implications of this on DC metabolism.

DC Lipid Metabolism

Metabolite tracing studies have shown that the rapid catabolism of glucose in TLR-stimulated DCs is closely linked with a number of biosynthetic pathways including the preferential generation of citrate through the TCA cycle (16, 20). Citrate production is understood to support fatty acid synthesis required for the expansion of endoplasmic reticulum and Golgi body cellular structures associated with DC activation (16, 20, 39, 40). While mTOR signaling is known to promote lipid biosynthesis (29), the explicit role of mTOR in regulating the citrate and fatty acid biosynthesis in stimulated DCs remains poorly defined. Nevertheless, the regulation of lipid metabolism in DCs has clear immunological relevance as there are notable instances where parasite infection of DCs leads to significant changes in lipid metabolism (41). In addition, LPS stimulation leads to specific increases in cellular ceramide concentration in DCs and immunogenic DCs and tolerogenic DCs display unique intracellular lipid profiles (42). With respect to cholesterol lipid metabolism, it is clear that this too is a highly important process in DCs. Liver X receptor, an important regulator of cholesterol metabolism, is implicated in promoting both DC differentiation and immune activation (43). Cholesterol hydroxylase activity is specifically upregulated by Type-I interferon signaling in macrophages and DCs (44), and both PRR and MHC molecules are associated with cholesterol-enriched lipid raft microdomains in the plasma membrane of DCs (45, 46). While it is logical that mTOR activity is involved in regulating these processes based on its role in other cell types, further work delineating mTOR's role in DC lipid metabolism is an important area for future investigation.

mTOR REGULATION OF DC EFFECTOR FUNCTION

Because pharmacological inhibitors of mTOR function attenuate lymphocyte proliferation, mTOR signaling has classically been considered to play a broadly pro-inflammatory role in the immune system and has been used extensively for its systemic tolerogenic properties in the clinic. Recent studies at the cellular level have revealed that mTOR can exert both inflammatory and anti-inflammatory effects depending on the physiological context and cellular subsets in question. A summary of these findings for mTOR's documented impact on various aspects of DC effector function are delineated below.

DC Maturation and Co-stimulatory Molecule Expression

While some studies have concluded that mTOR inhibition has minimal or contrasting effects on aspects of DC activation (47–49), other studies have argued that mTOR inhibition dramatically influences DC maturation in either a positive or negative direction. Many studies in both the mouse and human system have shown a negative impact on DC maturation (47, 50–52), while others have shown that mTOR inhibition can actually augment DC activation (10, 11, 15, 50). In one study, treatment with 1,25-dihydroxyvitamin D_3 was shown to induce an mTOR-dependent tolerogenic phenotype in monocyte-derived human DCs (moDCs) that was characterized by decreased surface expression of CD80, HLA-DR, and CD86, and increased production of IL-10 (53). In mouse bone marrow -derived DCs (BMDCs), shRNA knocking down AMPK, a negative regulator of mTOR activity, increased costimulatory molecule expression while AMPK agonists decreased DC maturation (10). These studies show a rapid de-phosphorylation of AMPK upon LPS stimulation and that IL-10 attenuates LPS-mediated AMPK de-phosphorylation (10). Taken together, these findings are consistent with a model whereby reduced AMPK activity and concomitant mTOR induction serve as a "master switch" to promote DC maturation and immune function. Consistent with this, we and others have shown that CD40 and CD86 expression on mouse BMDCs and subsets of human DCs can be enhanced by mTOR inhibition during TLR stimulation of these cells (11, 15, 50). One of the most informative studies for resolving the published discrepancies on the role of mTOR in DC maturation, published by Haidinger et al. showed that mTOR inhibitors negatively regulate IL-4/GM-CSF -differentiated moDC activation, but augment maturation of freshly isolated myeloid DCs from human peripheral blood (50). In accordance with this, multiple studies have reported that the requirement for mTOR signaling in DC development and function varies with respect to the DC subsets in question (50, 54, 55). This intriguing idea, that mTOR exhibits disparate roles in unique DC subsets is further supported by the finding that different DC subsets engage distinct metabolic signatures to support their specialized function (56, 57). To this point, tolerogenic DCs are reported to exhibit an increased dependence on mitochondrial metabolism, in contrast to the glycolysis-centric phenotype observed for many inflammatory DC subsets (56, 57). The fact that mTOR activator is typically considered an upstream promoter of protein translation, it is interesting that mTOR inhibitors can actually augment proinflammatory molecule production in certain DC subsets (11, 15, 50). This phenomenon is not restricted to the role of mTOR in regulating iNOS expression as it has also been reported for circulating human DCs that do not produce NO upon activation (11, 50).

Cytokine Production

The production of cytokines has been the most widely studied DC effector function with respect to the role of mTOR in these cells. Freshly isolated mouse splenic pDCs exhibit an mTOR -activated phenotype, and rapamycin-treated mouse and human pDCs show impaired secretion of multiple cytokines including Type I interferons, TNF-alpha, and IL-6 (58). In *L. monocytogenes* infected mice, rapamycin treatment protected animals from lethal challenge, and led to increased serum concentrations of IL-12p70, IFN-γ, and IL-6 (59). Treatment of human moDCs with the phytochemical cytopiloyne, which is reported to preferentially target mTORC2 signaling, lowers LPS-driven costimulatory molecules expression and inflammatory cytokine production (60). Another study identified a beta-Catenin/mTOR signaling axis as a primary driver of DC IL-10 production responsible for CD8+ T lymphocyte activation (61). Multiple studies have shown that inhibition of mTOR leads to decreased IL-10 production, often concomitant with increased production of inflammatory cytokines such as IL-12 and IL-6 (50, 62). Consistent with this, mTORC1 signaling by intestinal DCs has been shown to be required for IL-10 production and tolerance homeostasis in the gut (62). The connection between mTOR signaling and DC IL-10 production is particularly noteworthy because IL-10 has been shown to antagonize long-term glycolysis commitment in BMDCs (10). Upon LPS stimulation of whole blood from kidney transplant patients treated with rapamycin, IL-12p40, IL-6, TNF-α, and IL-1β levels were increased while IL-10 levels were decreased, compared to patient controls (63). Furthermore, rapamycin treatment of human CD14+ monocytes was shown to enhance IL-12p40, IL-12p70, and TNF-α production, and decrease IL-10 production, upon LPS stimulation (63). Interestingly, in a murine sepsis model, while dexamethasone treatment led to 100% survival, rapamycin treatment led to ∼40% survival, and combined treatment led to ∼50% survival, providing *in vivo* evidence that the pro-inflammatory impact of mTOR inhibition by rapamycin supersedes the anti-inflammatory impact of dexamethasone stimulation of the glucocorticoid receptor (63). With respect to cytokine production, there is a fair amount of consistency regarding the role of mTOR-dependent promotion of IL-10 production as an important brake on the inflammatory cytokine output by DCs.

Antigen Presentation and T Cell Stimulation

An important prerequisite for a DC's ability to stimulate T cells *in vivo* is its capacity to traffic to secondary lymphoid organs upon activation. While one study has shown that rapamycin limits DC lymph node trafficking in a mouse psoriasis model (64), other studies have shown no impact of rapamycin treatment on CCR7 expression or *in vivo* migratory capacity (47). In general, further studies are needed to better define the role of mTOR in regulating DC chemotaxis and migration, particularly given the high metabolic demand that these processes likely require. With regard to mTOR's impact on DC T cell stimulatory capacity, the published literature indicates that this phenotype is highly dependent on the DC subset in question. One study reported that rapamycin treatment enhanced the ability of TLR7-stimulated human pDCs to promote both the proliferation of CD4+ T cells and the induction of T regulatory cells (48), while other studies support the idea that rapamycin treatment

globally suppresses DC capacity to stimulate T lymphocytes (49, 53, 58, 65). A more nuanced look at mTOR's role in T lymphocyte activation has shown an important role for mTOR-mediated Th1/Th2 skewing of CD4+ cells, with the majority of studies demonstrating that mTOR preferentially supports Th2 lymphocyte activation, presumably through its function in promoting IL-10 production by DCs (62, 66, 67). Additionally, mTOR inhibition by rapamycin has been shown to promote T regulatory cell induction both *in vitro* and *in vivo* through DC-dependent action (68).

In contrast to the studies above, we and other have shown that mTOR inhibition can enhance T cell stimulatory capacity in certain contexts (11, 15, 50, 69). mTORC2 deficiency has been documented to augment CD8+ lymphocyte -mediated graft rejection in mice (70), while mice given autologous DCs simulated with LPS in the presences of rapamycin led to a negative impact on T lymphocyte activation and improved graft vs. host survival (71). In GM-CSF -differentiated BMDCs, both mTOR inhibitors enhance LPS-driven DC activation and T cell stimulatory capacity, at least in part through attenuation of mTOR-dependent nitric oxide generation (11, 15). In multiple mouse models of autologous DC vaccination, rapamycin conditioning of DCs enhanced vaccine efficacy to both melanoma tumor challenge (11) and tuberculosis infection (69). This phenotype is not restricted to mouse cells as freshly isolated CD1c+ human myeloid DCs have also been shown to exhibit enhanced T cell proliferation with rapamycin conditioning (50). Nevertheless, whether or not rapamycin treatment can enhance DC autologous vaccination regimens in humans remains to be determined.

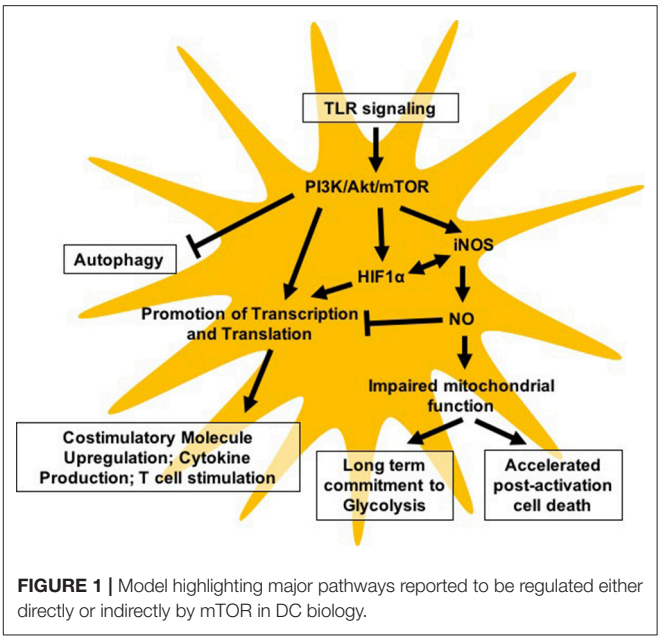

FIGURE 1 | Model highlighting major pathways reported to be regulated either directly or indirectly by mTOR in DC biology.

mTOR Control of Mouse iNOS Expression and NO Production

We have previously reviewed the profound impact that DC iNOS expression and NO production has on the metabolism, survival, and immune function of these cells (32). TLR activation induces iNOS expression in mouse BMDCs, and the long-term metabolic commitment to glycolysis in these cells is driven by reversible NO-mediated inhibition of mitochondrial respiration in these cells (12, 15). Furthermore, iNOS inhibition or deletion leads to prolonged post-activation survival and enhanced immunostimulatory capacity in mouse BMDCs (11, 12, 15). Interestingly, several recent studies have shown that mTOR promotion or *Leishmania* infection can downregulate iNOS expression in macrophages *in vivo*, suggesting that regulation of iNOS expression may depend critically on complex factors *in situ* (72, 73). A recent study, investigating the role of innate immune receptor signal strength in modulating DC metabolism and function, showed that stronger stimuli induce higher iNOS expression, NO production, and more dramatic inhibition of mitochondrial respiration (21). In these studies, even though early activation is associated with mTORC1 activity, only strong inflammatory stimuli induce sustained mTORC1 and mTORC2 activation (21). Other studies have suggested that mTOR-driven glycolysis regulates iNOS expression itself (30). These studies showed that both glucose depletion and rapamycin inhibition led to decreased HIF1α and iNOS expression, and promotion of HIF1α activity induced *Nos2* (iNOS) mRNA expression (30). Interestingly, a reciprocal relationship between iNOS and HIF1α was observed as iNOS inhibition or deletion also led to diminished HIF1α expression (30). These studies showed that the relationship between mTOR -mediated metabolic changes and iNOS activity is complicated, but defined an anti-inflammatory effect of glucose metabolism on DC immune function that is dependent on an mTOR/HIF1α/iNOS signaling circuit (30). Furthermore, there is evidence to suggest that T cell depletion of local glucose levels in the tissue microenvironment can impact mTOR/HIF1α/iNOS activity (30). While the contribution of mTOR-mediated iNOS expression and function to DC metabolism is striking, it is noteworthy that even in DC subsets that do not produce NO, mTOR inhibition can augment DC immune function (11, 15, 50).

It noteworthy to consider that only specific subsets of DC populations express iNOS in both mice and humans. GM-CSF -differentiated mouse BMDCs classically induce iNOS expression when stimulated by LPS and IFN-γ (74). From an *in vivo* perspective, monocyte-derived inflammatory DCs (originally termed TNF-a/iNOS-producing-DCs, or "TipDCs") are potent NO producers and are required to control a number of different types of both bacterial and viral infections (75–77). However, conventional tissue-resident DC subsets in secondary lymphoid organs rarely express iNOS in mice (12, 78, 79), and GM-CSF/IL-4—cultured monocyte-derived human DCs (moDCs) also do not express iNOS (12, 80). Despite these differences, it is clear that human DC populations can express iNOS *in vivo* including blood circulating $CD1a^{+}$ DCs (81) and DCs found in psoriatic skin lesions (82–84). Given the heterogeneity of iNOS expression in DC subsets discussed above, it is clearly important to consider the subsets of DCs under investigation

when interpreting the literature and this heterogeneity alone may explain key discrepancies among various studies. Nevertheless, even as a relatively rare subset, iNOS-expressing DCs have important immunological and metabolic consequences in the inflammatory tissue microenvironment that is important to consider (32).

mTOR REGULATION OF DC LIFESPAN AND SURVIVAL

While mTOR -dependent NO production has been strongly implicated in promoting DC cell death in iNOS-expressing DC subsets (11, 15), there is significant evidence to suggest that mTOR also plays a role in promoting DC survival in other contexts. Treatment of C57BL/6 splenic CD11c+ mature dendritic cells with AMPK activators, which directly antagonize mTOR activity, leads to increased pro-apoptotic molecule expression (85). In addition, rapamycin treatment of splenic CD11c+ mature dendritic cells increased apoptosis, indicating that mTOR promotes cell survival in this system (85). Interestingly, CCR7 expression induced by DC activation leads to inhibitory phosphorylation of AMPK and subsequent activation of mTOR signaling, which supports the post-activation survival of these cells (85). In further support of mTOR serving as a survival promoter in DCs, rapamycin-treated CD34+ hematopoietic progenitor cells resulted in impaired interstitial DC development, indicating that PI3K/mTOR regulates proliferation and survival (86). In addition, human moDCs treated with rapamycin induces decreased expression of anti-apoptotic protein mcl-1 and drives moDC apoptosis (87).

mTOR CONTROLS AUTOPHAGY IN DCs

The process of autophagy, whereby cytoplasmic components are ingested, degraded, and their molecular components recycled, plays important roles in DC antigen presentation [recently reviewed in (24)]. It has been elegantly shown that autophagy is a constitutive process in DCs that actively contributes endogenous peptide antigens to MHC-II complexes in the resting state (88). Upon TLR stimulation, the increase in mTOR activity inhibits the formation of the autophagy initiation complex, thereby restricting autophagy rates from the basal state (89). Experts in the field have argued that mTOR-dependent autophagy inhibition leads to a decreased emphasis on endogenous antigen presentation and increased presentation of exogenous antigen (22, 89). In support of this model, IL-4 -mediated induction of autophagy has been shown to augment endogenous antigen presentation on MHC-II molecules (90). Interestingly, while mTOR attenuates autophagy-dependent contributions to antigen presentation, it concomitantly promotes the presentation of exogenously acquired antigens by supporting lysosome acidification and endolysosomal trafficking of MHC-II/peptide complexes to the cell surface (91–93). These findings have clear clinical significance as kidney transplant patients on mTOR-inhibitor therapy exhibit higher levels of alloreactive T cells, possibly due to enhanced autophagy-dependent presentation of donor-endogenous antigen (94). While it seems clear that activated DCs downregulate autophagy in an mTOR -dependent manner, the contribution of autophagy to the nutrient compartment of resting DCs and its counter-regulation by AMPK remains an underexplored topic.

CONCLUDING REMARKS

Given the importance of metabolic changes in supporting the immune activation of DCs, it is not surprising that the central metabolic regulator mTOR plays a critical role in coordinating activation-associated changes in DC metabolism and function (**Figure 1**). While mTOR has well-documented impacts on DC development, immune effector function, and survival, the challenge in the field rests in understanding the complex and nuanced role that mTOR plays in distinct DC subsets and specific immunological contexts. To this point, it is evident that mTOR can influence DC biology in either a pro-inflammatory or anti-inflammatory direction, which can complicate the interpretation of data where global inhibition of mTOR is employed. Significant aspects of mTOR-mediated regulation of DC biology that would benefit from further investigation include the role of mTOR in nutrient flux in both basal and activated conditions, the cross-regulation of these processes by AMPK, the contribution of mTOR signaling to lipid metabolism, and a further delineation of differential mTOR signaling in distinct DC subsets in both mouse and human cells. We look forward with interest to the ongoing work in the field that will help resolve some of these discrepancies and better clarify the distinct contribution of mTOR signaling to the heterogeneous family of DCs in the mammalian immune system.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Metabolic Regulation of Dendritic Cell Differentiation

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Dendritic cells (DCs) are important antigen-presenting cells (APCs) that play essential roles in bridging innate and adaptive immune responses. Differentiation stages of DC subsets from bone marrow progenitor cells have been well-defined during the past decades. Features that distinguish DC progenitor cells from each differentiation stages, related signaling pathways and transcription factors that are crucial for DC lineage commitment have been well-elucidated in numerous studies. Recently, growing evidence are showing that cellular metabolism, as one of the most fundamental process of cells, has essential role in the modulation of immune system. There have been multiple reports and reviews that focus on the metabolic modulations on DC functions, however little attention had been paid to the metabolic regulation of DC development and differentiation. In recent years, increasing evidence suggests that metabolic regulations also exert significant impact on DC differentiation, as well as on the homeostasis of tissue resident DCs. The focus of this review is to summarize the findings from recent studies on the metabolic regulation of DC differentiation and to discuss the impacts of the three major aspects of metabolism on the processes of DC development and differentiation, namely the changes in metabolic pathways, the molecular signaling pathways that modulate cell metabolism, and the effects of metabolites and nutrients. The aim of this review is to draw attentions to this important and exciting research field where the effects of metabolic process and their regulation in DC differentiation need to be further explored.

Keywords: dendritic cell (DC), cell differentiation, metabolic regulation, glycolysis, fatty acid (FA), mitochondria function, mTOR pathway, nutrients

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INTRODUCTION

Dendritic cells (DCs) are specialized cells that not only recognize the pathogens by the various pattern recognition receptors (PRRs) and initiate the innate immune response, but also can uptake, process and present antigens to naïve T cells, thus promote the activation of adaptive immune response (1). Based on the expression of distinct cell surface molecules, the requirement for specific transcription factors essential for their development, the origins, and their tissue localizations, DCs can be classified into four types: the plasmacytoid DCs (pDCs), the conventional DCs (cDCs) which can be further divided into cDC1 and cDC2 subsets, the monocyte-derived DCs (moDCs), and Langerhans cells (LCs). DC subsets, especially cDCs acquire distinct features in different tissue environments. Characterizations of distinct functions of these DC subsets (2–4), and the molecular

regulation network for their development and differentiation (5–7) have been well-summarized in many review articles. The major features of these DC subsets are summarized in **Table 1** (7–11).

Apart from Langerhans cells which were shown to have an embryonic origin, most DC subsets are derived from hematopoietic stem cells (HSCs) (12). A series of DC progenitors have been identified based on their surface expression of molecules of hematopoietic progenitors, such as CD117, CD135, and their differentiation potential *in vitro* and *in vivo*. In mouse, both common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) can give rise to all the DC subsets (13, 14). The common DC progenitors (CDPs) are the committed precursors for both pDCs and cDCs (15, 16). Within the bone marrow, CDPs differentiate into pre-cDCs and pre-pDCs, and pre-pDCs further differentiate into pDCs. Both pDCs and pre-cDCs then migrate from the bone marrow to the lymphoid and non-lymphoid tissues, where pre-cDCs terminally differentiate to cDC1 and cDC2 subsets (17–19). Similarly, human granulocyte macrophage progenitors (GMP) can be divided into three sub-populations, based on their differentiation potential determined by clonal analysis at single cell level: one give rise to granulocyte, monocyte and DCs, defined as hGMDP, which is equivalent to murine CMP; one produce monocytes and DCs, defined as hMDP; the hCDP subpopulation can only differentiate into DC subsets, equivalent to murine CDP (20, 21). The differentiation capacity of these DC progenitors and their relationships help to define the lineage map of DC development. In *in vitro* culture system, murine pDC, cDC1, and cDC2 subsets can be generated from the bone marrow cells in the presence of fms-like tyrosine kinase 3 receptor ligand (Flt3L); bone marrow cells can also differentiate into $CD11c^{hi}$ MHC-II^{hi} $CD11b^{+}$ DCs in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 (22, 23). Human monocyte-derived DC (moDCs) can be obtained from purified blood $CD14^{+}$ monocyte or total peripheral blood mononuclear cells in the culture system supplemented with GM-CSF and IL-4 (24). And human myeloid DCs or Langerhans cells can also be generated from human $CD34^{+}$ hematopoietic progeniter cells with different cytokines (25–28). As shown in **Figure 1B**.

As metabolism is the essential process in all cell types, the effects of metabolic pathways on immune cell differentiation and functions have recently attracted great attention (29–32). Although limited, increasing numbers of studies are now revealing the importance of metabolic pathways involved in the modulation of DC development and differentiation. In this review, we will summarize the findings from recent studies on the metabolic regulation of DC differentiation and discuss the three major aspects that impact the processes of DC development and differentiation: the changes in metabolic pathways, the molecular signaling pathways that modulate cell metabolism, and the effects of metabolites and nutrients. Aiming to draw attentions to this promising research field where the effects of metabolic process and their regulator mechanisms in DC differentiation need to be further investigated.

ROLE OF GLYCOLYSIS AND MITOCHONDRIA FUNCTION

Glycolysis is one of the most important components in glucose metabolism which converts glucose into pyruvate in the cytoplasm. Pyruvate then either transforms into lactate as metabolite of anaerobic glycolysis in the cytoplasm or enters Krebs cycle in mitochondria. Regulation of glycolysis in immune cell development, differentiation and/or activation has been well-characterized in T cells (33), B cells (34, 35), and macrophages (36). Growing evidences have shown that function of glycolysis is essential for DC activation (31), but its role during DC differentiation is less well-investigated. Recently Kratchmarov et al. showed that blockage of glycolysis by 2-deoxyglucose (2-DG) *in vitro* led to defects in Flt3L-induced mouse DC progenitor proliferation, indicating that glycolysis is required for DC development (37).

Under hypoxia condition, the conversion of pyruvate into lactic acid is favored, and ATP is generated for cellular energy supply. It was reported that lactic acid accumulated in DC cultures with high cell density induced reprogramming of human moDC differentiation, which vanish their ability to produce inflammatory cytokines and chemokines upon activation compared with moDCs developing at low cell culture density, instead they tend to produce the anti-inflammatory cytokine IL-10 upon activation (38). Another study showed that hypoxia condition suppressed the generation of pDCs from bone marrow progenitor cells in Flt3L supplemented culture system, and knockout of HIF-1α in monocyte/DC progenitors (MDP) in LysM-cre HIF-$1\alpha^{fl/fl}$ mice can reverse the defects caused by hypoxia condition. Although not stressed by the authors, it is notable that the number of cDC1s ($CD24^{+}$ cDCs) other than cDC2 ($SIRP\alpha^{+}$ cDCs) also reduced under hypoxia condition (39).

Under the condition when oxygen is sufficient, pyruvate enters Krebs cycles whose products participate in oxidative phosphorylation (OXPHOS) to generate ATPs in mitochondria. This process generates more ATPs but at a slower rate compared with glycolysis. Compared to their precursor monocytes, larger number of mitochondria, higher endogenous respiratory activity, increased activity of the mitochondrial marker enzyme citrate synthase and a robust ATP production were observed in *in vitro* generated human moDCs. Inhibition of complex I in electron transport chain (ETC) by Rotenone resulted in an impaired differentiation of human moDCs accompanied by glycolysis compensation and compromised ATP production (40). Moreover, elevated mtDNA copy number and a rapid increase of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) followed by upregulation of Mitochondrial transcription factor A (TFAM) and Nuclear respiratory factor 1 (NRF-1) were also observed during *in vitro* generation of human moDC (41). It is also reported that during GM-CSF induced bone marrow-derived mouse moDC generation, upregulation of mitochondrial fusion-related proteins was also observed, indicating an active

TABLE 1 | Murine and human dendritic cell subsets are outlined with their surface phenotype, major transcription factors required for their development and their main functions (7–11).

DC subset		Surface maker					Specific transcription factors	Common function	
		Murine			Human			Antigen presentation and cytokine production	Downstream effect
cDC	cDC1	$CD11c^{hi}$, $MHC\text{-}II^{+}$, $CD45RA^{-}$, Siglec H^{-}, $PCDA\text{-}1^{-}$	$XCR1^{+}$, $Clec9A^{+}$, $DEC205^{+}$	$CD8\alpha^{+}$ (lymphoid tissues) $CD103^{+}$ (non-lymphoid tissues)	$HLA\text{-}DR^{+}$, $CD11c^{+}$, $CD123^{-}$	$XCR1^{+}$, $CD141^{+}$, $Clec9A^{+}$	Irf8, Id2, Batf3	Direct and cross-presentation IL-12, IL-6, Type III IFN	$CD8^{+}$ T cell activation Th1 activation
	cDC2		$Sirp\alpha^{+}$, $CD11b^{+}$	$Esam^{hi}$, $CD4^{+}$, $Clec4a4^{+}$ (lymphoid tissues) $Esam^{lo}$, $Clec12A^{+}$, $CD103^{+/-}$ (non-lymphoid tissues)		$Sirp\alpha^{+}$, $CD1c^{+}$, $CD301^{+}$	Irf4, Notch2, Klf4	Direct presentation IL-6, TNF-α IL-23 (intestinal and lung)	Th2 and Th17 activation
pDCs		$CD11c^{int}$, $MHC\text{-}II^{-}$, $CD45R^{+}$, $CD45RA^{+}$, Siglec H^{+}, $PCDA\text{-}1^{+}$			$CD11c^{lo}$, $CD123^{+}$, $CD45RA^{+}$, $BDCA2^{+}$, $BDCA4^{+}$		E2-2, Irf8, Bcl11a, Runx2, SpiB	Type I, Type III interferons IL-12, IL-6	Antiviral immunity
moDCs		$CD11c^{+}$, $MHC\text{-}II^{+}$, $CD11b^{+}$			$CD11c^{+}$, $HLA\text{-}DR^{+}$, $CD14^{+}$, $BDCA1^{+}$, $Fc\varepsilon RI^{+}$, $CD206^{+}$		Klf4, Irf8	TNF-α, IL-12, IL-23, iNOS	Th1 and Th17 response
Langerhans cells		$EpCAM^{hi}$, $MHC\text{-}II^{+}$, $CD11b^{+}$, $CD11c^{+}$			$CD11c^{lo}$, $CD1a^{hi}$, $Langerin^{+}$		Id2, Runx3	Dermal and epidermal antigen presentation IL-23, IL-6, IL-1β	Th17 response

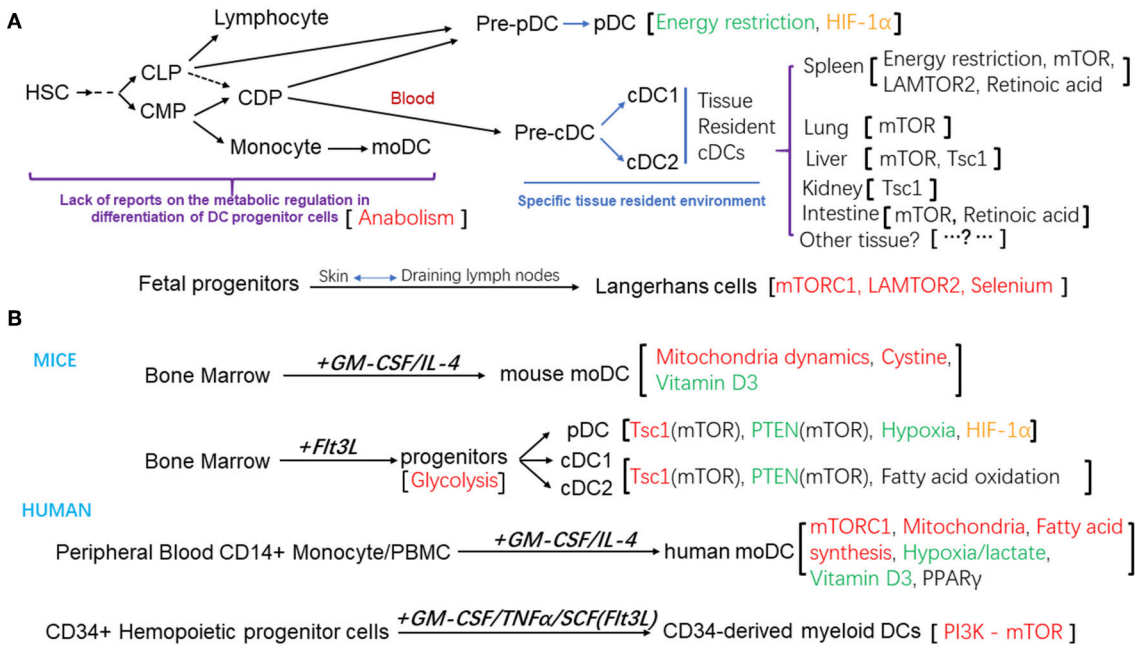

FIGURE 1 | (A) Schematic diagram of the differentiation and development of DC subsets and the metabolic regulation factors that modulate these processes. **(B)** Different *in vitro* culture system for the generation of DCs from mouse bone marrow progenitors, or human peripheral blood mononuclear cells, CD14+ monocytes, or CD34+ Hemopoietic progenitor cells. The metabolic regulation factors were also listed. Positive regulators were in the red color, negative regulators were in the green color, regulators that affected the homeostasis of DC subsets were in the black color, regulator that is controversial for its role were in the orange color.

mitochondrial dynamic during DC differentiation (42). These evidences implied an active state of mitochondria during DC differentiation. Recent systematic analysis on the differences of transcriptomics, proteomics and phosphoproteomics between CD8α$^+$ DCs (cDC1) and CD8α$^-$ DCs (cDC2) showed that CD8α$^+$ DCs exhibit much stronger oxidative metabolism indicated by higher oxygen consumption rate (OCR). This indicated that aberrant mitochondria function may affect the expansion of CD8α$^+$ DCs which hold great importance in CD8$^+$ T cells mediated anti-tumor function *in vivo* (43).

Collectively, glycolysis is essential for the maintenance of DC progenitor cells, while proper function of mitochondria is required during the differentiation process of monocyte derived DCs both in human and in mouse. Researches described above all implied that that mitochondria function or oxidative metabolism, which produces more ATPs than anaerobic glycolysis in glucose metabolism, is favored in the development or expansion of the DCs subsets responsible for proinflammatory functions or antigen presentation to CD8$^+$ T cells. As pDCs are the main source of Type I and Type III interferons among all DC subsets (**Table 1**) and cDC1s hold great importance in CD8$^+$ T cells mediated anti-tumor function *in vivo*, these results may also point out that the hypoxia condition in the micro-environment of tumor mass may suppressed the expansion and function of the DC subsets which promote anti-tumor processes. Also avoiding hypoxia condition or inhibition of HIF1α in *in vitro* culture system would help to gain pDCs or cDC1s with proper functions for their clinical application more efficiently.

ROLE OF FATTY ACID METABOLISM

Fatty acids can also serve as fuel for energy production in many types of cells (44). DC development from human PBMC precursors was diminished by blockade of fatty acid synthesis. *In vivo* experiments in mice suggest that dendropoiesis was also hampered after injection of fatty acid synthesis inhibitor to mice, as demonstrated by reduced CD11c$^+$ cell numbers in liver, primary and secondary lymphoid organs (45). The nuclear receptor peroxisome proliferator activated receptor-γ (PPARγ), which is important in fatty acid metabolism is significantly up regulated in human monocyte derived DCs induced by GM-CSF and IL-4 *in vitro* and plays important role in human moDC generation (46–48).

Intriguingly, a recent report on the regulation of mTOR on metabolic adaption of DCs during allergic inflammation in lung using CD11c-cre mTOR$^{fl/fl}$ mice indicated that the fatty acid metabolism, especially the fatty acid oxidation played important roles in the function and the expansion of inflammatory CD11b$^+$ DCs in lung upon HDM induced allergic inflammation (49). This *in vivo* data indicated that the fatty acid oxidation that may be mediated by mTOR is essential for the generation of inflammatory DCs.

Moreover, Kratchmarov et al. reported that inhibition of catabolism-associated fatty acid oxidation with an inhibitor etomoxir did not affect the development of total cDCs and pDCs, but led to significantly increased frequency of IRF4 dependent cDC2 and decreased frequency of IRF8 dependent cDC1 cells in Flt3L-supplemented culture system (37). The homeostasis of

changes of cDC subsets and their distinct functional features dependent on specialized signaling pathways and transcription factors (**Table 1**). This study implied that different cDC subsets may prefer specific metabolic status for their distinct functions, and the metabolic pathways may crosstalk with these signaling pathways and affect the differentiation of certain DC subsets. Crosstalk between fatty acid metabolism and glucose metabolism can be bridged by NADPH and acetyl-CoA. Defects in fatty acid oxidation will lead to the aberrant level of acetyl-CoA which may in turn affect the Krebs cycles in mitochondria. Notably, increasing evidences indicate that high concentrations of etomoxir may have off-target effects on inhibiting adenine nucleotide translocase (ANT) and the electron transport chain (ETC) in macrophage and T cells (50). Concentration of etomoxir used by Kratchmarov et al. was relatively high and off-target effects may also exist. *In vivo* DC specific knockout of Cpt1a the target of etomoxir is needed to validate the observation *in vitro*. However, together with the *in vivo* systematic research that cDC1 exhibit higher oxidative metabolism (43), both researches demonstrated that proper mitochondria function and oxidative metabolism is essential for the expansion of cDC1s.

On the other hand, fatty acid metabolisms such as the fatty acid synthesis and oxidations are frequently activated in tissue resident immune cells especially in liver and lung with distinct resident metabolic environments. The results of effect of fatty acid synthesis on the generation of moDCs and liver resident DCs, as well as fatty acid oxidation on the expansion of $CD11b^+$ inflammatory DCs under allergic status in lung indicated that tissue resident environment maybe the major factor that affect the metabolism tendency during DC differentiation. Further studies on the effects of environmental factors on the metabolic pathways favored for the differentiation of various tissue resident DCs should provide new insights into how the differentiation of tissue resident DC is regulated by metabolism, and potential targets maybe identified for modulating these processes in certain disease settings.

ROLE OF MAMMALIAN TARGET OF RAPAMYCIN (mTOR)

The mTOR pathway responds to various environmental cues such as nutrients and growth factors and controls numerous cellular processes that related to cell growth and metabolism. The mTOR protein is a serine/threonine protein kinase in the PI3K-related kinase (PIKK) family and mainly forms two functional protein complexes, mTORC1 and mTORC2 (51). In recent years growing evidence has shown that the mTOR pathway, especially mTORC1, plays essential role in the development and differentiation of DCs (52). Deletion of mTOR using *CD11c-Cre* system disturbed the homeostasis of tissue resident DC subsets in lung, spleen, liver as well as white adipose tissue and large intestinal lamina propria etc. (49). mTORC1 controls terminal myeloid differentiation by affecting population of the mature circulating monocytes and the development of neutrophils and DCs trough mTORC1-Myc pathway (53), and is essential for the development of cDCs, pDCs as well as Langerhans cells (52–58). In DC culture system that generate DCs for human hematopoietic precursors, the PI3K-AKT-mTOR pathway was stimulated during the GM-CSF and IL-4 induced monocyte-derived DCs differentiation. Inhibition of mTORC1 with rapamycin disrupted the GM-CSF signaling pathway and induced apoptosis of human moDCs in *in vitro* differentiation system (54, 55). The PI3K-mTOR pathway is also required for generation of pDCs and myeloid DCs from human $CD34^+$ hematopoietic progenitor cells in *in vitro* culture system supplemented with different cytokines (56, 57). In mice, rapamycin which inhibits the mTOR pathway was shown to block the Flt3L induced generation of all DC subsets both in culture and *in vivo* (58, 59). And the homeostasis of Langerhans cells was proved to be depend on the mTORC1 pathways other than the mTORC2 pathways (60).

Activation of PI3K-AKT-mTOR pathway by deleting an intrinsic inhibitor—phosphatase and tensin homolog (Pten), greatly accelerated DC development in Flt3L-supplemented bone marrow (BM) culture system and can partially restore the defects caused by the presentation of rapamycin. The DC-specific loss of Pten (*Cd11-Cre* system) resulted in cell-intrinsic expansion of $CD8^+$ or $CD103^+$ cDC1 *in vivo*. However, Pten deletion showed little effect on DCs generated in GM-CSF supplemented BM cultures, indicating a diverse regulatory mechanism of Pten and mTOR pathways in different DC progenitors (59). Ablation of the tuberous sclerosis 1 (Tsc1), another negative regulator of mTORC1, using $Tsc1^{f/f}$*-ERCre* system, led to more rapid expansion of BMDCs and bigger cell size than that of control cells in GM-CSF supplemented BM cultures (61). However, almost at the same time Wang et al. reported that knockout of Tsc1 using Rosa26-Cre-ER^{T2} system up-regulated cell metabolic programs including glycolysis, mitochondrial respiration and lipid synthesis, but significantly impaired DC development *in vivo* and in Flt3L-supplemented culture system. The mechanistic study revealed that a Tsc1-mTOR and Myc axis orchestrated metabolic programming during DC development (62). Myc, a critical transcription factor for stem cell and cancer cell proliferation was demonstrated to be one of the downstream effectors of mTORC1 (62). One of the paralogues of Myc, L-Myc was specially upregulated in DC progenitors and affect cDC subsets especially cDC1 in lung and liver, and it can be regulated by GM-CSF and IRF8. Furthermore, overexpression of c-Myc in $Flt3^+$ CMPs reduced the proportion of mature cDCs and pDCs in Flt3L supplemented cultures (63). Although discrepancies exist in the regulatory mechanism of Pten and Tsc1, these results all suggest that the mTOR-Myc pathway is important for proliferation of DC progenitors and for expansion of DC subsets.

Discrepancies of results from studies described above implied a complicated and precise regulatory mechanism of mTOR pathway in the development and differentiation of DCs. On one hand, downstream effectors of Flt3L activated signaling pathway that mainly rely on activation of STAT3 and Flt3L supplemented mouse BM culture supports the generation cDCs and pDCs, while downstream effectors of GM-CSF activated pathway that mainly activates STAT5 which supports the generation of

monocyte derived DCs, but suppress the pDC development from mouse BM (64, 65). It is still not clear how activation of PI3K-mTOR may cross talk with JAK-STATs pathways. Pten or Tsc1 may be involved differently in the activation of STAT3 or STAT5. On the other hand, deletion of Pten or Tsc1 may also activate other signaling or metabolic pathways that may be differently involved in the downstream of Flt3L or GM-CSF activated pathway, but more evidences are required to elucidate these possibilities.

Attenuating mTORC1 pathway by depleting Raptor, an essential component of mTORC1 pathway, in DCs resulted in expansion in splenic $CD8^+$ cDCs and intestinal $CD11c^+CD11b^+$ cDCs (66). Another mTOR positive regulator, the late endosomal/lysosomal adaptor and MAPK and mTOR activator 2 (LAMTOR2) is a member of the Regulator/LAMTOR complex and regulates mTOR and extracellular signaling-regulated kinase (ERK) cascade. Deletion of LAMTOR2 in CD11c expressing cells (*Cd11c-Cre*) led to significant reduction of Langerhans cells in the epidermis soon after birth by impairing mTOR and ERK signaling (67). However, enlarged spleen and lymph nodes were observed with expanded cDCs and pDCs in aged mice with conditional knockout of LAMTOR2 in DCs (*Cd11c-Cre*). Since LAMTOR2 is also important for the endosome function, the accumulation of Flt3 on cell surface and downstream super-activated mTOR signal in LAMTOR2 knockout aged mice may be caused by a feedback regulation (68). Since LAMTOR2 has functions other than modulating the mTOR pathway, the consequences caused by LAMTOR2 knockout might be different in different DC subsets and a feedback regulatory mechanism may also contribute to the different outcomes in aged LAMTOR2 knockout mice.

On balance, these results all point out that precise regulatory network of mTOR is essential in DC development and differentiation. Although it was observed by Sinclair et al. that mTOR modulates the homeostasis of DC subsets in different tissues in quite diverse manners (49), the regulatory mechanisms of mTOR and the metabolic changes in various tissue resident DC differentiation warrant further investigation.

ROLE OF NUTRIENTS

General Effect of Nourishment

Energy restriction (ER) which is also known as calorie restriction was shown to inhibit the mTOR pathway. However, ER induces cell metabolic changes not only through inhibition of mTOR, but also through its principal upstream regulators—AMPK and Akt and its downstream targets p70S6K and 4E-BP1 (69, 70). NIH-31 is a rat and mouse diet standard set up by the National Institutes of Health that takes the nutrient loss during autoclaved sterilization in account. Comparing to mice that consumed NIH-31 diet *ad libitum* (have free access to food or water), ER mice that consumed 40% energy-restricted NIH-31 diet had significantly reduced bone marrow CDP, pre-DC populations and splenic $CD8^+$ cDC and pDC populations (71).

Vitamins

Vitamin A and vitamin D3 were shown to have crucial impact on DC differentiation.

By feeding mice with vitamin A-deficient diet or high vitamin A diet, Beijer et al. demonstrated that Vitamin A was specifically necessary for the development of $RelB^{high}$ Notch-dependent $CD4^+$, and $CD8^-CD4^-$ cDCs (72). Meanwhile, Klebanoff et al. reported that pan-retinoic acid receptors (pan-RARs) antagonist treatment caused a selective loss of the splenic $ESAM^{high}$ cDC2 population and the developmentally-related intestinal $CD11b^+CD103^+$ cDCs (73). In addition to the terminal differentiation of cDC2, retinoic acid signaling was also shown to modulate the generation of gut-tropic migratory DC precursors—pre-mucosal DCs (pre-μDCs) from bone marrow progenitors both *in vitro* and *in vivo* (74).

1,25-Dihydroxyvitamin D3 (calcitriol) is the active form of vitamin D3. In 2000, four groups reported the inhibitory role of calcitriol in DC differentiation from murine or human monocyte *in vitro*. Addition of calcitriol impeded human and murine moDC differentiation from human PBMC or monocyte and murine bone marrow cells, respectively (75–78). While vitamin D receptor, the nuclear hormone receptor for vitamin D3, is repressed by IL-4 induced GATA-1 during human moDC differentiation, its expression is induced by TGF-β1 and has positive impact during human Langerhans cell lineage commitment (79). However, the cell-specific influence of VD3 on DC differentiation *in vivo* has not been properly addressed yet.

Amino Acids

Amino acids as important components of proteins also take part in many metabolic processes. Glutathione was reported to play a crucial role in protecting cell from oxidative stress and it also has a protective role for DCs. The cystine/glutamate antiporter transports cystine (oxidized form of cysteine) into the cell for the glutathione biosynthetic pathway in exchange for glutamate. D'Angelo et al. reported that blocking cystine/glutamate antiporter activity impeded human moDC differentiation but did not affect LPS-induced DC maturation (80). The roles of other amino acids in DC differentiation are yet to be uncovered.

Dietary Minerals

Selenium (Se) is an essential micronutrient that is important for metabolism process like proper thyroid hormone metabolism and has non-negligible effects on the immune system through its incorporation into selenoproteins. Inadequate intake of Se has been reported to compromise immune responses in animals and in human (81). Five weeks of Se-deficient diet treatment can decrease the epidermal Langerhans cell numbers by half in mice (82). The role of Se during DC differentiation has also been studied in chicken. Addition of inorganic Se (sodium selenite) in the culture system was reported to accelerate the differentiation of chicken DCs from chicken peripheral blood monocytes (83).

Studies above pointed out diverse effect of vitamins, amino acids and selenium on DC development. Other nutrients were also reported to have important effect on function or survival of DC. For example, vitamin C and vitamin E inhibits activation of human moDC upon proinflammatory cytokine stimulation

(84); Zn^{2+} triggers murine moDC apoptosis through stimulating ceramide formation (85). Whether other nutrients influence differentiation of DCs awaits further study. Distinct tissue resident DC subsets with different functions are regulated by different tissue environments. Metabolic environment in different tissues may significantly impact the differentiation of pre-DCs to resident DC subsets. For example, in lung the cells have better access to oxygen in adipose tissue the fatty acid metabolism is more active, whereas in intestine, the metabolism of various carbohydrates, peptides and small nutrients are highly active. Intestinal DCs, for instance, are among the first line of immune cells that encounter dietary nutrients, thus, it is highly possible that these nutrients function as major regulators in the differentiation of intestinal DC from pre-DCs. Based on the finding that retinoic acid was involved in regulation of intestinal DC differentiation (73, 74), as well as the study showing that gut microbiota-derived short chain fatty acids could serve as competitive regulators for intestinal DC differentiation (86, 87), it is reasonable to assume that homeostasis of tissue resident DC subsets may also be susceptible to distinct metabolic pathways in other tissues. Further exploration of the exact roles of different metabolites and nutrients in the differentiation of different tissue resident DCs should provide new knowledge for better understanding the importance of metabolic regulation of DC differentiation and function, and the potential correlations between immune alterations and some metabolic diseases.

DISCUSSION AND CONCLUSION

In recent years, emerging evidence has revealed that the metabolic modulation is essential for the development and function of immune system. Some evidence also suggested that the differentiation and activation of DCs might also be under metabolic modulation. A better understanding of the metabolic regulation of DC development and differentiation will not only help to establish the crucial network amongst various molecular regulatory mechanisms and metabolic regulations, but also help to elucidate the potential association of altered DC differentiation and activation with some metabolic diseases. However, current knowledge in this field is still limited. In this review we summarized these findings from published studies as shown in **Figure 1**. As reviewed by O'Neill et al. multiple metabolic inhibitors have been used in studies to validate the role of specific metabolic pathways in immune system (88). However, only few inhibitors were tested to determine the roles of metabolic regulation in DC differentiation. Most of the published studies were done with the *in vitro* culture systems supplemented with Flt3L or GM-CSF, although they provided useful information for these mentioned metabolic pathways in DC differentiation, clear and definitive conclusions can only be drawn from properly designed *in vivo* studies, and those should be the major focus of the further studies. Furthermore, little is known about the regulatory mechanisms of these metabolic pathways and their interplay/cross talk with other molecular or epigenetic regulation pathways known important for DC differentiation, such as regulations by transcription factors, cytokines and microRNAs. The impacts of other metabolic pathways including the pentose phosphate pathway (PPP) and nitrogen metabolism pathways on DC differentiation are yet to be determined. In addition, apart from the mTOR pathway, the effects of other molecular signaling pathways that regulate metabolism such as AMPK pathway on DC differentiation are not yet clearly elucidated. The role of other nutrients including minerals in DC differentiation also needs more attention for their easy access in daily diets.

Immunotherapy has shown a bright future for cancer treatment. The functions of DCs are crucial for the effectiveness of these therapies. Impairment of DC homeostasis or function are related to many diseases, such as inflammatory diseases (89, 90), autoimmune diseases (91) and cancer (92–94). The DC vaccines also hold a promising potential for developing more effective approaches for the treatment of various immune related diseases. The *in vitro* generation of various DC subsets from hematopoietic progenitor cells is non-substitutable in the studies of human DC differentiation. They can also serve as the main source of DCs for DC related therapies or DC vaccines. Modulation of specific metabolic pathways or addition of particular nutrients during the generation of DCs according to their metabolism requirements, may help to obtain specific DC subsets desired for various clinical applications. More extensive studies of the metabolic regulation of DC development and differentiation should be one of the priorities in the field of DC biology and the new knowledge gained from these studies will facilitate the clinical applications of DCs in the treatment of some immune-related diseases.

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LW supervised the writing, analyzed, and edited this manuscript. ZH wrote, organized and edited the manuscript. XZ, ZS, and TW wrote part of the review.

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FcγR-TLR Cross-Talk Enhances TNF Production by Human Monocyte-Derived DCs via IRF5-Dependent Gene Transcription and Glycolytic Reprogramming

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Antigen-presenting cells (APCs) such as dendritic cells (DCs) are crucial for initiation of adequate inflammatory responses, which critically depends on the cooperated engagement of different receptors. In addition to pattern recognition receptors (PRRs), Fc gamma receptors (FcγRs) have recently been identified to be important in induction of inflammation by DCs. FcγRs that recognize IgG immune complexes, which are formed upon opsonization of pathogens, induce pro-inflammatory cytokine production through cross-talk with PRRs such as Toll-like receptors (TLRs). While the physiological function of FcγR-TLR cross-talk is to provide protective immunity against invading pathogens, undesired activation of FcγR-TLR cross-talk, e.g., by autoantibodies, also plays a major role in the development of chronic inflammatory disorders such as rheumatoid arthritis (RA). Yet, the molecular mechanisms of FcγR-TLR cross-talk are still largely unknown. Here, we identified that FcγR-TLR cross-talk-induced cytokine production critically depends on activation of the transcription factor interferon regulatory factor 5 (IRF5), which results from induction of two different pathways that converge on IRF5 activation. First, TLR stimulation induced phosphorylation of TBK1/IKKε, which is required for IRF5 phosphorylation and subsequent activation. Second, FcγR stimulation induced nuclear translocation of IRF5, which is essential for gene transcription by IRF5. We identified that IRF5 activation by FcγR-TLR cross-talk amplifies pro-inflammatory cytokine production by increasing cytokine gene transcription, but also by synergistically inducing glycolytic reprogramming, which is another essential process for induction of inflammatory responses by DCs. Combined, here we identified IRF5 as a pivotal component of FcγR-TLR cross-talk in human APCs. These data may provide new potential targets to suppress chronic inflammation in autoantibody-associated diseases that are characterized by undesired or excessive FcγR-TLR cross-talk, such as RA, systemic sclerosis, and systemic lupus erythematous.

Keywords: Fc gamma receptor (FcγR), interferon regulatory factor 5 (IRF5), dendritic cells, macrophages, glycolytic reprogramming, tumor necrosis factor (TNF), rheumatoid arthritis (RA), chronic inflammation

INTRODUCTION

Protection against different classes of pathogens requires the activation of antigen-presenting cells (APCs) such as dendritic cells (DCs). A crucial step for shaping both innate and adaptive immunity by DCs is the production of various pro-inflammatory cytokines. DCs produce these cytokines upon detection of pathogens or endogenous danger signals via activation of different families of receptors, which collectively are referred to as pattern recognition receptors (PRRs). Well-known examples of PRRs include the families of Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectins, and RIG-I-like receptors (RLRs). However, the list of receptor families that control cytokine production is still expanding.

In recent years, it has become clear that also the family of Fc gamma receptors (FcγRs), which are receptors for the Fc region of immunoglobulin G (IgG), play an important role in the induction of cytokines by DCs. While individual stimulation of FcγRs elicits little cytokine production, FcγRs synergize with PRRs such as TLRs to strongly but selectively amplify pro-inflammatory cytokine production. FcγRs synergize with TLRs that are expressed both intracellular (TLR3, TLR7/8) and extracellular (TLR2, TLR4, TLR5), as well as other receptors such as NLRs and particular cytokine receptors (1, 2). Combined, modulation of cytokine production by FcγRs thereby tailors immune responses to the immunological context (3, 4).

In human APCs such as DCs, the best studied cytokine-inducing FcγR is FcγRIIa. FcγRIIa has a low affinity for IgG, and is therefore able to discriminate between unbound IgG and IgG immune complexes (i.e., antigen-bound). While unbound IgG, as present under homeostatic conditions, induces inhibitory signaling (5), stimulation of FcγRIIa with immune complexes, as present on opsonized pathogens, strongly enhances cytokine production induced by TLRs (1, 6). Although monocytes and macrophages are known also to express other FcRs such as FcγRI, FcγRIIa is the main IgG receptor responsible for amplifying TLR responses (2).

The physiological function of FcγR-TLR cross-talk is to counteract infections with various classes of pathogens. For example, upon IgG opsonization of bacteria, the simultaneous activation of FcγRIIa and TLRs specifically amplifies the production of pro-inflammatory cytokines TNF, IL-1β, IL-6, and IL-23 by human DCs, which in turn promote human T helper 17 (Th17) skewing, thereby tailoring immune response to counteract extracellular bacterial infections (1, 6). However, in addition to its physiological function, FcγR-TLR cross-talk can also be induced undesirably by immune complex formation of autoantibodies. This pathological role of FcγR-TLR cross-talk contributes to the pathogenesis of various autoimmune diseases including rheumatoid arthritis (RA) (7).

Remarkably, while FcγR-induced cytokine production plays an important role in both host defense and various autoimmune diseases, still very little is known about the underlying molecular mechanisms. Similar to other FcγR-mediated functions such as phagocytosis and ADCC, FcγR-induced cytokine production is dependent on the upstream kinase Syk (8). However, recent findings indicate that the downstream signaling events required for FcγR-induced cytokine production are distinct from other FcγR-mediated functions such as phagocytosis (3). Compared to FcγR signaling, relatively more is known about the signaling pathways that are induced by individual stimulation of TLRs. TLRs signal via adaptor proteins such as MyD88 and/or TRIF to activate various transcription factors including NF-κB and MAP kinases, which are important for the transcription of pro-inflammatory cytokines such as TNF (9). Yet, how TLR and FcγR signaling pathways collaborate to synergistically amplify pro-inflammatory cytokine production is still largely unknown.

In this study, we identified that FcγR-TLR cross-talk-induced cytokine production critically depends on activation of the transcription factor interferon regulatory factor 5 (IRF5), which results from collaborative IRF5 activation by both FcγRs and TLRs. While TLR stimulation induced IRF5 phosphorylation, FcγR stimulation was required for IRF5 nuclear translocation. Moreover, we identified that IRF5 activation by FcγR-TLR cross-talk amplified pro-inflammatory cytokines production by both increasing cytokine gene transcription and by inducing glycolytic reprogramming, thereby identifying FcγRs as a new family of receptors that can induce metabolic reprogramming.

MATERIALS AND METHODS

Cells and Stimulation

This study was done according to the ethical guidelines of the Academic Medical Center and human material was obtained in accordance with the AMC Medical Ethics Review Committee according to the Medical Research Involving Human Subjects Act. Buffy coats obtained after blood donation (Sanquin blood supply) are not subjected to informed consent, which is according to the Medical Research Involving Human Subjects Act and the AMC Medical Ethics Review Committee. All samples were handled anonymously. Ethical review and approval was not required for this study in accordance with the local legislation. Monocytes were isolated from buffy coats by density gradient centrifugation on Lymphoprep (Nycomed) and Percoll (Pharmacia). DCs or macrophages were generated by culturing monocytes for 6 days in IMDM (Lonza) containing 5% FBS (Biowest) and 86 μg/mL gentamicin (Gibco), supplemented with 20 ng/mL GM-CSF (Invitrogen) and 2 ng/mL IL-4 (Miltenyi Biotec) for DCs or 50 ng/mL recombinant human M-CSF (BioLegend) for macrophages. At day 2 or 3, half of the medium was replaced by new medium containing cytokines.

For silencing at day 3, cells were harvest by resuspending (DCs) or by using TrypLE Select (Invitrogen) (macrophages). Cells were microporated in the presence of 500 nM IRF5 si-RNA or control si-RNA (Dharmacon) and cultured for 3 more days in IMDM without gentamicin with supplemented cytokines.

DCs were harvested at day 6 by putting the cells on ice for 30 min and macrophages were harvested at day 6 by TrypLE Select. For cIgG stimulation, 96-well high-affinity Maxisorp plates (Nunc) were coated with 2 μg/mL IgG from pooled IgG (Nanogam; Sanquin Blood Supply) diluted in PBS overnight at 4°C, followed by blocking with PBS containing 10% FBS for 1 h at 37°C. Cells were stimulated (30,000–50,000 cells per well) with 10 μg/mL Pam3CSK4 (Invivogen). Co-stimulation experiments

were performed by simultaneous exposure of the cells to cIgG and Pam3. Syk was inhibited with 1 μM R406 (Selleckchem), TBK1/IKKε was inhibited with 2 μM BX795 (Invivogen) and glycolysis was blocked using 10 mM 2-Deoxy-D-glucose (2DG; Sigma Aldrich). Cells were incubated with the inhibitor or the corresponding volume of DMSO (Sigma-Aldrich) or medium for 30 min at 37°C before stimulation.

Quantitative RT-PCR

For mRNA-level analysis, cells were lysed at the indicated time points, after which mRNA extraction was performed using RNeasy Mini Kit (Qiagen) and cDNA synthesis using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas). Quantitative RT-PCR (StepOnePlus Real-Time PCR System; Thermo Fisher Scientific) was performed using Taqman Master Mix and the following Taqman primers (all from Thermo Fisher Scientific): *GAPDH* (4310884E), *IRF5* (Hs00158114_m1), and *TNF* (Hs00174128_m1). mRNA levels were normalized to the geometric mean of the Ct-values of housekeeping gene *GAPDH* $[2^{\mathrm{Ct(housekeeping)-Ct(target)}}]$, and folds were calculated compared with an unstimulated control sample ($t = 0$ h).

ELISA

For analysis of cytokine production, supernatants were harvested after overnight stimulation and stored at −20°C. Cytokine levels in supernatants were measured by ELISA, using antibody pairs for TNF (eBioscience), IL-Iβ, IL-6, and IL-23 (U-CyTech Biosciences).

Fluorescence Microscopy

For analysis of IRF5 translocation, DCs or macrophages were stimulated as indicated in Maxisorp plates. After 2 h stimulation, cells were washed with PBS, fixed with 3.7% formaldehyde (Sigma-Aldrich) for 15 min at room temperature, washed in PBS and stored in PBS containing 0.5% bovine serum albumin (BSA; PAA) and 0.1% sodium azide (Merck) at 4°C. Cells were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 5 min at room temperature and blocked for 30 min in PBS containing 0.5% BSA and 0.1% sodium azide. Cells were then stained with a rabbit-anti-human-IRF5 antibody (1:400) (Cell Signaling) or rabbit-anti-human NF-kB p65 antibody (1:100) (Cell Signaling) for 45 min at room temperature, washed with PBS and stained with a Cy3-labeled goat-anti-rabbit-IgG antibody (1:50) (Jackson ImmunoResearch). Cells were again washed with PBS and nuclei were stained using 1 μg/mL Hoechst (Immunochemistry Technologies) for 1 min at room temperature. Cells were imaged using a DM IRB inverted fluorescence microscope (Leica), combined with a DFC 300FX digital color camera (Leica).

Flow Cytometry

For analysis of TBK1/IKKε phosphorylation, DCs or macrophages were stimulated as indicated in 48-well plates (Greiner Bio-One) for 30 min and fixed using Lyse/Fix buffer (BD Biosciences) for 10 min at room temperature. For analysis of IRF5, unstimulated DCs were also lysed and transferred in a 96-well plate following the same protocol as TBK1 phosphorylation. Cells were harvested by gentle scraping, transferred to a 96-well round-bottom plate (Greiner Bio-One), washed in PBS, and permeabilized using Perm III buffer (BD Biosciences) for at least 30 min at −20°C. Cells were then washed in PBS containing 0.5% BSA and 0.1% sodium azide and stained for 1 h at RT with a rabbit-anti-human-IRF5 antibody (1:200) (Cell Signaling) or a rabbit-anti-human-pTBK1 antibody (1:50) (Ser172; Cell Signaling), which also reacts to pIKKε, followed by a 30 min staining at room temperature with Alexafluor488-labeled goat-anti-rabbit-IgG antibody (1:400) (Molecular Probes). Fluorescence was determined by flow cytometry (Canto II, BD Biosciences).

Metabolic Assays

Real-time analysis of the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) of DCs were analyzed using an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience). 30,000 DCs were plated per well. To trigger FcγR on DCs XF-96 cell culture plates were coated with 4 μg/ml IgG prior to seeding of the cells. DCs were plated in glucose-free medium after which glucose was added (10 mM) to the cells during the assay to be able to determine true glycolysis-driven ECAR. Thirty minutes after glucose addition cells were stimulated with 10 μg/mL Pam3CSK4 during the essay after which OCR and glycolysis-driven ECAR were determined 30 min post stimulation.

Western Blot

For analysis of IRF5 phosphorylation, DCs were stimulated as indicated in 6-well plates (1,250,000–2,000,000 cell per well) (Costar) for 30 min. Cells were gently scraped and collected in cold PBS. After washing, cells were lysed on ice for 10 min using RIPA lysis buffy (Cell signaling) supplemented with protease and phosphatase inhibitors (both from Roche). Lysates were briefly sonificiated for 10 s at 30% and centrifuged for 10 min at 14,000 × g. BCA assay was performed (Thermo Scientific) and samples were boiled with 4x Laemmli Sample Buffer (Bio-Rad) for 15 min at 95°C. Cell lysates were run on a 4–12% Bis-Tris protein gel (Invitrogen) using MES-running buffer (Invitrogen). Proteins were transferred to a PVDF membrane (GE healthcare) using transfer buffer (Invitrogen) and blocked with 2% milk (Bio-Rad) afterwards. Membrane was incubated in TBS Tween o/n at 4°C with indicated antibodies: Phospho-IRF5 (Ser437) polyclonal antibody (1:1000) (Thermo Scientific), IRF5 (1:1000) (E1N9G) rabbit mAb (Cell Signaling), or Actin antibody (I-19) (1:2000) (Santa Cruz). Afterwards membrane washed with TBS Tween and incubated for 1 h at room temperature with polyclonal swine anti-rabbit immunoglobulins HRP (1:3000) (Dako).

Data Analysis

Co-localization quantification of the fluorescence microscopy data was done using Huygens Professional software (SVI, Hilversum, The Netherlands) calculating the Manders Coefficients. Western blots were analyzed using ImageJ. Data were analyzed for statistical significance using student's *t*-test with GraphPad Prism version 7 software (GraphPad Software).

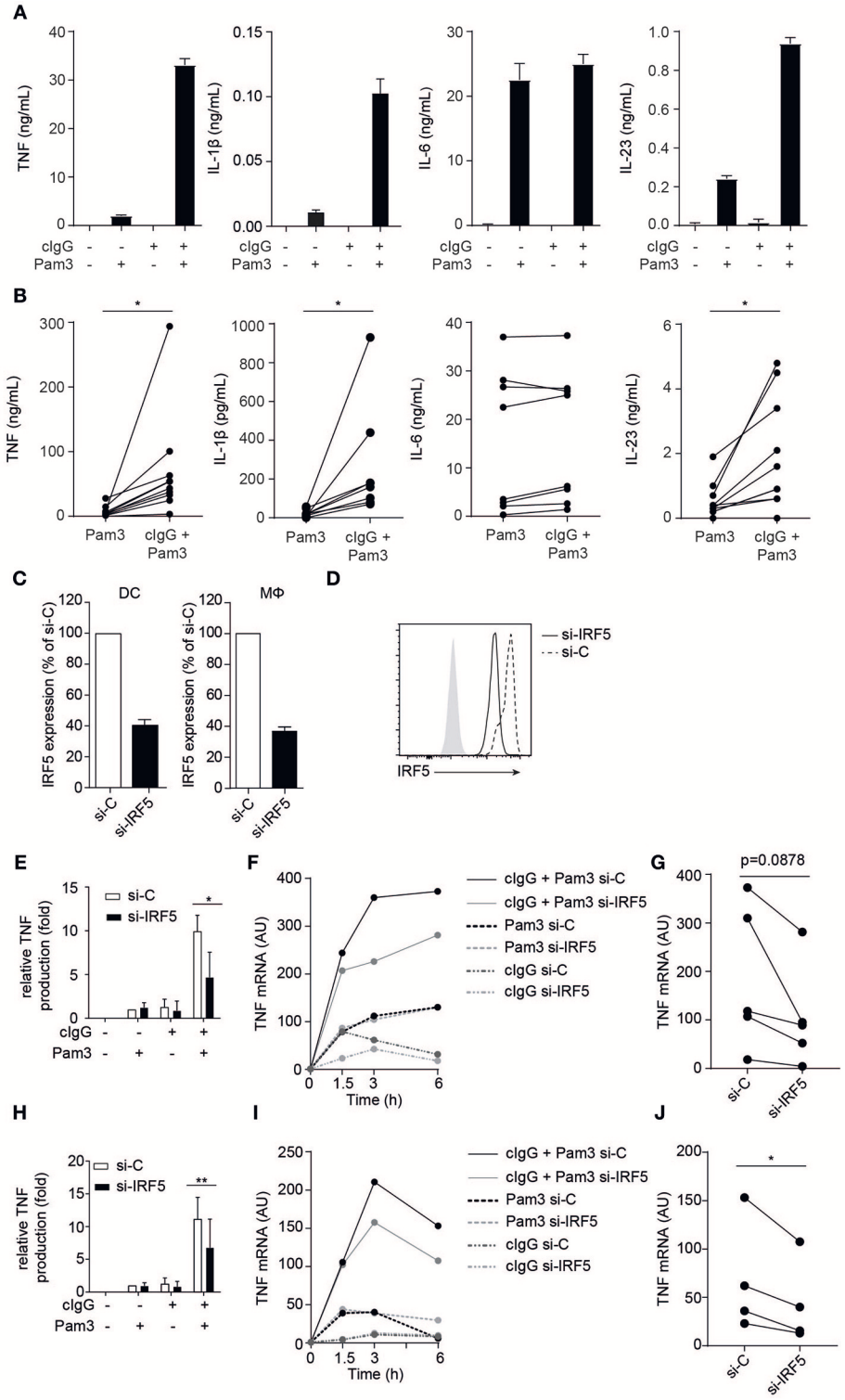

FIGURE 1 | FcγR-TLR cross-talk in human moDCs and macrophages is dependent on IRF5. **(A,B)** Human monocyte-derived dendritic cells (moDC) were stimulated with Pam3CSK4 (Pam3), cIgG, or the combination for 24 h. Protein production was determined by ELISA. **(A)** Representative examples of experiments performed in triplicate (mean + SEM). **(B)** Protein production of multiple donors, each pair of dots represent one donor. **(C,D)** IRF5 in human moDCs and macrophages was silenced using specific si-RNA. **(C)** *IRF5* mRNA expression of unstimulated moDCs or macrophages (Mϕ), after IRF5 silencing (si-IRF5) or non-targeted control silencing (si-C). Data shown is *IRF5* mRNA expression as percentage of control of *IRF5* mRNA expression in si-C conditions. Mean + SD of three (moDC) or eight

(Continued)

FIGURE 1 | experiments (Mϕ). **(D)** IRF5 protein expression of unstimulated moDCs after IRF5 silencing or non-targeted control silencing measured by flow cytometry. **(E,H)** Control or IRF5-silenced moDCs **(E)** and macrophages **(H)** were stimulated with Pam3, cIgG, or the combination for 6 h. Protein production was determined by ELISA. Data shown is protein production normalized to Pam3-induced TNF production for each experiment (set to 1), mean + SD of three **(E)** or six **(H)** experiments using different donors. **(F,I)** Control or IRF5-silenced moDCs **(F)** and macrophages **(I)** were stimulated with Pam3, cIgG, or in combination and *TNF* mRNA expression (normalized to housekeeping gene expression) was determined at indicated time points by quantitative RT-PCR. Representative examples of four experiments. **(G,J)** *TNF* mRNA expression after 6 h co-stimulation of control or IRF5-silenced moDCs **(G)** and Mϕ **(J)** of multiple donors. Each pair of dots represents one donor. $^{*}p < 0.05$, $^{**}p < 0.01$, Student's *t*-test.

RESULTS

FcγR-TLR Cross-Talk in Human moDCs and Macrophages Is Dependent on IRF5

FcγR-TLR cross-talk plays an important role in inducing inflammation during both bacterial infections and autoimmune diseases (1, 2, 6, 8). As illustrated in **Figures 1A,B** (representative donor and multiple donors, respectively), FcγR-TLR cross-talk synergistically amplifies the production of key pro-inflammatory cytokines TNF, IL-1β, and IL-23, while other cytokines such as IL-6 are not affected. Here, we set out to identify the molecular mechanisms underlying this response, using TNF production as a main read-out for FcγR-TLR cross-talk. FcγR-TLR cross-talk is known to amplify TNF production at the level of gene transcription (1, 8). Here, we hypothesized a role for IRF5, since this transcription factor is known to be involved in enhancing *TNF* transcription (10–14), is highly expressed in human myeloid APCs (15), and since *IRF5* polymorphisms are a known risk factor for several autoimmune diseases (16–22).

To study the role of IRF5 in FcγR-TLR cross-talk, we made use of a small interfering (si)-RNA approach, which on average resulted in a 60 % reduction of *IRF5* mRNA expression and a similar reduction in IRF5 protein in monocyte-derived DCs (moDCs) (**Figures 1C,D**). For stimulation of FcγRs and TLR2 we used plate-bound complexed IgG (cIgG) and Pam3CSK4 (Pam3), respectively. While individual stimulation with cIgG or Pam3 induced moderate amounts of TNF, combined stimulation strongly and synergistically amplified TNF production (**Figure 1E**). However, strikingly, silencing of *IRF5* specifically reduced TNF protein production by FcγR-TLR cross-talk, without affecting cytokine production induced by the individual ligands (**Figure 1E**). In addition, we assessed whether IRF5 is also responsible for FcγR-TLR cross talk-induced gene transcription. Indeed, (partial) silencing of IRF5 reduced *TNF* mRNA production upon FcγR-TLR co-stimulation (**Figure 1F** for kinetics of representative donor, **Figure 1G** for multiple donors). In contrast, *TNF* mRNA induced by TLR stimulation alone was not affected by IRF5 silencing (**Figure 1F**), indicating that IRF5 specifically controls *TNF* transcription induced by FcγR-TLR cross-talk.

To determine whether IRF5 is only essential for FcγR-TLR cross-talk in moDCs, or whether it is also required for FcγR-TLR cross-talk in other cell types, we assessed the effect of IRF5 silencing on human macrophages, which are the main source of TNF in inflamed synovia of RA patients (23). Similar to moDCs, silencing of IRF5 in monocyte-derived macrophages (**Figure 1C**) specifically reduced TNF production induced by FcγR-TLR synergy, both on protein (**Figure 1H**) and mRNA (**Figures 1I,J**).

Combined, these data demonstrate that the synergistic induction of TNF by FcγR-TLR cross-talk in human moDCs and macrophages is dependent on IRF5.

FcγR Stimulation Induces IRF5 Nuclear Translocation

The transcription factor IRF5 is constitutively expressed by myeloid APCs (15), but to regulate gene transcription IRF5 needs to be translocated to the nucleus (24). Therefore, we assessed IRF5 localization in human moDCs by fluorescence microscopy upon FcγR-TLR co-stimulation. IRF5 contains two nuclear localization signals (NLS) as well as a nuclear export signal (NES) and therefore continuously shuttles in and out of the nucleus (25–27). Indeed, in unstimulated moDCs IRF5 was present throughout the cell, both in the nucleus and the cytoplasm (**Figure 2A**). Similar to unstimulated cells, TLR2-stimulated moDCs also displayed an even distribution of IRF5 (**Figure 2A**). In contrast, stimulation with cIgG, either combined with TLR stimulation or not, resulted in near exclusive accumulation of IRF5 in the nucleus (**Figure 2A**; quantified in **Figure 2B**). As a control we also ascertained that (individual) TLR stimulation of moDCs results in nuclear translocation of NF-κB subunit p65 (**Figures 2C,D**), which is responsible for TLR-induced pro-inflammatory cytokine production. Very similar to moDCs, FcγR stimulation induced IRF5 nuclear translocation in human macrophages (**Figures 2E,F**), suggesting that nuclear translocation of IRF5 induced by FcγR stimulation is a general mechanism in myeloid APCs.

Since FcγR-TLR cross-talk is known to depend on signaling through the kinase Syk, we next assessed whether Syk is required for IRF5 nuclear translocation. As shown in **Figure 2G**, Syk inhibition by therapeutic small-molecule inhibitor R406 indeed suppressed IRF5 nuclear translocation both upon individual stimulation with cIgG and upon cIgG+Pam3 co-stimulation.

These data indicate that, in human moDCs and macrophages, stimulation with IgG immune complexes is responsible for nuclear translocation of IRF5.

FcγR-TLR Cross-Talk Is Dependent on TLR-Induced Phosphorylation of TBK1/IKKε and IRF5

While individual FcγR stimulation induced IRF5 translocation into the nucleus, it is not sufficient to induce *TNF* transcription (1, 6, 8). Importantly, in addition to nuclear translocation, IRF5 needs to be activated by phosphorylation in order to be transcriptionally active (14, 25, 26, 28, 29). Therefore, we determined IRF5 phosphorylation upon (co-)stimulation of human moDCs by Western blot. Interestingly, while Pam3

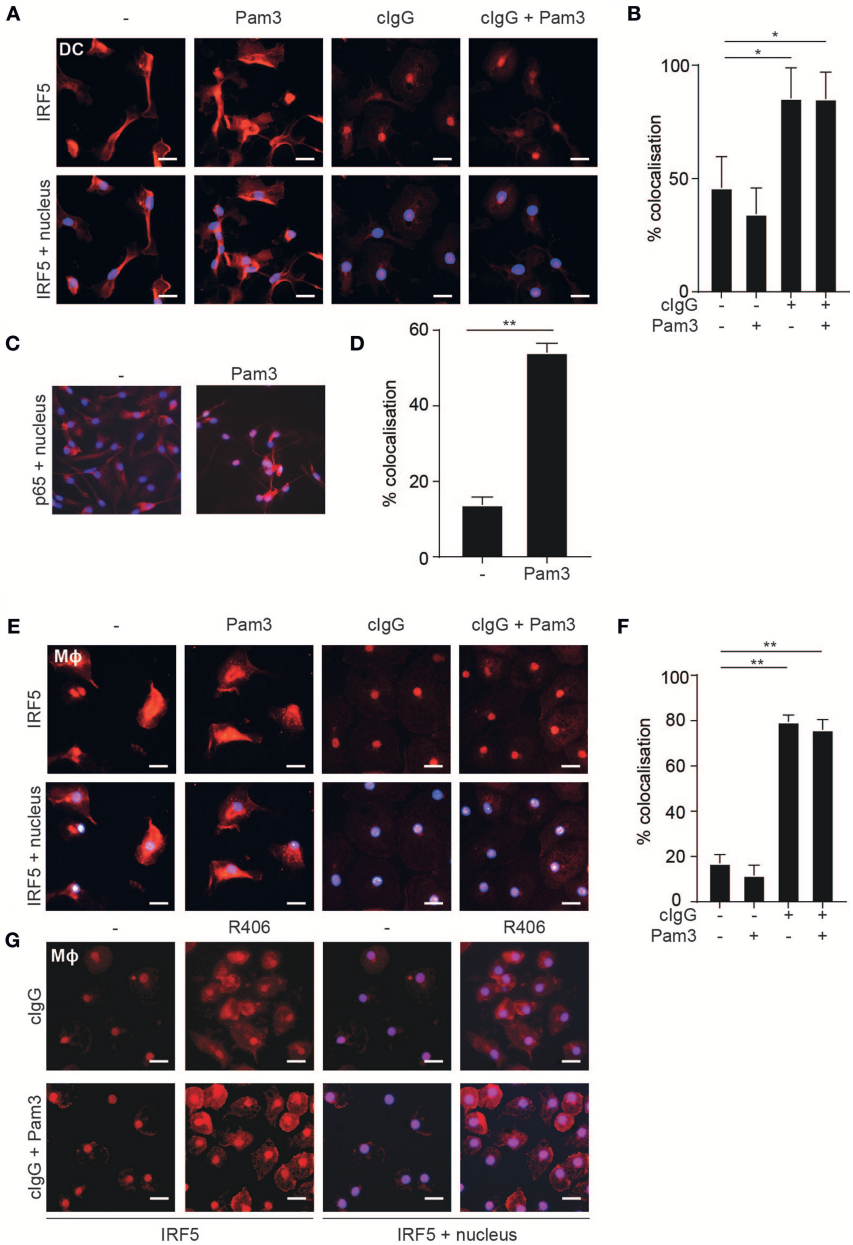

FIGURE 2 | FcγR stimulation induces IRF5 nuclear translocation. **(A,E)** Human monocyte-derived dendritic cells (moDC) **(A)** and macrophages (Mϕ) **(E)** were stimulated with Pam3CSK4 (Pam3), cIgG, or the combination for 2 h and stained for IRF5 (red) and nuclei (Hoechst, blue). Representative images of three independent experiments, bar: 20 μm. **(B,D,F)** Quantification of the microscopy data showing percent co-localization of three experiments (mean + SD). $*p < 0.05$, $**p < 0.01$, Student's *t*-test. **(C)** moDCs were stimulated with Pam3 and stained for p65 (red) and nuclei (Hoechst, blue). Representative image of three independent experiments. **(G)** Human monocyte derived macrophages were pre-incubated with the Syk inhibitor R406 and stimulated with Pam3, cIgG, or the combination for 2 h and stained for IRF5 (red) and nuclei (Hoechst, blue). Representative images of three independent experiments, bar: 20 μm.

stimulation induced IRF5 phosphorylation, stimulation with cIgG did not (**Figure 3A**, quantified as pIRF5/IRF5 ratio for multiple donors in **Figure 3B**). These data indicate that while FcγR stimulation induces IRF5 nuclear translocation, TLR stimulation is required for IRF5 phosphorylation.

IRF5 phosphorylation can be induced by TBK1, a member of the Iκ kinase (IKK) family that shares larges structural and functional similarity to IKKε (25, 26, 30). Since TBK1/IKKε also needs to be phosphorylated in order to execute kinase activity (31), we assessed TBK1/IKKε phosphorylation by flow cytometry after (co-)stimulation. Similar to IRF5 phosphorylation, we found that stimulation with Pam3 induced TBK1/IKKε phosphorylation, while stimulation with cIgG did not (**Figure 3C**, quantified for multiple donors in **Figure 3D**).

To determine whether TBK1/IKKε is required for cytokine production by FcγR-TLR cross-talk, we inhibited TBK1/IKKε

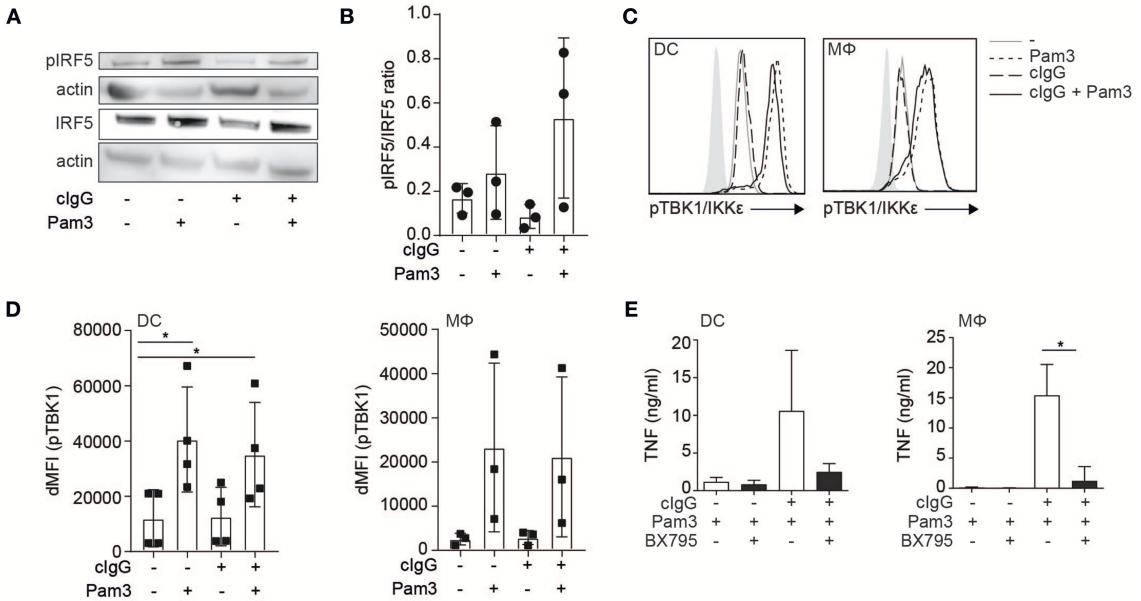

FIGURE 3 | FcγR-TLR cross-talk is dependent on TLR-induced phosphorylation of TBK1/IKKε and IRF5. **(A)** Human monocyte-derived dendritic cells (moDC) were stimulated with Pam3CSK4 (Pam3), cIgG, or the combination for 30 min. IRF5 phosphorylation at Ser437 and total IRF5 expression was assessed by Western blot. Data shown is representative example of three independent experiments. **(B)** Quantification of the three independent Western blot experiments using ImageJ (mean + SD). First bands were corrected for actin, after which the pIRF5/IRF5 ratio was calculated. **(C)** Human moDCs and macrophages (Mϕ) were stimulated with Pam3, cIgG, or the combination for 30 min and stained for p-TBK1/IKKε and analyzed by flow cytometry (^{10}log scale, light gray indicates background staining). Representative example of four (DCs) and three (Mϕ) experiments. **(D)** ΔMFI of pTBK1 of four (moDCs) and three (Mϕ) independent experiments (mean + SD). **(E)** After pre-incubation with 2 μM BX795 or the corresponding volume of DMSO, moDCs, and macrophages were stimulated with Pam3, or cIgG combined with Pam3 for 24 h and TNF production was determined by ELISA. Mean + SD of four independent experiments. *$p < 0.05$, Student's *t*-test.

using small-molecule inhibitor BX795. Indeed, BX795 abrogated FcγR-TLR cross-talk-induced TNF production (**Figure 3E**).

Thus, while FcγR stimulation induces nuclear translocation of IRF5, TLR stimulation induces phosphorylation of TBK1/IKKε and IRF5, which combined results in nuclear translocation of phosphorylated IRF5 to modulate cytokine gene transcription.

FcγR-TLR Cross-Talk Induces Glycolytic Reprogramming via IRF5

Amplification of cytokine production can be orchestrated at both the transcriptional and translational level. Interestingly, upon FcγR co-stimulation of moDCs, the fold increase in expression of *TNF* mRNA was lower than that fold increase at the protein level (**Figure 4A**), suggesting that increased translation also contributes to the amplified cytokine response. In DCs, increased cytokine mRNA translation in response to TLR stimulation has been shown to be underpinned by a rapid increase in glycolytic rate, to serve as a carbon source for *de novo* fatty acid synthesis to support expansion of the endoplasmic reticulum required for increased cytokine gene translation (32, 33). This, together with the recent finding that IRF5 is able to increase the glycolysis in macrophages (34), led us to hypothesize that FcγR (co-) stimulation induces a similar metabolic reprogramming via IRF5 to support increased translation. To this end, we stimulated moDCs and analyzed them for changes in rates of extracellular acidification (ECAR), as a measure of lactate production (a proxy for the glycolytic rate), and the rate of oxygen consumption (OCR), as a measure of oxidative phosphorylation. Notably, stimulation with cIgG indeed increased the ECAR, which was even further enhanced upon co-stimulation with cIgG and Pam3 (**Figure 4B**). In contrast, the OCR was not affected by individual stimulation with cIgG or Pam3, and only moderately increased upon co-stimulation (**Figure 4C**).

Next, we set out to investigate whether the amplification of the glycolytic response by FcγR-TLR cross-talk was also dependent on IRF5. While silencing of IRF5 did not affect the ECAR induced by individual stimulation with cIgG or Pam3, IRF5 silencing did inhibit the increased ECAR induced upon co-stimulation (**Figure 4D**). These data indicate that FcγR-TLR cross-talk amplifies the glycolytic response via IRF5.

To assess whether the increased glycolysis by FcγR-TLR cross-talk indeed contributes to the induction of cytokine responses, we stimulated moDCs in the presence of 2-deoxyglucose (2DG), which blocks glycolysis by inhibiting hexokinase activity (35). In line with previous findings, 2DG suppressed cytokine production induced by individual TLR stimulation (**Figure 4E**). In addition, 2DG also strongly suppressed cytokine production upon co-stimulation with cIgG and Pam3 (**Figure 4E**). Interestingly, while 2DG strongly impaired FcγR-TLR cross-talk-induced TNF protein production, blocking of glycolysis had very little effect on FcγR-TLR cross-talk-induced *TNF* gene transcription (representative donor **Figure 4F**, multiple donors **Figure 4G**). These data indicate that

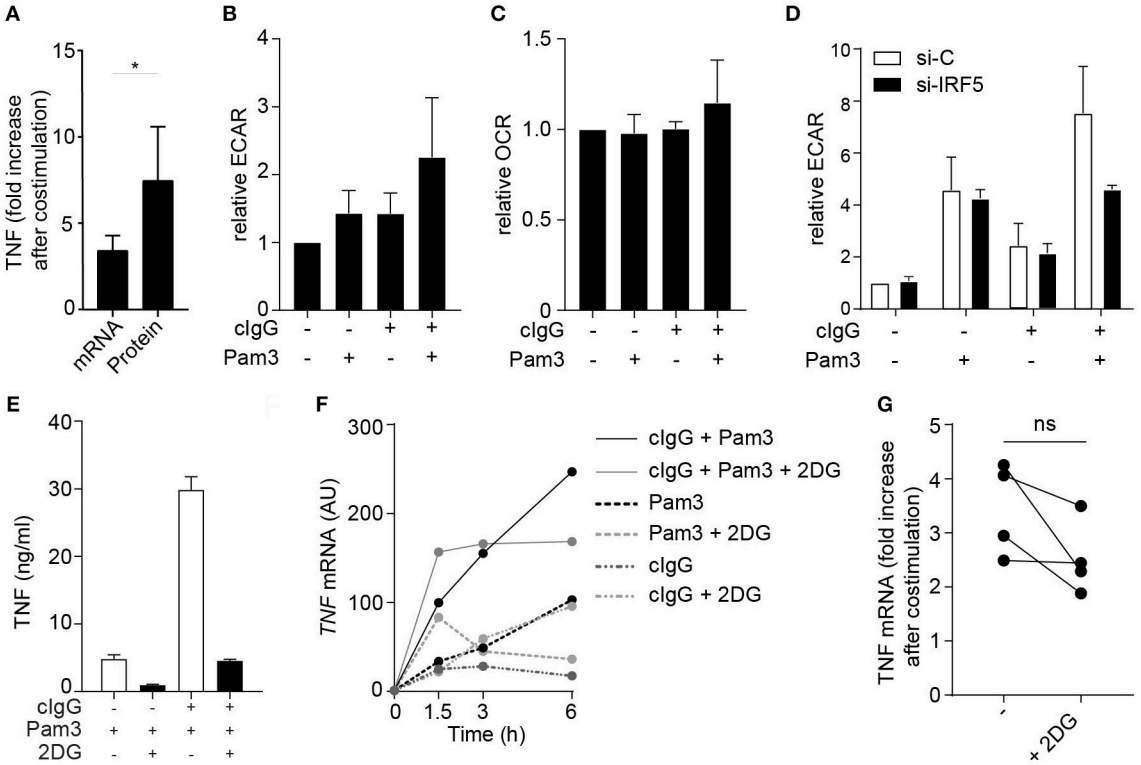

FIGURE 4 | FcγR-TLR cross-talk induces glycolytic reprogramming via IRF5. **(A)** TNF fold increase in human monocyte derived DCs (moDCs) after co-stimulation with Pam3 and cIgG for mRNA and protein. Fold increase of TNF was determined by setting Pam3 stimulation at 1 and calculating fold increase after co-stimulation at $t = 3$ h (mRNA) or $t = 24$ h (protein). Mean + SD of four (mRNA) or eight (protein) experiments. **(B,C)** moDCs were stimulated for 30 min with Pam3, cIgG, or the combination and extracellular acidification rate (ECAR) **(B)** and oxygen consumption rate (OCR) **(C)** was determined. Values are normalized to unstimulated moDCs for each experiment (set to 1). Mean + SD of four experiments. **(D)** Control or IRF5-silenced moDCs were stimulated with Pam3, cIgG, or the combination for 30 min and ECAR was measured. Representative experiment in triplicate of three independent experiments. **(E,F)** After 30 min pre-incubation with 10 mM 2-Deoxy-D-glucose (2DG), moDCs were stimulated with Pam3, cIgG, or in combination. **(E)** TNF production after 24 h was determined by ELISA; representative example in triplicate of eight independent experiments. **(F)** *TNF* mRNA expression was determined at indicated time points (normalized to housekeeping gene expression) by quantitative RT-PCR; representative example of four independent experiments. **(G)** TNF fold increase after co-stimulation with Pam3 and cIgG with and without 2DG. Fold increase of TNF was determined by setting Pam3 stimulation at 1 and calculating fold increase after co-stimulation at $t = 3$ h. Mean + SD of four experiments. Each pair of dots represent one donor. *$p < 0.05$, Student's *t*-test.

the glycolytic changes induced by FcγR-TLR cross-talk, although essential for protein production, have little effect on cytokine gene transcription.

Taken together, these data identify that IRF5 activation by FcγR-TLR cross-talk does not only enhance cytokine gene transcription, but also boosts translation through glycolytic reprogramming that together account for the strongly increased pro-inflammatory profile of moDCs activated by FcγR-TLR cross-talk.

DISCUSSION

FcγR-TLR cross-talk in human myeloid APCs is an important initiator of inflammation during both infection and autoimmunity (1, 2, 6, 8). However, the molecular mechanisms underlying this cross-talk are still largely unknown. Here, we identified a crucial role for IRF5, which is activated by two different pathways during FcγR-TLR co-stimulation to synergistically amplify pro-inflammatory cytokine production (schematically depicted in **Figure 5**). While TLR stimulation induces IRF5 phosphorylation, FcγR stimulation results in IRF5 nuclear translocation. In addition, we identified that during FcγR-TLR cross-talk IRF5 amplifies cytokine production in at least two different ways. First, IRF5 increases cytokine gene transcription. Second, IRF5 induces glycolytic reprogramming, which amplifies cytokine production in a post-transcriptional manner.

IRF5 is a transcription factor that was originally identified to be involved in type I interferon (IFN) production and antiviral responses. Over the last decade, multiple additional functions of IRF5 have been identified (24). Of these, the role of IRF5 in promoting transcription of pro-inflammatory cytokines such as TNF is the most pronounced (10–14). In addition, IRF5 expression has been identified as a marker to discriminate between subsets of macrophages, since IRF5 expression is higher in inflammatory macrophage subsets (12). Although IRF5 expression levels differ between different immune cells, our data indicate that IRF5 is required for

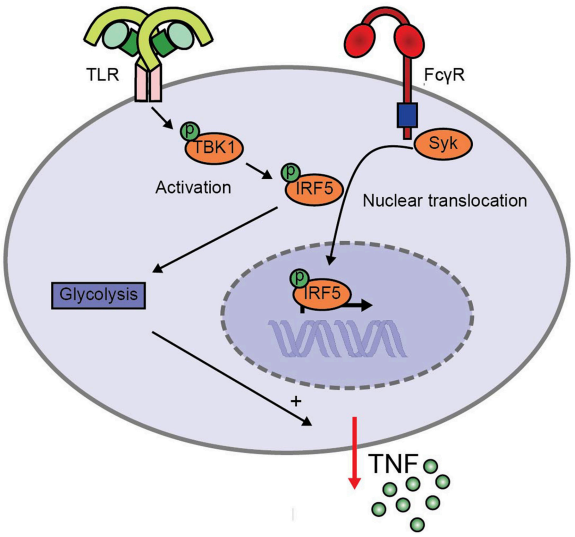

FIGURE 5 | Model for enhanced TNF production upon FcγR-TLR cross-talk via IRF5**.** TLR stimulation induces TBK1/IKKε phosphorylation that leads to IRF5 phosphorylation, while FcγR signaling induces IRF5 nuclear translocation. Simultaneous activation of IRF5 by TLRs and FcγRs amplifies pro-inflammatory cytokine production in two ways. First, IRF5 increases cytokine gene transcription. Second, IRF5 increases the glycolytic rate, which amplifies cytokine production in a post-transcriptional manner.

FcγR-TLR cross-talk in various human APCs, including DCs and macrophages.

We identified that IRF5 promotes inflammation by both enhancing gene transcription and by inducing glycolytic reprogramming. IRF5 is known to enhance gene transcription of pro-inflammatory genes such as TNF by both directly binding to IFN-stimulated response element (ISRE) regions in the TNF promoter, and by forming a complex with other transcription factors, specifically NF-κB subunit p65 (13). Transcriptional activation of IRF5 is strictly regulated by different and independent post-translational modifications, to ensure initiation of appropriate immune response and prevent unrestrained inflammation. On one hand, IRF5 needs to be phosphorylated, which enables dimerization that is required for DNA binding (14, 25, 26, 28, 29). On the other hand, IRF5 needs to be translocated into the nucleus, which is achieved via K63-ubiquitination of IRF5 (25, 26, 36). Hence, either phosphorylation or ubiquitination individually are generally not sufficient for full IRF5 activation (24–26). Based on our findings and current literature we here propose a cooperation model of IRF5-dependent gene transcription upon FcγR-TLR cross-talk (schematically depicted in **Figure 5**). In this model, TLR stimulation induces TBK1/IKKε-dependent phosphorylation of IRF5, which is required for IRF5 activation. Additionally, FcγR stimulation induces Syk-dependent nuclear translocation of IRF5. Together, these two pathways cooperate leading to activated IRF5 inside the nucleus, thereby amplifying cytokine gene transcription.

How FcγRIIa triggering induces IRF5 nuclear translocation is still speculative, but it may result from Syk-dependent activation of an E3 ligase that induces K63-ubiquitination of IRF5. Interestingly, Syk has been previously coupled to IRF5 activation, which was indeed independent of IRF5 phosphorylation (24, 37). In this regard, a relevant candidate E3 ligase is TRAF6 (38, 39), which has previously been identified to K63-ubiquinate IRF5 (36). Interestingly, also TRAF6 activation by Syk has been described to be dependent on K63-linked ubiquitination (40). Another candidate is Pellino-1, which additionally provides a connection between K63-ubiquitination of IRF5 and glucose metabolism (41).

In addition to increasing gene transcription, we identified that FcγR-TLR cross-talk also induces glycolytic reprogramming by IRF5. Interestingly, this finding corroborates a recent study by Hedl et al., which shows that IRF5 regulates the glycolytic rate in human and murine macrophages (34). IRF5 increases the glycolysis upon NLR stimulation via activation of the kinase Akt2, which upregulates the transcription of various glycolytic genes (34). However, remarkably, the phosphorylation of Akt2, which is essential for Akt2 activation, is independent of IRF5 phosphorylation (34), suggesting that also other posttranslational modifications of IRF5 are required for increasing glycolysis. Since FcγR stimulation induces IRF5 nuclear translocation, which is dependent on K63-ubiquitination (36, 41), the increased glycolysis by FcγR-TLR cross-talk may therefore depend on multiple posttranslational modifications of IRF5, which ultimately lead to increased Akt2 activation and glycolysis.

FcγRs such as FcγRIIa signal through an ITAM sequence in the cytoplasmic tail, which is a common signaling module used by a variety of receptors, including B cell receptors and T cell receptors, and other members of the Fc receptor family (42, 43). Interestingly, cross-talk with TLRs has previously been described for various other Fc receptor family members, including FcαRI (33, 44) and FcεRI (45, 46). In addition, Fc receptors have been shown to not only amplify cytokine responses induced by TLRs, but also by several other receptors such as NLRs, C-type lectins, IL-1R, and IFNγR (2, 33, 44). The fact that the cross-talk of different Fc receptors with various PRRs and cytokine receptors in different cell types all amplify pro-inflammatory cytokines in a similar manner suggests that the identified pathway may be a general mechanism of synergy between ITAM signaling receptors and PRRs, analogous to the previously described collaboration between the ITAM signaling module and JAK-STAT signaling pathways (42).

FcγR-TLR cross-talk provides protective immunity against various pathogens including bacteria and viruses (1, 3, 47), but is detrimental in various autoimmune diseases, since it strongly promotes the production of pathogenic pro-inflammatory cytokines (6, 8). Interestingly, IRF5 activation is also tightly associated with various chronic inflammatory disorders (17, 18, 21, 22). In addition, disease-associated *IRF5* polymorphisms have previously been shown to dramatically affect cytokine production by myeloid immune cells by both increasing gene transcription and glycolysis (34, 48). Disease-associated *IRF5* polymorphisms are generally associated with higher IRF5 expression, but some polymorphisms also give rise to novel IRF5 isoforms (49). For future research, it would be very interesting to determine whether disease-associated IRF5 polymorphisms also promote cytokine

production by enhancing FcγR-TLR cross-talk. In addition, targeting of IRF5, or its upstream activators such as TBK1/IKKε, may open a new avenue for therapeutic intervention (22, 49).

Taken together, we identified IRF5 as a key component of FcγR-TLR cross-talk in human antigen-presenting cells. Our data strengthen the concept of a powerful pro-inflammatory role of IRF5 through amplification of gene transcription and metabolic reprogramming. Because undesired activation by autoantibodies contributes to the pathogenesis of various chronic inflammatory disorders, targeting of FcγR-TLR signaling may be a valuable tool to suppress inflammation in diseases such as RA, systemic lupus erythematous (SLE), and inflammatory bowel disease (IBD).

AUTHOR CONTRIBUTIONS

JdD: conceptualization; WH, MN, LV, MK, BE, and JdD: methodology; WH, MN, LV, LS, IH, BE, and JdD: investigation; WH, LV, and JdD: writing the original draft; MK, DB, BE, and JdD: reviewing and editing the manuscript.

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Interplay of Na^+ Balance and Immunobiology of Dendritic Cells

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Local Na^+ balance emerges as an important factor of tissue microenvironment. On the one hand, immune cells impact on local Na^+ levels. On the other hand, Na^+ availability is able to influence immune responses. In contrast to macrophages, our knowledge of dendritic cells (DCs) in this state of affair is rather limited. Current evidence suggests that the impact of increased Na^+ on DCs is context dependent. Moreover, it is conceivable that DC immunobiology might also be influenced by Na^+-rich-diet-induced changes of the gut microbiome.

Keywords: dendritic cells, Na^+ balance, antigen-presentation, Nfat5, kidney, skin microenvironment

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Dendritic cells (DCs) represent important sentinel cells that continuously scan their microenvironment and play a key role in inducing immune responses and maintaining immunogenic tolerance [reviewed in (1–3)]. It is accepted that DCs are able to respond to a plethora of proteinaceous, lipid or carbohydrate molecules as well as nucleic acids via specialized receptors and signaling pathways [reviewed in (4–6)]. Recently, however, it emerged that the local Na^+ electrolyte abundance impacts on innate and adaptive immune cell function and vice versa [reviewed in (7, 8)].

EXTRARENAL Na^+ STORAGE

In general, body Na^+ and fluid homeostasis are known to be regulated in very narrow limits. Disturbing this balance by excessive dietary salt intake is linked to various diseases including hypertension and autoimmunity, which ultimately results in increased morbidity and mortality [reviewed in (9, 10)]. Traditionally, the kidney was seen as the sole organ that controls body salt content and fluid regulation. For that purpose, Na^+ concentrations of about 400 mM can be reached at the renal loop bend accompanied by osmolalities of up to about 1,200 mOsm/kg in the renal medulla (11). The remaining extracellular body fluids are thought to readily equilibrate with plasma. Therefore, extra-renal regulation of total body and certain tissue Na^+ content and concentration was largely ignored [reviewed in (7, 12–15)], even though evidence of interstitial salt storage was provided already in 1909, when chloride storage was found in the skin during pre-clinical studies (16, 17). Within the last twenty years, however, the interstitium of the skin has emerged as important organ involved in maintaining body Na^+ balance. For instance chemical analysis in rodents revealed that the effective osmolyte concentration in skin tissue (i.e., skin (Na^++K^+)/skin water) can reach levels of about 190 mM which is substantially higher than the effective osmolyte concentration in plasma of about 145 mM (18). Recent evidence using ^{23}Na MRI and mathematical modeling demonstrate that very high Na^+ concentrations are present at the epidermal and dermal junction zone (19, 20). Of note, chemical analysis of skin biopsies confirmed that the skin may serve as

a Na^+ buffer also in humans (21). This Na^+-storage is reversible by dialysis (22, 23) and is able to strengthen the innate immune barrier by invigorating macrophage-dependent responses against intruding pathogens (24).

Elevated Na^+ deposition is paralleled by changes in the gel-like cutaneous collagen matrix (25–27). Upon Na^+-rich diets, there is an increased sulfation of glycosaminoglycan (GAGs) which might enable cutaneous Na^+ storage [reviewed in (15)]. In addition to high Na^+ containing diets (18, 27–29), it emerged that superficial skin infections (24) and chronic inflammatory processes (30) are able to trigger local Na^+ accumulation. The mechanisms underlying both, the diet-dependent and diet-independent Na^+ accumulation in the skin are, however, unknown. It is tempting to speculate that soluble or cell-bound mediators are able to modulate the GAG network's ability to serve as a negative charge capacitor facilitating local Na^+ accumulation (27). Moreover, aldosterone and glucocorticoids may play an important role in this state of affair (31).

DENDRITIC CELLS AS POTENTIAL REGULATORS OF CUTANEOUS Na^+ STORES

While the mechanisms that allow for local cutaneous Na^+ accumulation remain elusive, depletion of mononuclear phagocytes using clodronate liposomes unraveled that these cells play an important role in regulating cutaneous Na^+ stores (18, 28). In addition, targeting the osmoprotective transcription factor *nuclear factor of activated T cells 5* (*Nfat5*) in myeloid cells using Lyz2 (Lysozym2)/ LysM-Cre deleter mice revealed that this transcription factor plays an important role in sensing Na^+-rich diet-induced local hypertonic environments (29). This myeloid cell specific osmoprotective response included the upregulation of the *Nfat5* target gene vascular endothelial cell growth factor C (*Vegfc*) which ultimately leads to lymphcapillary hyperplasia facilitating removal of Na^+ from the skin (18, 29). Recent evidence also suggests that local Na^+ storage additionally increases lymph flow in muscle and skin (32).

However, clodronate liposomes are known to deplete various mononuclear phagocytes in the skin including monocytes, macrophages and DCs (33). Moreover, although Lyz2 Cre primarily induces recombination in granulocytes, monocytes and macrophages, there is some recombination occurring in DCs (34, 35). In the Immgen Database (www.immgen.org), DCs, for instance, from skin draining lymph nodes (LN) ($CD11c^+$, $MHCII^{hi}$, $Langerin^-$, $CD11b^-$ $CD103^-$ $CD8a^-$ $CD4^-$; $CD11c^+$, $MHCII^{hi}$, $Langerin^-$, $CD11b^+$ $CD103^-$ $CD8a^-$ $CD4^-$; $CD11c^+$, $MHCII^{hi}$, $Langerin^+$, $CD11b^{low}$, $CD103^+$, $CD8a^-$, $CD4^-$; $CD11c^+$, $MHCII^{hi}$, $Langerin^+$, $CD11b^+$ $CD103^-$ $CD8a^-$ $CD4^-$) express very high *Nfat5* levels, suggesting that these cells might be involved in organization and regulation of cutaneous Na^+ balance. To the best of our knowledge, the relative contribution of different mononuclear phagocyte subtypes including various DC subtypes in this state of affair is, however, unexplored. The use of novel DC- and macrophage-specific (transcriptional) reporter mouse strains and ablation strategies might be useful to uncover the relative contribution of distinct mononuclear phagocyte subtypes [reviewed in (36–38)]. It is likely that, in addition to macrophages, DCs might fulfill distinct tasks in regulating cutaneous Na^+ balance. Recently, Randolph and colleagues demonstrated that lymphatic vessel permeability is controlled by DCs in a G protein-coupled homing receptor CCR7-dependent manner. Further analysis revealed that this task is fulfilled by IFN regulatory factor 4-positive DC subset (39). Taking these observations and the data from the Immgen database into account it is possible that DC-mediated regulation of the lymphatic vessels might be involved in facilitating the drainage of excess Na^+ from cutaneous interstitial space (**Figure 1**).

DENDRITIC CELLS AS POTENTIAL REGULATORS OF RENAL Na^+ HANDLING

In addition to regulating local Na^+ balance in the skin, it is conceivable that DCs play a key role in orchestrating renal electrolyte handling. It is well-established that there is a dense network of mononuclear phagocytes including macrophages and DCs throughout the kidney. These cells play an important role in various inflammatory and fibrotic kidney injury models [reviewed in (40–43)]. Furthermore, they are able to change their shape and motility upon tissue damage (44, 45) and are involved in curtailing and/ or promoting inflammatory responses after various insults (46–52). Under *steady state*, the mononuclear phagocyte compartment of the mouse kidney mainly consists of $CD103^+$ and $CD11b^+$ renal mononuclear phagocyte subsets [reviewed in (41, 43, 53)]. The $CD103^+$ mononuclear phagocytes are derived from *bona fide* DC precursors and these renal DCs play an important anti-inflammatory role upon renal damage (52, 54). The $CD11b^+$ renal mononuclear phagocytes represent over 90% of the renal mononuclear phagocyte population and comprise DCs and macrophages [reviewed in (41, 43, 53)]. In contrast to the $CD103^+$ renal mononuclear phagocytes/ DCs, the DC subset of these $CD11b^+$ mononuclear phagocytes exerts proinflammatory functions (54). Of note, recent evidence using a transcriptional reporter mouse for DCs (zinc finger and BTB domain containing 46 [*Zbtb42*]-GFP; visualizing both $CD103^+$ and $CD11b^+$ DCs) demonstrates that $CD103^+$ and $CD11b^+$ renal DC subsets are round-shaped and located around blood vessels while in contrast counterintuitively most of the other renal mononuclear phagocytes (i.e., macrophages) are dendritically shaped (54).

While there is substantial evidence that these DCs are involved in inflammatory responses in the kidney it is currently unclear whether DCs contribute to the regulation of renal Na^+ excretion. Recent data indicates that renal mononuclear phagocytes play an important role as accessory cells in regulating Na^+ transport of renal tubular cells. Crowley and co-workers uncovered that IL-1-signaling modulates tubular Na^+ excretion via mononuclear phagocytes in mice (55). Moreover, using a CD11b-Cre deleter mouse strain, Zhang et al. reported that prostaglandins derived from renal mononuclear phagocytes modulate the activity of renal Na^+-Cl^- cotransporters (56). As the CD11b-Cre deleter mouse strain recombines in DCs as well (35), it is tempting to speculate that renal DCs are

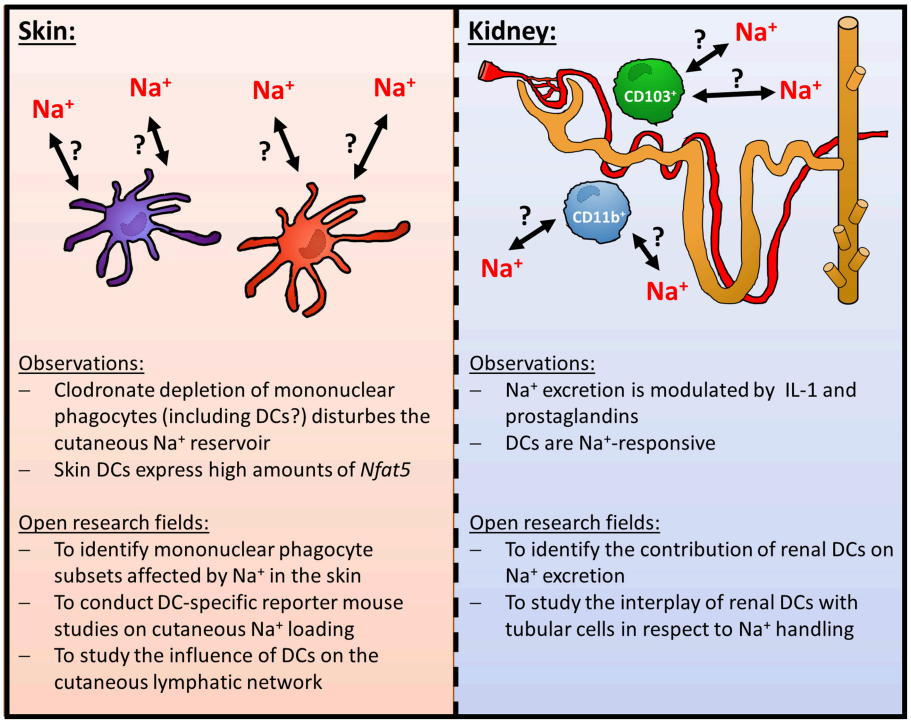

FIGURE 1 | Role of DCs in homeostatic cutaneous and renal Na^+-handling.

involved in this state of affair. This idea is further supported by the fact that murine DCs express specific molecules that facilitate the transport of Na^+ and thus sensing of increased extracellular Na^+ levels such as the sodium-potassium chloride cotransporter-1 (NKCC1), chloride cotransporter (NCC), the sodium-calcium exchanger (NCX) and the α and γ subunits of the epithelial sodium channel (ENaC) (57). Murine DCs are able to express gap junction proteins such as Connexin 43 (58), which are able to facilitate Na^+ entry in addition to other molecules (59). It is tempting to speculate that DCs are able to form functional syncytial cell aggregates with tubular cells and thereby regulate renal Na^+ handling. However, the contribution of these molecules in electrolyte physiology is unexplored and warrants further studies.

IMPACT OF Na^+ ON DENDRITIC CELL IMMUNOBIOLOGY

DCs might not only be important regulators of local Na^+ balance. For instance there is robust evidence that increases in Na^+ levels limits the anti-inflammatory capacity of macrophages while promoting their proinflammatory status (24, 60–65). Enhanced induction of proinflammatory macrophage activation required the activity of the osmoprotective transcription factor *Nfat5* (24, 64). Recently, Buxade et al. reported that *Nfat5* regulates the expression of MHCII molecules under standard cell culture conditions (i.e., normal salt conditions) and thereby regulates $CD4^+$ T cell responses (66). This regulatory circuit only operates in macrophages but not in DCs (66). Surprisingly, the impact of increased Na^+ levels on DC immunobiology has been studied in less detail and the data available are controversial (**Figure 2**).

Jörg et al., for instance, reported that high Na^+ levels do not impact the generation, maturation or function of mouse DCs but rather directly impact on T cells (67). In contrast to these findings, Chessa et al. demonstrate that increasing extracellular Na^+ levels, found in the renal medulla during DC development, skews murine DCs to a macrophage-like regulatory phenotype and suppresses the release of the Th1 priming cytokine IL-12p70 (68).

In line with this, Popovic et al. reported that the ability of mouse DCs to cross-present the model antigen ovalbumin is severely impaired (69). Decreased cross-presentation was recorded despite enhanced antigen uptake, processing, and presentation. Of note, increased Na^+ levels resulted in enhanced expression of co-inhibitor and co-stimulatory molecules. Using knock out strategies and blocking antibodies the authors exclude that enhanced expression of co-inhibitory/ -stimulatory molecules or reduced production of IL-12 underlies this phenotype. The authors provide evidence that the suppressive effect of high salt conditions (HS) on cross-presentation is dependent on TIR-domain-containing adapter-inducing interferon-β (TRIF) regulated process. However, the TRIF-dependent mechanism that ultimately results in impaired cross-presentation requires further investigation (69). Recently, Zhang et al. reported that exposure of virally infected mouse macrophages to increased Na^+ levels boosts the release of Type 1 interferon (65). Since TRIF and type 1 interferon production are intertwined [reviewed in (70)] and type 1 interferon signaling has the potential to inhibit antigen-presentation (71), it is

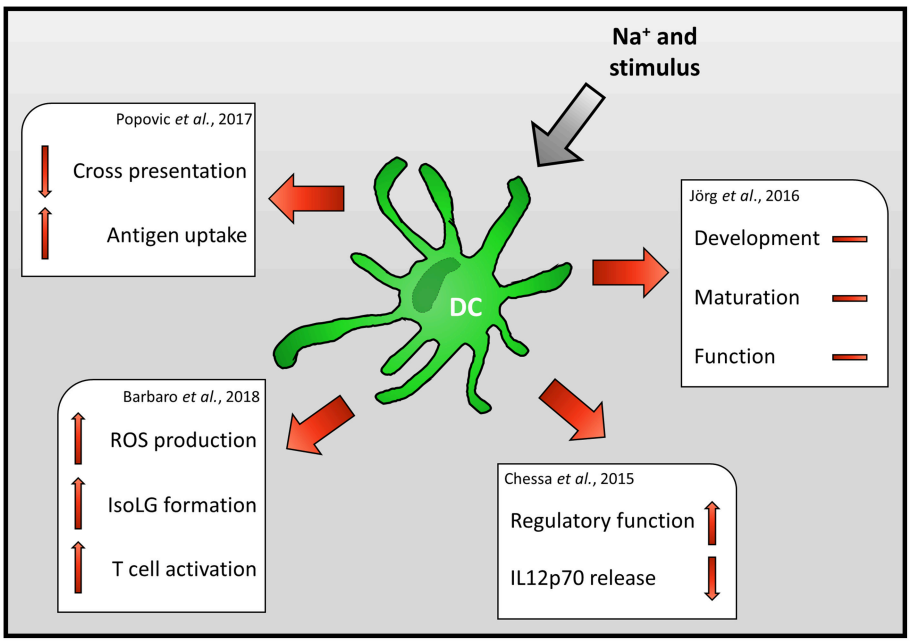

FIGURE 2 | Impact of Na^+ on DC immunobiology.

conceivable that exposure to increased Na^+ levels triggers an overshooting type 1 interferon response which ultimately inhibits cross-presentation by DCs.

In line with enhanced degradative activity of DCs upon HS exposure (69), Barbaro et al. found that increasing extracellular Na^+ levels result in enhanced ROS production and formation of isolevuglandin (IsoLG)-protein adducts in mouse DCs. However, in contrast to the study using the model antigen ovalbumin, Barbaro et al. reported increased frequencies of IFN-γ and IL-17 producing T cells after co-incubation of DCs with T cells. Moreover, transfer of DCs exposed to high Na^+ environments, increased the blood pressure of mice subjected to low levels of angiotensin II (57). These findings suggest that increased local Na^+ levels enhance the inflammatory potential of DCs and, thus might propagate inflammatory circuits that ultimately result in arterial hypertension and cardiovascular death.

Of note, increases in dietary Na^+ might not only directly influence the immunobiology of dendritic cells. Recently, Wilck et al. demonstrated that dietary high salt conditions change the composition of the microbiome by removal of *Lactobacillus murinus* (72). Depletion of *Lactobacillus* was accompanied by reduction of the tryptophan metabolites such as indole 3-lactic acid (ILA) and indole 3-acetic acid. Increased levels of ILA directly inhibit the proliferation of T_H17 cells *in vitro* (72). In addition, it is possible that these tryptophan degradation products are impacting on gut dendritic cells, which in turn orchestrate e.g., regulatory T cell, T_H22 and T_H17 effector cell balance (73, 74). In line with this, there are several reports that Na^+-rich diets increases the production of cytokines that are key players in screwing the induction of T_H1 and T_H17 cells in inflamed gut tissue such as *Il12b* and IL-23 (75, 76).

CONCLUSION

Na^+ availability emerges as a new factor of tissue microenvironment which on the one hand is regulated by immune cells and on the other hand is able to impact on their immunological function. In contrast to macrophages, our knowledge regarding DCs is rather limited. Current evidence suggests that the impact of increased Na^+ levels on DCs is context dependent. However, the role of DCs in regulating local Na^+ stores is unexplored and warrants further studies.

DATA AVAILABILITY

Publicly available datasets were analyzed in this study. This data can be found here: "www.immgen.org."

AUTHOR CONTRIBUTIONS

PN and JJ: conception and writing of the manuscript. AS and DM: contributed to the writing.

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