AMPK signaling in dendritic cells: a metabolic sensor controlling the balance between immunity and tolerance

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Part 4

Discussion


AMPK

8

General discussion

Introduction

The metabolic status of an individual at both systemic as well as tissue level has a significant impact on the immune system. For example, excess of nutrients leads to chronic inflammation and metabolic dysfunction, while children suffering from undernutrition have a higher risk of dying from infectious diseases (1,2). Furthermore, the tumor micro-environment (TME) exerts immunosuppressive effects through local dysregulation of nutrient availability (3,4). This thesis aimed to generate new mechanistic insights into this intricate link between nutrient availability and immune responses by focusing on how nutrient-sensor AMPK in dendritic cells (DCs) translates metabolic cues from the environment to intracellular metabolic changes, thereby affecting the immune response. Through assessment of AMPK activity in tissue-resident DCs under various (pathological) conditions, by studying the effects of AMPK signaling in DCs *in vivo*, and through in depth analysis of the consequences of AMPK modulation in DCs *in vitro*, we now have a better understanding of how AMPK signaling in DCs contributes shaping the balance between immunity and tolerance. A summary of the key findings from each research chapter can be found in box 1.

Box 1: summary of main findings presented in this thesis

- The LKB1-AMPK/SIK signaling axis in hepatic DCs protects against obesity-induced metabolic dysfunction by limiting Th17 responses (chapter 4)
- AMPK activation induces tolerogenic moDCs that promote differentiation of regulatory T cells through RALDH activity, phospholipid breakdown, mitochondrial-fission induced fatty acid oxidation, and glucose catabolism (chapter 5)
- AMPK signaling in tumor-associated DCs promotes tumor growth and limits anti-tumor immunity (chapter 6)
- Retinoic-acid induces tolerogenic moDCs through AMPK-FoxO3 signaling (chapter 7)
- AMPK drives $CD103^+$ DCs to induce Treg responses *in vivo* (chapter 7)

AMPK signaling in DCs, a double-edged sword

The studies presented in this thesis reveal a role for AMPK signaling in promoting tolerance and suggest that AMPK activation in DCs could compromise the immune system in nutrient-restricted conditions. Hence, heightened AMPK signaling in DCs can potentially weaken the immune system and contribute to increased susceptibility to infectious diseases during under nutrition, and impaired anti-tumor immunity during tumor growth. In these contexts, inhibition of AMPK signaling in DCs could be of clinical interest. However, diminished AMPK signaling may not be beneficial in other settings, as absence of AMPK in DCs limits parasite expulsion and control of associated pathology (5,6) and increases susceptibility to developing inflammatory bowel disease (unpublished data). Hence, AMPK signaling in DCs is a double-edged sword, which has to be taken into consideration if this pathway were to be explored as target for clinical purposes.

Also in other immune cells AMPK has a dual role. For example, AMPK signaling dampens the primary antibody response of B cells, while supporting the function of memory B cells (7), AMPK signaling in tumor-infiltrating T cells can have anti- (8) and pro- tumorigenic (9) effects, and while most studies show an anti-inflammatory role for AMPK in macrophages (10,11), pro-inflammatory polarization can also depend on AMPK signaling (12). Taken together, these examples highlight the crucial role of AMPK in controlling the balance between immunity and tolerance.

Consequences of AMPK activation in DCs across tissues

Given the important role of AMPK in adapting cellular metabolic states to the tissue metabolic environment, and thereby DC function, a better understanding of how AMPK activation is regulated in different tissues and pathological contexts is key. Here, I aim to provide an overview of AMPK activity in DCs in murine tissues and how this affects immune responses in health and disease.

Given the fact that AMPK is activated during metabolic stress, AMPK signaling in DCs is expected to be low during homeostasis. In line with this, $CD11c^{\Delta AMPK}$ mice do not have any observable phenotype (data not shown). In chapter 7 we show that AMPK activity in DCs during homeostasis is similar among a variety of tissues, including the spleen, lung, and liver, with the notable exception of the colon and small intestine, where DCs display high levels of AMPK activity (Fig. 1A). Intestinal homeostasis requires a fine balance between inflammatory responses to pathogens and tolerance to commensal microorganisms, and it has been suggested that AMPK activity in gut-residing DCs may contribute to the immunosuppressive environment (13). Indeed, we showed that AMPK signaling is required for maintenance of, and RALDH activity in intestinal $CD103^{+}CD11b^{+}$ DCs, a subset of DCs with known tolerogenic properties. We hypothesize that AMPK activity in intestinal DCs is driven by retinoic acid (RA), derived from dietary Vitamin A, as we have seen that RA in *in vitro* moDCs induces AMPK activity (Chapter 7).

In addition to the immunosuppressive environment of the intestine, we showed in chapter 6 that the immunosuppressive TME can also promote AMPK signaling in DCs (Fig. 1B). AMPK signaling suppressed expression of various metabolic enzymes, lowered expression of CD86 on cDC1s, and prevented the accumulation of $GLUT1^{+}$ cDC1s with high expression of activation markers. Furthermore, tumor-infiltrating $CD8^{+}$ T cells secreted lower levels of TNF in $CD11c^{WT}$ compared to $CD11c^{\Delta AMPK}$ mice. Although the exact mechanisms are yet to be discovered, these studies suggest that AMPK signaling in tumor-associated (TA)-DCs contributes to immunosuppression, thereby supporting tumor growth. We have not explored the signals that induce AMPK activity, but a variety of metabolic characteristics of the TME can induce AMPK activation (4), including low glucose levels (14), hypoxia (15), and lactate (16).

In contrast to the presumably nutrient-deficient TME, limited AMPK activity is expected in nutrient-rich inflamed tissues of high fed diet (HFD)-fed mice (2). In chapter 4 we compared AMPK signaling in DCs from adipose tissue and liver from low fat diet (LFD)- and HFD-fed mice, and indeed, AMPK activity in DCs from these metabolic tissues remained low following a HFD (Fig. 1C). Correspondingly, loss of AMPK signaling in CD11c-expressing cells did not affect the metabolic dysfunctions caused by HFD, including weight gain, impaired glucose

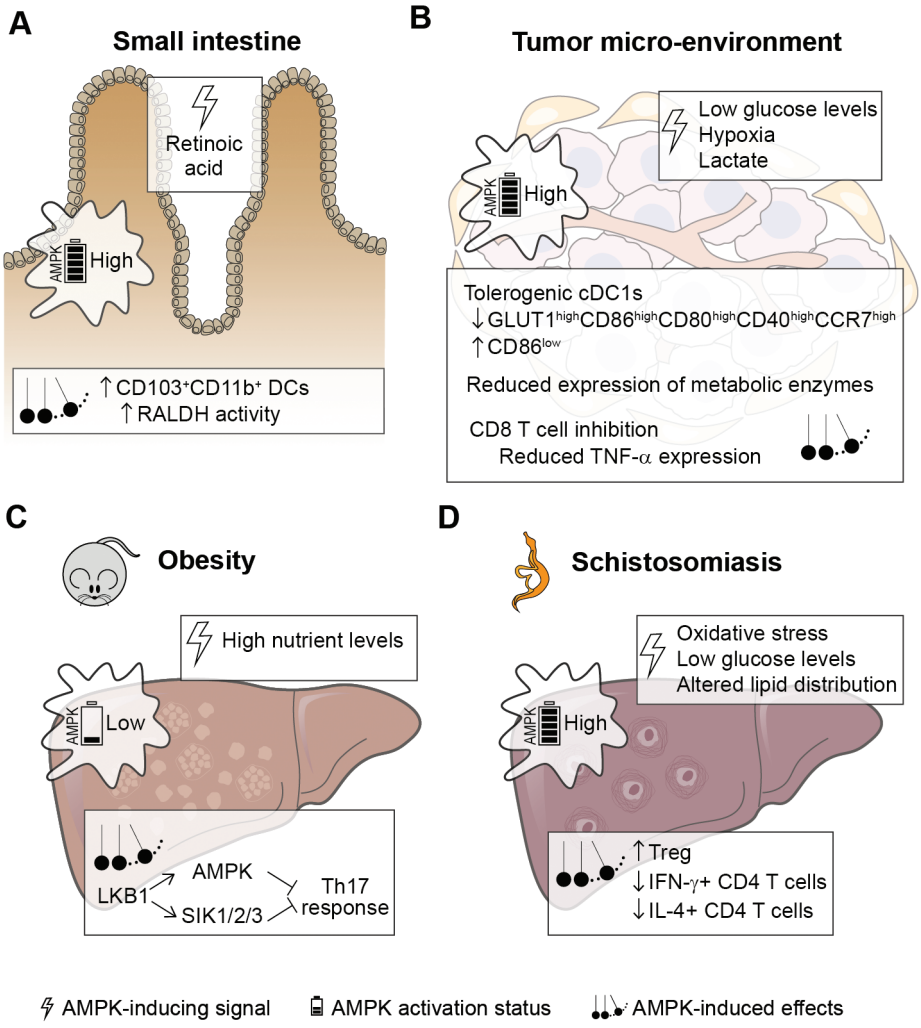

Figure 1: Dendritic cell AMPK activation *in situ*. Putative AMPK-inducing signals, AMPK activation status, and AMPK-induced effects in DCs residing in **(A)** the small intestine, **(B)** the TME, **(C)**, the liver during obesity, and **(D)** the liver during schistosomiasis.

homeostasis, and hepatic steatosis. In contrast, we did show that LKB1 in DCs, a direct upstream activator of AMPK, was required for limiting metabolic dysfunctions during obesity. Interestingly, we identified this effect to be dependent on LKB1-driven AMPK/SIK signaling to limit hepatic pathogenic Th17 accumulation. These data suggest that even in conditions where AMPK activity is not notably elevated, AMPK can apparently play important role in relaying signals from upstream kinase LKB1, albeit in combination with other AMPK-related kinases.

While DCs in the pro-inflammatory, nutrient-rich liver of obese mice do not exhibit high AMPK activity, hepatic DCs during schistosomiasis did show elevated AMPK activity compared to hepatic DCs from naïve mice (Fig. 1D) (chapter 7). Hence, AMPK activation in DCs is not restricted to certain tissues, but highly dynamic and dependent on the (metabolic) conditions. AMPK signaling in hepatic DCs contributed to regulatory T cell (Treg) accumulation, dampened IFN-γ and IL-4 secretion by CD4 T cells, and reduced attenuated egg granuloma size during schistosomiasis. Intriguingly, both HFD and schistosomiasis increased FoxP3$^+$ Tregs in the liver, but only during schistosomiasis AMPK signaling in DCs was involved in the Treg response. The Th2 response to egg-derived antigens in the liver (17) is accompanied by oxidative stress, elevated glycolytic enzyme expression in hepatocytes (possibly leading to reduced glucose levels in the environment), and altered lipid metabolism (18), potential inducers of the observed AMPK activation in hepatic DCs during schistosomiases (19).

The conclusions that can be drawn from studying AMPK signaling in DCs across various tissues under different pathological conditions are summarized in box 2. The tissue-specific factors that drive AMPK activation in DCs have not been addressed in this thesis. It is likely that the metabolic cues that promote AMPK signaling are diverse and context dependent. Within the TME, AMPK-induced tolerogenicity and metabolic stress-induced immunosuppression could be a side effect of the high metabolic demands of tumor cells. However, it can also be argued that it is favorable for tumors to have limited vascularization and scarce nutrient levels, accompanied by a metabolically immunosuppressive environment. Tumor cells, however, will most likely also outcompete DCs for nutrients in nutrient-rich conditions, thereby further increasing their proliferation rates. Additionally, taking into account that tumor cells also developed other strategies to suppress the immune system (e.g. MHC-I downregulation and secretion of anti-inflammatory molecules) (20), AMPK-induced suppression of anti-tumor immunity is most likely a beneficial side effect of metabolically highly active cancer cells, rather than a mechanism that evolved to promote tumor growth. Further understanding of this balance between nutrient supply and immune suppression, for example by comparing nutrient availability in high immunogenic vs. poor immunogenic tumors, may help to find therapeutic strategies to change the metabolic, immunosuppressive TME at the cost of tumor growth.

It is important to keep in mind that AMPK signaling is a dynamic process. AMPK activation status in intestinal DCs might be affected by food intake for example and AMPK activity may also differ at various locations within one tissue, e.g. nutrient gradients caused by poor vascularization in the tumor micro-environment may lead to various degrees of AMPK activity within tumor-associated DCs. Spatiotemporal control of AMPK activation in different (pathological) settings is a yet unexplored area that warrants further investigation, to fully understand the biology of AMPK signaling in DCs and to implement this knowledge for therapeutic purposes.

The downstream effects of AMPK signaling in DCs

Thus far it remained largely unknown how AMPK activation affects DC biology. In chapter 5 we explored the immunological consequences and downstream mechanisms of AMPK activation in DCs using human monocyte-derived DCs (moDCs), treated with AMPK-activator 991 (21). AMPK activation induced RALDH^{high} tolerogenic DCs that primed T cells towards a regulatory phenotype. Using an unbiased approach we showed that breakdown of phospholipids, mitochondrial fission-induced fatty acid oxidation, and glucose catabolism are important

Box 2: Dendritic cell AMPK signaling in murine tissues: main findings.

- Without a (metabolic) environment that induces AMPK activation, DC homeostasis and function are not affected by loss of AMPK
- AMPK activation in DCs is associated with, but not restricted to, immunosuppressive environments
- AMPK activation in DCs can regulate Treg, Th17, and Th2 responses, but always favors a tolerogenic rather than an immunogenic response.
- In conditions that do not elevate AMPK activation, AMPK can still control DC function in combination with other AMPK-related kinases

for AMPK-induced tolerogenicity. However, when AMPK activation *in situ* is driven by low glucose levels, it is unlikely that glycolysis would be upregulated. In line with this, we observed that AMPK-activated TA-DCs, residing in the TME of highly glycolytic B16 melanoma cells, expressed lower levels of enzymes involved in glycolysis and the TCA cycle, suggesting a reduction rather than increase in glucose oxidation (chapter 6). Discrepancies between *in vitro* and *in vivo* studies may be explained by the nutrient-rich medium in which moDCs are cultured, which does not reflect the physiological conditions of AMPK activation. On the other hand, AMPK activity in TA-DCs suppressed expression of ACC1, a key enzyme of fatty acid synthesis, suggesting that *in vivo* AMPK activation suppresses anabolic metabolism, while *in vitro* AMPK activation boost catabolic metabolism. This points towards a consistent shift from anabolic to catabolic metabolism upon AMPK activation. This shift can, depending on the context, be mediated by inhibition of fatty acid synthesis through downregulation and/or inhibition of ACC1, promotion of fatty acid oxidation through inhibition of ACC2 or induction of mitochondrial fission, enhanced glucose oxidation, and/or elevated catabolism of phospholipids.

Within the tumor, AMPK signaling in DCs affected cDC1s and CD8 T cells, but no difference in the intra-tumoral Treg compartment could be detected. On the other hand, DC-specific AMPK activation during schistosomiases promoted Treg abundance and was, interestingly, associated with higher RALDH activity, which is important for Treg induction by AMPK-activated moDCs *in vitro*. Although we did not address CD8 T cell priming using our human moDC model, this may indicate that the observed metabolic changes and tolerogenic properties induced by AMPK activation in moDCs *in vitro*, may reflect how AMPK regulates DC biology in the context of schistosomiasis, rather than cancer. In depth metabolic analysis of hepatic DCs during schistosomiasis may reveal whether increased phospholipid, fatty acid, and glucose catabolism could also play a role in promoting Treg responses by AMPK-activated DCs *in vivo*.

Our *in vivo* models show that loss of AMPK in DCs shifts the balance towards pro-inflammatory responses rather than tolerance. As AMPK is activated by metabolic stress, AMPK-deficient DCs still reside in a presumably metabolically harsh environment, which raises the question of which nutrient sources and metabolic pathways are used for DC activation upon AMPK loss. In the TME of CD11c$^{\Delta AMPK}$ mice we observed an increase in cDC1s with high expression of activation markers and elevated GLUT1 levels. Upregulation of nutrient transporters, in this case a glucose transporter, may allow these cells to more efficiently compete for nutrients, to better fuel the metabolic programs underpinning their activation.

Furthermore, intracellular lipid, protein, or glycogen storages may be used, mechanisms also described during early DC activation (22).

Taken together, our data provide new and functional information on the consequences of AMPK activation in DCs. How these findings translate to other immune cells, and how the metabolic environment affects these downstream events remains to be explored.

Future perspectives

The work in this thesis expands the field of immunometabolism with novel insights on the role of AMPK signaling in DCs. To ultimately be able to exploit these insights for DC-based immunotherapies continued efforts are needed that further address cellular and systemic metabolism. Key future research areas are summarized in box 3 and include further studies on spatiotemporal regulation of AMPK signaling in DCs *in situ*, tissue-specific metabolic demands of DCs, metabolic rewiring during development and activation of the different DC subsets, and metabolic crosstalk (e.g. nutrient exchange) between DCs and other (immune) cells. To drive this research forward we need to take full advantage of the latest models and methods that allow a deeper understanding of the interplay between metabolism and immune cells.

Box 3: Future research directions

- Spatiotemporal control of AMPK activity *in situ*
- Mechanisms of AMPK controlled immune responses
- AMPK as target for inflammatory and tolerogenic DC-based therapies
- Differences in metabolic reprogramming necessary for the activation and function of distinct DC subsets
- Tissue-specific metabolic demands of DCs
- Metabolic crosstalk between DCs and other (immune) cells
- Modulation of the metabolic environment for therapeutic purposes

Methods to study immunometabolism

Tools to study immunometabolism can be roughly divided into functional assays, including extracellular flux analysis and enzyme activity assays, flow cytometry approaches, and omics-based methods (transcriptomics, proteomics, metabolomics) (23). The scarcity of dendritic cells *in situ* make *ex vivo* functional assays difficult and therefore the quickly expanding flow cytometry and -omics toolbox is a boost for this field. Single cell transcriptomics data has been of great value for understanding immune cell ontogeny and inter- and intra-tissue heterogeneity, and also is a useful tool to obtain metabolic information (24). Mass spectrometry allows for quantification of metabolites, lipids, and proteins and has proven to be an important method for unravelling immunometabolic mechanisms, especially when these different omic-datasets are integrated (chapter 5). Furthermore, C13/N15 isotope labelling techniques can provide information about fluxes of various nutrients in different metabolic pathways, both *in vitro* and *in vivo*, and may help to define DC subset-specific metabolic rewiring. Imaging mass spectrometry can provide spatiotemporal information to connect nutrient availability to immune cell activation and metabolic status, which is particularly important for understanding

the dynamics of nutrient-sensing enzymes such as AMPK, that are highly-dependent of microenvironment (25). Spatial transcriptomics also contributes to this understanding, although AMPK is a kinase and hence addressing the phosphorylation of target proteins using imaging mass spectrometry provides better information on the AMPK activation status than a transcriptional profile (25). A highly valuable resource for system-based immune cell profiling are publicly available datasets, that are sometimes integrated in easy-to-access online databases. Examples include mouse single cell atlas with data from all major mouse organs called Immgen (24), a database containing transcriptomics data from murine mononuclear phagocytes derived from 38 different tissues (26), and the Chan Zuckerberg Cell by Gene discover that allows easy access to human single cell datasets (27).

Unlike to omics-based approaches, flow cytometry is limited by the number of markers and biased by the selected markers that can be analyzed. On the other hand, it is better accessible and can also provide data on dynamic processes, including activity of the metabolic pathways using SCENITH (28). Spectral flow cytometry allows for panels up to 40 markers and in combination with either SCENITH or Met-Flow, which contains antibodies targeting key metabolic enzymes and proteins, in depth metabolic information on multiple immune cell subsets can be obtained in one run (28,29). Flow cytometry can also be used to measure mitochondrial activity with metabolic dyes and to assess uptake of nutrients (30). A disadvantage of fluorescent labeling of nutrients is that it may affect their structure and thereby the cellular import route (31). Click-chemistry based staining, which only requires minor metabolite modifications and allows intracellular fluorescent labelling, may overcome this problem (32,33). Another interesting method is imaging flow cytometry. Adding subcellular spatial information to the fluorescently labelled markers can for example visualize DC-T cell interactions (34), which in combination with a met-flow panel can potentially lead to new insights into the metabolic crosstalk required for this interaction.

The quickly expanding immunometabolism toolbox, particularly those methodologies that provide spatial information or allow assessment of dynamic processes on single cell level, are key to better understand the metabolic processes inside immune cells, and are needed to improve immunotherapies (Fig. 2).

Models to study DC biology

In addition to innovating methods, proper model systems are needed. The extremely low numbers of DCs in blood and tissues complicate *ex vivo* experiments to study these cells. Hence, several *in vitro* human and murine DC models have been developed. Human moDCs, differentiated *in vitro* from PBMC-derived $CD14^+$ monocytes using GM-CSF and IL-4 are the most commonly used model for human DC studies (35). moDCs exhibit phenotypical and functional similarities with cDCs, but are transcriptionally related to $CD1c^+CD1a^+CD14^+$ inflammatory DCs and may not be representative for DC biology during homeostasis (36,37). Interestingly, recent developments in the immunometabolism field contributed to differentiation of highly immunogenic moDCs, induced through GM-CSF and inhibition of metabolic regulators PPARγ and mTORC1 (38). This study shows that in depth knowledge of developmental trajectories can lead to improved *ex vivo* differentiation methods that will hopefully lead to culture conditions that better resemble the different *in vivo* DC subsets.

DCs can also be generated from $CD34^+$ cells derived from human umbilical cord blood (39). $CD34^+$ hematopoietic progenitors can differentiate into cDC1s, cDC2s, and pDCs that closely resemble peripheral blood DC, using a cocktail of cytokines, growth factors, and Notch

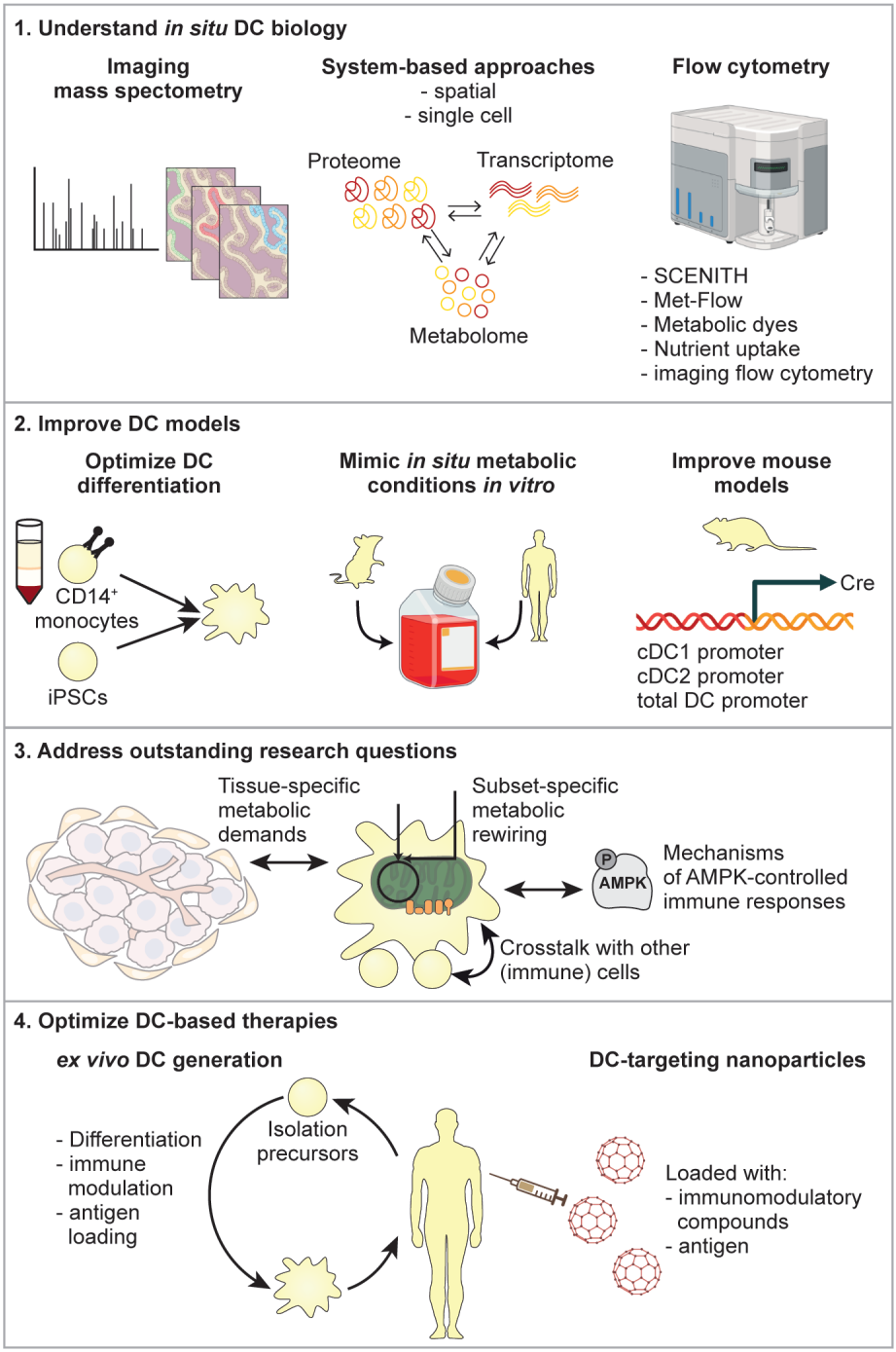

Figure 2: Tools to study DC metabolism and function. Methods and models to strengthen our fundamental knowledge on DC metabolism and immunity, to ultimately improve DC-based therapies. iPSCs = induced pluripotent stem cells.

ligand-expressing stromal cells. Unfortunately, in addition to a challenging cell culture protocol, differentiation can take up to 4 weeks, and cell numbers are low (40,41). Representative cDC1s (42), cDC2s (43), and regulatory DCs (44) can also be differentiated from induced pluripotent stem cells (iPSCs) that can be cultured in high cell numbers, but also require complicated cell culture conditions (45). Another possibility to achieve high cell numbers is the use of immortal cell lines. Several human myeloid leukemia cell lines have been used for DC differentiation, but the leukemic-origin of these cells results in several defects with regards to antigen presentation capacity (46,47).

An important step towards better understanding of how metabolism and immune cell function are linked is the use of cell culture conditions that better reflect the *in situ* environment of cells. Tissue-specific oxygen levels can be mimicked using microfluidic devices (48) and physiological medium allows for exposure to physiological relevant concentrations of carbon sources, salts, and other plasma components. Culturing moDCs in physiological oxygen concentrations (5%) did increase IL-12p40 secretion by DCs, but had no effects on CD8 T cell priming (49), suggesting that the use of atmospheric oxygen levels might be sufficient to mimic *in situ* DC biology *in vitro*. The effects of physiological medium on DC function are yet to be determined, but various studies show that metabolism and inflammatory responses observed in T cells *in vitro* are distinct and more similar to *in vivo* conditions when standard culture medium is replaced for physiological medium (50–52). Mimicking physiological conditions *in vitro* may lead to better representative models, in particular when addressing the role of nutrient-sensing enzymes such as AMPK.

Mouse models are extremely useful for *in vivo* studies. Adoptive transfer of DCs allows for *ex vivo* treatment and genetic manipulation of DCs, and the Cre-loxP system facilitates *in situ* targeting of DCs, albeit with limitations. The relatively new Xcr1-Cre strain is the best model for genetic manipulation of cDC1s (53), but a similar model for cDC2s does not exist, due to the variable expression of lineage-defining markers across tissues (54). CD11c-Cre is used to target total DCs and while the largest Cre-induced effects are indeed in the DC compartment, contribution of other CD11c-expressing cells, including macrophages or T cells, to an obtained phenotype cannot be excluded (55).

To move the DC field forward it is important to keep optimizing models and methods (Fig. 2). As monocyte to DC differentiation is relatively easy, commonly used, and enables high cell yields, newly obtained knowledge on immunological markers, metabolic conditions, and cytokine requirements for the distinct developmental trajectories should be used to optimize this model. iPSC-derived DCs are also a highly valuable model to study DC biology, but the use of this model has not received much traction yet amongst immunologists. Nevertheless, adapting to new protocols that better take into account the specific metabolic context in which DCs are cultured are likely to be critical to improve efficacy of DC-based therapies.

DCs as therapeutic intervention: targeting AMPK signaling?

The field of immunotherapy, the treatment of disease through stimulation or suppression of the immune system, is quickly expanding since the FDA approval of the first immune checkpoint inhibitor in 2011, but has a long history with earliest reports regarding the disappearance of tumors after infection, from ancient Egypt, 3000 years ago (56,57). The basis for modern science started in the 18th century and led to one of the first major immunotherapeutic breakthroughs in 1796: the discovery of protective immunity against smallpox after inoculation with common cowpox virus. Today, there is a broad spectrum of immunotherapeutic treatments, including

prophylactic and therapeutic vaccinations, immunosuppressants, antibody-based therapies, cytokine-treatments, and cell-based therapies (56,57). As antigen-presenting cells, DCs have the capacity to initiate a highly specific immune response and are therefore a very interesting target for cell-based therapies.

In 2010 the FDA approved the first (and thus far only) DC vaccination therapy, which aimed to treat prostate cancer. Autologous PBMC-derived APCs were activated *ex vivo* with a fusion protein consisting of a tumor antigen and GM-CSF and although clinical trial results were promising, it did not improve disease outcome in clinical settings (56,58). Since then, multiple approaches have been developed to improve DC therapy. *Ex vivo* generation of DCs from monocytes and *in vivo* targeting of DCs using nanoparticles are strategies that use immunomodulatory compounds to change the *in situ* immune response (Fig. 2) (59).

Clinical trials have proved the safety of tolerogenic moDCs for treatment of inflammatory diseases (59,60) and immunogenic moDCs for treatment of cancer (61,62), but efficacy is generally still limited. Problems include reduced fitness of patient-derived monocytes, limited duration of the tolerogenic state, insufficient antigen presentation capacity, and impaired migratory abilities (59–62). Delivering antigens and immunomodulatory compounds to DCs *in vivo* would overcome some of these problems. Although various nanoparticles are already approved for therapy (63), therapies using DC-based nanoparticles are still in early stages (64). Results from clinical trials are promising, but optimization of the nanoparticles itself, the delivery methods, and the desired cell-specificity, are examples of aspects that need to be improved (64). Important additional variables that impact efficacy for both approaches include effects of the micro-environment (e.g. the immunosuppressive TME may overrule the enhanced inflammatory capacity of the treated-DCs) and the selection of the type of antigen, adjuvant, or immunomodulatory compound.

Results from this thesis indicate that AMPK activation may be a promising strategy for the generation of therapeutic tolerogenic DCs. Furthermore, inhibiting AMPK activity may boost inflammation of DC-based cancer therapies by providing protection against metabolic stress in the TME. Further studies into AMPK signaling in DCs, as part of larger efforts to better delineate how nutrient availability, DC metabolism, and DC function are connected, will hopefully contribute to improvement of DC-based therapies and better treatment of immune-driven diseases.

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