Role of metabolic pathways and sensors in regulation of dendritic cell-driven T cell responses

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10

General discussion

Introduction

The establishment of a successful immune response depends on the timely coordination of multiple innate and adaptive immune cells; with divergent infectious agents in different locations leading to distinct combinations of immune cells [1-5]. Dendritic cells (DCs) are the quintessential antigen presenting cell type that integrates and relays danger signals from the peripheral tissues to activate and inform naïve T cells in secondary lymphoid tissues (reviewed in [6]). DCs (reviewed in **Chapter 2**) and other immune cells (reviewed in **Chapter 6**) update their metabolism to match the functional output that is required by a specific situation (reviewed in [7]) and understanding these changes to reveal new therapeutic opportunities can be considered the goal of 'immunometabolism'.

Cell culture systems have been indispensable for the development of key concepts in the field of immunometabolism. However, even if we would put the appropriately interacting immune cells in a dish, at exactly the right ratio of cells, with a carefully chosen media, this would still not recapitulate the metabolic gradients and zones that we can expect in tissues and organs (discussed in [8]). The liver is probably the best studied example of metabolic zonation [9, 10], but also secondary lymphoid organs - although more irregular in organization - have been described to show zones of respective pH [11] and oxygen [12, 13] and gradients of respective antigens [14] and cytokines [15]. All which can modify the metabolism of an immune cell (reviewed in [16]). In line with this, immune cells adapt their metabolism to the tissue they take residence in (reviewed in [17]) and can display further metabolic heterogeneity depending on their microanatomical position within the tissue. This has been shown most clearly in the context of tumours [18, 19]. A final layer of complexity is provided by the ability of immune cells to compete for nutrients (reviewed in [20]). The capacity of activated CD8 T cells to deprive adjacent DCs of glucose during their interaction [21] is an example of this that is most relevant to the establishment of successful DC mediated anti-viral and anti-tumour CD8 T cell responses (reviewed in [22]). This last point raises the question: if immune cell function is integrally linked to immune cell metabolism, but the nutrients that fuel these pathways are limited in the microenvironment, how do interacting immune cells coordinate their competing metabolisms in space and over time?

I will here discuss the findings presented in this thesis, what we have learned from it and where I envision the field of DC metabolism is heading. To do so, I will provide an in-depth summary of recent models of immune cell coordination during the generation of adaptive T cell responses. Particularly those that are driven by conventional DCs (cDCs) in secondary lymphoid organs. I will

then give an update on the often-overlooked metabolism of plasmacytoid DCs (pDCs) and then try to combine these findings and a decade of other immunometabolic knowledge with the previously discussed models of immune cell coordination. Moreover, I will look at what is known about the understudied metabolome of lymph fluid that bathes immune cells in the lymph node. Finally, I will present my view on which tools are needed to better understand the topics considered here and can potentially further mature the field DC metabolism.

CD4 T cell help and innate immune cell assistance during DC mediated T cell priming

CD4 T cell help to CD8 T cells is critical for the generation of maximal CD8 T cell responses in many settings of DC driven immune responses (reviewed in [23]). This by itself implies that a co-culture of antigen loaded DCs and CD8 T cells is limited in its explanatory value. Another layer of complexity arises from the fact that DCs need to move across multiple different environments before they find their antigen specific naïve T cell partner. They can therefore potentially interact with numerous cells along the way. Prime examples for this have recently come from studies that investigated the role of type I cDCs (cDC1s) in the establishment of anti-viral CD8 T cell responses [1-3, 5]. All these interactions might modulate the metabolism of a DC, which traditionally has been investigated using homogenous cell preparations in extracellular flux (XF) analysers (described in **Chapter 3**). However, before we can discuss what to expect in terms of DC metabolism in vivo, we first must introduce which immune cells are likely to interact with antigen loaded DCs and at what step of the immune response.

Professional antigen presenting DCs can broadly be divided into cDC1s and cDC2s. The former is best known for its superior ability to cross present exogenous antigens on major histocompatibility complex I (MHCI) to CD8 T cells and the latter for its interaction with CD4 T cells in the context of MHCII (reviewed in [6]). The expression of X C motif chemokine receptor 1 (XCR1) - a chemokine receptor that upon triggering causes migration towards high concentrations of its ligands XCL1 and XCL2 – is restricted to cDC1s and can therefore be used as a surface marker to distinguish cDC1s from other cells. This has also led to generation of mice with fluorescently labelled XCR1 and mice with XCR1 specific conditional deletion systems [24] that have drastically improved our ability to study cDC1 biology in vivo. cDC2s can be distinguished from cDC1s based on surface markers such as SIRPα and transcription factors such as IRF4 [25], but no cDC2 exclusive protein is known currently. The interaction between DCs, T cells and other immune cells during DC driven immune responses is therefore best understood from the perspective of cDC1s and the anti-viral CD8 T cells they expertly instruct (reviewed in [22])

Naïve T cells continuously enter lymph nodes at the level of the paracortex through high endothelial venules (HEVs) that connect the lymph nodes to the blood circulation. In contrast, in response to local infection, antigen loaded migratory cDCs (migDCs) enter draining lymph nodes via the afferent lymphatics and pass the lymph node capsule, subcapsular sinus (SCS), cortical sinus and intrafollicular area (IFA) before finally moving into the deeper paracortex. The paracortex is especially rich in T cells and is therefore also known as the T cell zone. The cortical sinuses and IFAs together with the B cell follicles they separate are known as the cortex. Alternatively, during systemic infection, lymph borne pathogens and their antigens flow into the lymph node and are captured by SCS resident macrophages and DCs. Under the guidance of inflammatory cues DCs and T cells will then meet at some of these locations and interact. Most activated T cells leave the lymph node via the medullary sinus, lymph node medulla and efferent lymphatics. A minority of activated T cells differentiate into follicular B helper T cells (Tfh cells) and remain in the lymph node to interact with B cells in their follicles. Blood borne pathogens and their antigens are filtered from the circulation by the spleen. They are captured by macrophages and DCs residing in the marginal zones of splenic white pulp, which are isolated areas filled with white immune cells and that are distributed throughout the far more abundant red pulp. Importantly, like DCs in peripheral tissues, DCs in spleen marginal zones need to migrate to T cell areas. These are also located in splenic white pulp and are called the periarteriolar lymphoid sheath (reviewed in [6, 26]).

Studies with the model pathogen vaccinia virus (VV) have shown that after infection with VV by subcutaneous (s.c.) injection into the footpad, both virus infected type 1 migDCs (migDC1s) and migDC2s were found in the SCS of the lymph node, where they clustered with CD8 T cells in the absence of CD4 T cells, within the first 8-12 hours after infection [2]. Importantly, both DC subsets were able to activate CD8 T cells ex vivo at this point. During later stages of infection (38-40 hours), these activated CD8 T cells were found in the paracortex, where they clustered with different DC and CD4 T cells. This third subset of DCs were elucidated to be XCR1+ resident cDC1s (resDC1s) and without them, CD8 T cells did not form clusters with CD4 T cells and the number of activated CD8 T cells dropped. Mice that lacked resDC1 also failed to mount a robust memory recall response against *Listeria monocytes*, another intracellular pathogen. These findings together led to the notion that CD8 and CD4 T cells are initially activated separately in different intranodal locations on distinct DCs, but that they later interact with each other on resDC1s. Resident cDC1s therefore serve as a 'critical platform' for CD4 T cell help to CD8 T cells [2], which is important for the optimal induction of CD8 T cell responses in multiple settings (reviewed in [23]).

This concept was expanded by showing that pDCs translocate to these initial migDC-CD8 T cell clusters (10 hours) in a C C motif chemokine receptor type 5 (CCR5) dependent manner and failure to do so impaired later CD8 T cell responses [1]. Mechanistically, expression of the type I IFN receptor was required on resDC1s, but not on migDCs, for the maturation of resDC1s and their enhanced capacity to induce CD8 T cell responses [1]. This indicates that pDCs -through their canonical production of type I IFNs - activate the resDC1 platform and prepare it for optimal interaction with CD8 T cells and potentially also for interaction with CD4 T cells. It was also shown that CD8 T cells are likely to organize both clusters of migDCs-CD8 T cells-pDCs in the SCS [1] and CD8 T cell-resDC1s-CD4 T cells in the paracortex [2], or potentially a super cluster of CD8 T cell-resDC1s-pDC-CD4 T cells. This is because they produced robust amounts of CCL3 - a CCR5 ligand - in vitro and their production of XCL1 was needed for the formation of CD8 T cell-CD4 T cell clusters in vivo [1]. These findings together indicate that the optimal induction of CD8 T cell responses requires assistance of innate immune cells other than antigen loaded DCs and platform resDC1s.

Infection with herpes simplex virus (HSV) seems to share some features of this intranodal immune cell coordination during VV infection. Like VV infection, during later stages of HSV infection (42 hours), CD8 T cells were found to cluster in the paracortex around an activated non-migratory DC that expressed XCR1 [5]. In addition, these CD8 T cells were found to be frequently visited by CD4 T cells. However, unlike VV infection, CD4 T cells rather than CD8 T cells were the first to reduce velocity, cluster around migDCs and become activated. Furthermore, these cells were the first to leave the lymph node and to arrive in the spleen [5]. It should be noted that this model of infection was performed by scarification of epidermal flank skin and therefore local. Upon switching to s.c. injection - as was done in the VV model - initial CD8 T cells and CD4 T cell activation was synchronous as well rather than staggered. This opens the possibility that the reported differences between VV and HSV infection are the result of divergent infection strategies rather than the actions of distinct virus families. The transfer of antigen from CD103+ migDC1s to CD8+ resDC1s is necessary for the induction of anti-HSV immune responses [27, 28] and both these DC subsets express XCR1. The importance of resDC1s as a critical platform for CD4 T cell help to CD8 T cells during HSV infection [5] could therefore not be formally tested by conditional deletion of XCR1+ cells as was done in the VV model [2]. Still, CD4 T cells interact with CD8 T cells on resDC1s during HSV infection [5], CD4 T cells are needed for the production of CTL priming IL-15 by CD8+ DCs [29] and CD8+ DCs are required for the establishment of a successful immune response to HSV [30]. These findings together indicate that resDC1s are also a critical platform for CD4 T cell help to CD8 T cells

during HSV infection. The production of IL-15 by CD8+ DCs needed both CD4 T cells and stimulation with type I IFNs [29], but pDCs were not required for protective immunity [31]. This suggests that perhaps another innate immune cell - through its production of type I IFNs - assists the activation of the resDC1 platform during HSV infection. For example, host protection against lymph borne vesicular stomatitis virus (VSV) depends on the early production of type I IFNs by SCS macrophages and not pDCs [32].

In response to mouse cytomegalovirus (MCMV) infection, natural killer cell (NK cell) distribution in the spleen changes from a homogenous spread throughout the red pulp to a more concentrated localization around the marginal zone of the white pulp [3]. Here cDC1s were found to cluster with these NK cells (40 hours) before they moved into deeper white pulp structures, such as the bridging channels (44 hours) and T cell areas (48 hours). Whole body deletion of XCR1 decreased the number of NK cell-cDC1 clusters at the marginal zone and the migration of cDC1s into the T cell zone. This was associated with a failure of cDC1s to upregulate their expression of the chemokine receptors GPR183 and CCR7. This was additionally associated with a strong decrease in IL-12 production by DCs and type II IFN (i.e. IFNγ) production by NK cells. The expression of costimulatory molecules on cDC1s was not affected. Reciprocal interaction between cDC1s and NK cells was revealed as XCR1 specific deletion of IL-15 significantly reduced the number of NK cells (4 days) and splenic NK cells isolated from MCMV infected mice induced the upregulation of CCR7 expression on immature type 1 Flt3L differentiated in vitro DCs (FLDC1s). The latter was dependent on stimulation of FLDC1s with granulocyte-macrophage colony-stimulating factor (GM-CSF), which NK cells were the major producers of during MCMV infection (40 hours). In turn, NK cells isolated from naïve mice produced GM-CSF after ex vivo stimulation with recombinant IL-12, of which cDC1s were the dominant source in vivo (40 hours; [3]). CCR7 [33] and GPR183 [34-36] are involved in the intrasplenic homing of cDC2s from the marginal zone into T cell areas. The authors proposed that during MCMV infection, splenic NK cells licence splenic cDC1s for migration towards T cell zones deeper in the white pulp. While NK cells were by far the major producers of XCL1 during both steady state conditions and in response to MCMV infection, NK cell specific deletion of XCL1 did not affect their clustering with cDC1s [3], suggesting that NK cells were are attracted to the cDC1s and not vice versa

Natural killer cells also seem to play a role in the induction of CD4+ Th1 responses to intradermally injected (i.d) *Mycobacterium smegmatis* [4], a facultative intracellular bacterium. In response to infection with *M smegmatis*, antigen loaded migDC2s (investigated on day 2) and large numbers of IL-12 producing monocyte derived inflammatory DCs (infDCs; day 2-3) appeared in

the draining lymph node, together with IFNγ producing NK cells (day 1-2) and IFNγ producing Th1 cells (day 5). These infDCs surpassed migDC2s in their production of IL-12, which in combination with IL-18 was entirely responsible for the appearance of IFNγ producing NK cells in the lymph node. The production of IFNγ was conversely required for the recruitment of monocytes into the lymph node and their differentiation into infDCs. The recruitment of monocytes was in turn necessary for optimal induction of Th1 responses. Together, although the exact kinetics of this immune response was not assessed, the authors suggested that - based on other published literature - a cooperation between migDC2s and infDCs is likely, in which migDCs provide antigen and co-stimulation for T cell activation and infDCs provide high levels of IL-12 for T cell differentiation into Th1 cells [4].

In **Chapter 9** we showed how long-term loss of mTORC1 signalling in cDCs in vivo by deletion of raptor in CD11c expressing cells (CD11cΔraptor mice) leads to a pronounced reduction in MHC complexes and costimulatory molecules on cDCs. Of these, MHCI was most strikingly affected. Nevertheless, CD8 T cell responses to primary infection with *L. monocytogenes* were intact in these mice and so were CD8 T cell memory responses and host protection. A comparison of several s.c. injection strategies with antigenic peptides and TLR adjuvants revealed that the frequencies of antigen specific CD8 T cells within the CD8 T cell pool could even be increased in these mice following immunization. No matter the combination of ovalbumin, human papillomavirus (HPV) E7 protein, TLR3 ligand PolyIC and TLR9 ligand CpG-B. The exact mechanism for this was not elucidated, but we did report a doubling of IL-12 producing CD11c+ skin migratory Langerhans cells (LCs) in draining lymph nodes. Our current understanding of murine LCs is that they have a relatively poor cross priming capacity [37]. However, they might have taken up the role of high IL-12 production assisting innate immune cells in CD11cΔraptor mice, thereby explaining the intact or even enhanced immune responses in mice with impaired cDCs.

Taken together, these publications provide evidence for the existence of a resDC1 platform on which CD8 T cells and CD4 T cells organize themselves for the optimal induction of immunity in response to viral infection. In some settings, pDCs provide type IFNs that assist in the activation and preparation of the resDC1 platform, while in other settings pDCs are not are not required, but the ability of cDCs to react to type I IFNs is still crucial for their activation. Natural killer cells are likely to assist cDCs in their migration towards the deeper T cell zones in secondary lymphoid organs and NK cells can also interact in a reciprocal manner with IL-12 and IL-15 producing cells. These are cDC1s in some settings and in other settings infDCs, which are likely to assist antigen

presenting cDCs in their interaction with T cells by providing additional IL-12. All of this suggests that complex clusters of several different immune cells interacting arise at multiple points along a spatiotemporal description of DC mediated T cell priming, in which these immune cells will need to coordinate their interplay for the optimal induction of immune responses and the establishment of protective immunity.

Nutrient competition between DCs and T cells

The concept of nutrient competition between immune cells is not new (reviewed in [20]) and especially the competition for tryptophan between DCs and T cells is well established (reviewed in [38]). However, the recent finding that DCs differentiated in vitro using GM-CSF (GMDCs) and were starved of glucose - which can occur during their interaction with glucose hungry activated CD8 T cells - perform better in T cell activation assays than their glucose satiated counterparts [21] is striking. This is because the metabolism of pro-inflammatory DCs has traditionally been associated with anaerobic glycolysis (reviewed in [39]. This competition for glucose between DCs and activated CD8 T cells occurred both in vitro and in vivo and mechanistically involved an immediate enhancement of costimulatory molecule expression and IL-12 transcription, but also the prolonged maintenance of this activated state. Low glucose availability inhibited mTORC1 activity in GMDCs and inhibited the establishment of a feed forward loop with mTORC1 mediated activation of hypoxia inducible factor 1 alpha (HIF1α). HIF1α mediates the synthesis of nitric oxide (NO) by inducing transcription of inducible NO synthase (iNOS). NO stabilizes HIF1α protein, thereby completing the loop [21]. Inhibition of mTORC1 activity in GMDCs had previously been shown to enhance GMDC immunogenicity after transfer in vivo [40-42], which was associated with increased autophagy [42] and improved survival [40] through prevention of mitochondria poisoning by NO [43]. This paper built upon those findings by showing the existence of a mTORC1-HIF1α-iNOS-NO-HIF1α feed forward loop that could also be initiated by exogenous NO. Most importantly in the context of immunometabolism, this paper visualized and pointed out that two DCs can be adjacent to each other and still display strikingly distinct nutrient sensing states. Dendritic cells that cluster with large numbers of T cells were found to be in a nutrient depleted “mTORC off” state, whereas DCs that were by themselves displayed active mTORC1 signalling. This highlights the need for tools that can “spatially resolve the metabolic configurations of single cells within their tissue microenvironment”, as was strongly argued for in an excellent recent review [44].

Although the glucose starvation of GMDCs by glucose greedy CTLs is the first example of nutrient competition that potentiates rather than limits DCs in their T cell priming capacity, it is possible that other combinations of DC

and T cell subsets use similar mechanisms. For example, blocking glycolysis in human moDCs using 2-DG enhances their transcription of the Th17 associated cytokine IL-23, if it is part of an overarching program that induces the unfolding protein response (UPR; [45]). In T cells, the UPR can be induced by simultaneous inhibition of glucose and glutamine uptake [46] and just recently, it was shown that skin migDCs become metabolically reprogrammed during obesity to increase IL-23 and IL-6 production in an UPR dependent manner [47]. This was reproduced in GMDCs by either glutamine deprivation or 2-DG exposure. Interestingly, deletion of phosphofructokinase, an enzyme lower in the glycolysis pathway than the one inhibited by 2-DG, had no effect on IL-23 transcription [47]. This hints at a possible involvement of the hexosamine biosynthesis pathway (HBP), which requires both glutamine and the upper glycolysis metabolic intermediate fructose-6-phosphate for pathway activity. Together, it is therefore imaginable that the differentiation of T cells towards a CTL or Th1 cell is reinforced by starving DCs of glucose and thereby boosts the secretion of IL-12, while the differentiation towards a Th17 cell is reinforced by starving DCs of both glucose and glutamine and thereby boosts the secretion of IL-23 and IL-6. Studies have shown that the in vitro differentiation of murine Th1 and Th17 cells are both dependent on the glutamine transporter SLC1A5, in contrast to Th2 cells and Tregs [48, 49]. However, one of these studies also showed that the transfer of glutamine transporter deficient T cells into a mouse with experimental allergic encephalomyelitis resulted in less Th17 cells recovered as expected, but surprisingly more Th1 cells [49]. This suggests that primary Th17 cells are in fact more dependent on glutamine than their Th1 cell counterparts and makes it imaginable that physiologically differentiated Th17 cells are more competitive for glutamine.

Our own work shows that the intracellular glutamine pool of moDCs conditioned with the Th2 associated antigen omega-1 is larger than that of immature moDCs (**Chapter 7**) and it is also larger in moDCs conditioned with a combination of the Th1 associated antigens LPS and Poly (I:C). In contrast, moDCs conditioned with the Th17 associated antigen zymosan have unchanged quantities of glutamine (Pelgrom, *unpublished*). Metabolite abundance does not distinguish between altered extracellular uptake and altered pathway flux, but it is tempting to speculate that Th17 priming DCs have adapted their metabolic machinery to match that of Th17 cells. For example, by limiting their intracellular glutamine pool they might make themselves susceptible to glutamine starvation and by the same token render them poised to produce Th17 priming cytokines, which may involve a decreased engagement of the HBP. This would position Th17 priming DCs differently from the Th2 and Th1 priming DCs we investigated, as the latter two were found to depend on O-GlcNAcylation and N-glycosylation respectively

for their T cell priming capacity (**Chapter 7**), which are both reactions that use the HBP end product uridine diphosphate N-acetylglucosamine (UDP-GlcNAc).

Taken together, in the microenvironment of DC-T cell interaction, T cells can starve DCs for nutrients, even when surrounded by culture media with supraphysiological concentrations of nutrients. Restriction of glucose can lead to CTL/Th1 priming IL-12 production associated with decreased mTORC1 activity, while restriction of glucose and glutamine flux through the HBP may lead to production of Th17 priming cytokines IL-23 and IL-6.

Instruction of DC metabolism through paracrine cytokines and metabolites
In the previous section we pointed out the importance of DC metabolism in the establishment of T cell responses. We additionally provided examples of how pDCs activate the lymph node resDC1 critical platform through their production of type I IFNs and how NK cells assist in migration of cDCs to T cell areas through their production of GM-CSF and potentially IFNγ. So, what do we know about the relationship between these cytokines and immune cell metabolism?

Type I IFNs stimulation was necessary but not enough for the activation of splenic cDCs and the acquisition of their T cell priming capacity after injection of Poly (I:C) in vivo [50]. This was associated with a switch from OXPHOS towards anaerobic glycolysis that involved HIF1α [50] in a manner that was disconnected from NO, as cDCs do not produce NO like GMDCs (reviewed in [51]). Lymph node resDC1s share many similarities with splenic cDC1s including their expression of CD8 (reviewed in [6]) and it is therefore imaginable that the lymph node resDC1s critical platform switches towards anaerobic glycolysis upon exposure to type I IFNs [1] like splenic cDCs and moreover, that this metabolic transition is required for their activation and platform function.

It has been shown that GM-CSF stimulation enhances glycolysis in macrophages [52, 53]. As glycolysis is necessary for the oligomerization of CCR7 in DCs [54] and splenic NK cell derived GM-CSF is thought to assists in the migration of splenic DCs [3], there will potentially be DCs in spleen marginal zones that are adjacent to each other but display strikingly distinct glycolytic pathway "on and off" states as a consequence of being in a cluster with GM-CSF producing NK cells (glycolysis on) or being by themselves (glycolysis off).

Although a clear role for NK cells in the intranodal priming of CD8 T cells during VV infection was excluded [2], GM-CSF induced glycolysis may still facilitate the migration of antigen loaded migDCs from the SCS to the T cell areas, as pDCs can also produce GM-CSF [55]. Alternatively, when SCS macrophages are delivered VV antigen and transfer this antigen to lymph node resDC1s [56],

NK cells may play the same role as facilitators of intra-organ DC migration as they did during splenic MCMV infection [3].

For an in depth description of NK cell metabolism we like to refer you to another review [57], but briefly, short-term stimulation of NK cells with cytokines such as IL-12 and IL-15 does not result in increased rates of glycolysis and OXPHOS [58], while overnight stimulation results in a substantial enhancement of overall cellular metabolism [59]. Moreover, while NK cells do not seem to require FAO [58] and glutaminolysis [58, 60] for their functions, they are highly depend on glycolysis [61] and are likely to import glutamine from the extracellular environment to stimulate vital mTORC1 signalling (reviewed in [57]). This would make them poor company for glucose and glutamine hungry activated T cells (reviewed in [20]) in T cell areas of the spleen and might explain why they did not take part in the late stage formation of DC-T cell clusters during VV infection [2].

IFNγ is produced by NK cells upon viral infection [3] but also by activated CD8 T cells [2]. It was shown recently that intertumoral production of IFNγ by CD8 T cells promoted the classical activation of tumour associated macrophages by stimulating AMPK activity. This in turn down regulated SREBP1 and lipid metabolism in these macrophages that would otherwise sustain their alternative activation [62]. We have discussed in the previous section how inhibition of mTORC1 in DCs after their initial activation can enhance their CD8 T cell priming capacity. AMPK is a well-known negative controller of mTORC1 (reviewed in [63]). It is therefore possible to imagine that highly immunogenic DCs with low mTORC1 activity are kept in this state through the paracrine action of IFNγ produced by activated CD8 T cells they interact with.

In **Chapter 8** we showed how absence of AMPK signalling in cDCs in vivo by deletion of its upstream kinase liver kinase B1 (LKB1) in CD11c expressing cells (CD11cΔLKB1 mice) leads to increased maturation, migration and T cell priming capacity of peripheral cDCs. That the loss of AMPK signalling in DCs (**Chapter 8**) and stimulation of AMPK activity in myeloid cells [62] both result in enhanced immunogenicity seems incongruent. However, this inconsistency is also seen with mTORC1. Deletion of raptor led to decreased maturation of cDCs (**Chapter 9**), while acute inhibition of mTORC1 increased the CD8 T cell priming capacity of GMDCs [21]. This suggests that DCs need to be able to switch back and forth between AMPK high/mTORC1 low and AMPK low/mTORC1 high states, for optimal functioning from their initial activation in the periphery to their final interaction with T cells in the T cell area. In addition to timing, location also influences the immunological outcome of AMPK/mTORC1 manipulation in DCs. In **Chapter 8** we showed that LKB1 deficient cDC2s in the thymus have a potientiated ability to differentiate thymocytes into natural Tregs, while others

provided further evidence that LKB1 deficient DCs in the periphery tend to promote the polarization of mature naïve T cells into Th17 cells [64]. The former relied on gain of phospholipase C β1 activity and intracellular calcium release, while the latter depended mostly on loss of AMPK mediated negative control on mTORC1. Together, these publications highlight the complexity of metabolic signalling in immune cells in situ and the value of more controllable deletion systems such as diphtheria toxin receptor mediated systems.

Tumour necrosis factor (TNF) is another cytokine that is produced by activated CD8 T cells and it can also promote the classical activation of macrophages, although in this setting by stimulation of SREBP2 [65] instead of inhibition of SREBP1 [62]. This in turn leads to the transcription of genes involved in sterol metabolism and in addition the transcription of pro-inflammatory genes in a manner that was independent of cholesterol synthesis [65]. Stimulation with the TLR3 and 4 ligands Poly (I:C) and LPS and also IFNα, IFNβ and IFNγ can induce the transcription of genes involved in cholesterol synthesis in GMDCs [66] and cholesterol accumulation induces inflammasome activation and production of pro-inflammatory cytokines in splenic cDC2s [67]. Conversely, acute inhibition of the mevalonate pathway involved in cholesterol synthesis using statins inhibits the production of type I IFNs by murine and human pDCs [68]. Together, this makes it imaginable that the stimulation of de novo cholesterol synthesis in DCs after exposure to CD8 T cell derived TNF supports the further development of DC mediated T cell responses in secondary lymphoid organs. This contrasts with de novo FA synthesis which seems to be dispensable for cDC function [69].

In this section we have described pro-inflammatory paracrine signals, but it is also important to have brakes in place that can prevent excessive inflammation which can lead to collateral host tissue damage. In this context, we have already referred to papers that showed how preventing autocrine production of NO by GMDCs could enhance their immunogenicity [21, 43], but importantly, NO produced by neighbouring cells can also institute a HIF1α-iNOS-NO-HIF1α feed forward loop that conversely supresses immunogenicity of DCs, even in iNOS deficient DCs [21]. It is noteworthy that NO serves as donor for the post-translational modification of proteins through nitrosylation and that in addition to prolyl hydroxylase (PHD), the enzyme that breaks down HIF1α, and nitrosylation of HIF1α itself, many other immunomodulatory transcription factors can be nitrosylated (reviewed in [70]). Moreover, a recent study showed that after high NO production, which was induced by acute kidney injury, the top nitrosylated proteins in kidney extracts were almost all metabolic enzymes [71]. This suggests that the effects of paracrine NO production extend well beyond that of on DCs and beyond that of HIF1α [21] and poisoning of mitochondria [72].

Classically activated macrophages are an obvious candidate for paracrine NO production because they are in part characterized by their production of NO (reviewed in [16]). However, SCS macrophages were not important for early-stage formation of DC-T cell clusters during VV infection [2] and in line with this, the contribution of SCS macrophages to host protection against VV infection seems limited [73]. Nevertheless, SCS macrophages are known to have important functions during other examples of viral infection [32, 74]. It is also noteworthy that in addition to production of arginine derived NO by classically activated macrophages, alternatively activated macrophages can secrete arginine derived polyamines, which can induce a tolerogenic program in neighbouring DCs [75]. In which in vivo settings this interaction occurs is currently unknown, although the tumour environment and its myeloid derived tumour suppressor cells seems a plausible scenario.

Monocytes are also recruited to the lymph node during viral infections in a type I IFN and CCL2 dependent manner (reviewed in [76]). These monocytes can be helpful in some situations (e.g. VSV infection [77]) and destructive in other circumstances (e.g. LCMV infection [77] & chikungunya virus [CHIKV; [78]). In the latter situations, protective immune responses could be restored by inhibition of NO production [77, 78]. This suggests that these pathogens recruit NO producing inflammatory monocytes to hijack a normally healthy form of negative feedback, in which IFNγ and TNF production by activated T cells stimulates lymph node reticular fibroblasts to produce NO, that in turn tempers further T cell proliferation [79]. Reticular fibroblasts are also known to alter the metabolism of T cells through production of cytokines such as IL-6 [80], while T cells in turn can alter the metabolism of reticular fibroblasts through production of IL-17 [81]. However, if these fibroblasts influence the activity and metabolism other immune cells such as DCs are yet to be elucidated. Moreover, although some lymph node reticular fibroblasts have the capacity to produce retinoic acid [82], if metabolites other than NO play a role in these processes remains unclear.

The anti-inflammatory cytokine IL-10 is known to control and resolve inflammation in part by controlling the cellular metabolism of macrophages [83], while the anti-inflammatory cytokine TFGβ can inhibit the activation and function of NK cells through mTORC1 dependent [84] and independent means [85]. However, while both cell types have been discussed above in the context of viral infections, if and how these cytokines play a role in the fine tuning of immune cell interactions during the establishment of an anti-viral immune response remains elusive for the moment.

Taken together, at every step of the DC-mediated immune responses discussed, be it the NK cell-DC cluster, the migDC-CD8 T cell-pDC cluster or the CD8 T cell-resDC1-CD4 T cell cluster, cytokines are being produced by non-DC cells that are very likely to alter the metabolism of the interacting DC. However, as there are no studies yet that have investigated the metabolism of DCs during the establishment of DC mediated immune responses in a spatiotemporal manner, the actual metabolic state of DCs at these junctions remains speculation.

Stocking up on nutrients before the long haul to the draining lymph node or inflamed tissue

In **chapter 4** we demonstrated that GMDCs have glycogen stores that are utilized in the production of citrate during the early stages of LPS induced metabolic reprogramming, when glucose transporters are not yet upregulated on the surface. This investigation was sparked by an older study showing that murine lymphoid cDCs and human adipose tissue cDCs also have glycogen stores and, in addition to that, also neutral lipid stores [86]. The conditioning of GMDCs with IL-4 resulted in the increased storage of glycogen and lipids, while the effects of LPS were not fully elucidated at the time. It was reported however that conditioning with LPS resulted in the increased storage of lipids [86] and we now know that stimulation with LPS conversely decreases glycogen stores (**Chapter 4**). Whether cDCs respond in a similar manner to these compounds as GMDCs remains to be seen. However, if this is the case, one might wonder if in response to these and other danger associated molecular patterns (DAMPs), DCs prepare themselves for different scenarios by outfitting themselves with distinct metabolic apparatus. Maybe the main function of the cDC that has recognized LPS is to travel to the draining lymph node, where it presents bacterial peptides to the T cells there and in line with the benefits of low mTORC1 activity in DCs during DC-T cell interaction [21] discussed in the previous section, has equipped itself with a metabolic machinery that avoids the consumption of mTORC1 activating glucose and glutamine and instead burn lipids. In contrast, the presence of IL-4 may indicate that an adaptive immune response has already been initiated and that the inflammatory moDCs must infiltrate an area of persistent infection and minimal nutrient availability, where low glucose levels can interfere with the oligomerization of the chemokine receptor CCR7 [54], something which might be overcome with the flux of glycogen derived carbons into glycolysis.

Stocking up on appropriate lipid species seems to be beneficial for DC immunogenicity in general (reviewed in [87]). Mouse and human liver DCs with a high lipid content produced more pro-inflammatory IL-6 and TNF at steady state conditions and after TLR triggering. These cells were shown to be nearly 10 times as efficient at cross presentation than their low lipid content

counterparts. This improved the interaction of high lipid content mouse liver DCs with CD8 T cells, CD4 T cells and NK cells both in vitro and in vivo in a manner that could be diminished by administration of the ACC inhibitor TOFA. Interestingly, the authors noted that these DCs were as good as splenic DCs regarding their T cell priming capacity but even better at activating NK cells [88]. Regarding splenic DCs, CD8+ cDC1s in the spleen had almost 40% more lipid droplets than their CD8- cDC2 counterparts. Deletion of Irgm3, a family member of the immunity related 47kDa GTPases (p47 GTPases) that localizes to endoplasmic reticulum (ER) and lipid bodies, diminished the accumulation of lipid bodies in spleen DCs and their capacity for cross presentation independent of co-stimulatory molecules expression and cytokine production. Moreover, these effects could be recapitulated using the diglyceride acyltransferase (DGAT) inhibitor xanthohumol [89]. Finally, these studies combined show that abundance of lipid stores can be increased in GMDCs in vitro using LPS [86] and IFNγ [89], while LPS and PolyIC injection can increase the abundance of lipid stores in liver DCs and splenic DCs in vivo [88, 89], with splenic cDC1s being more responsive than splenic cDC2s [89].

In **Chapter 9** we showed how long-term loss of mTORC1 signalling in cDCs leads to a pronounced reduction in MHCI surface expression on cDC1s, which was associated with a reduced uptake of the glucose analogue 2-NBDG by the same DCs. It was previously shown that glucose is used for de novo synthesis of fatty acids and subsequent lipid droplet formation in TLR-stimulated in vitro DCs [90]. As glycolysis and fatty acid synthesis are dependent on mTORC1 [91], it is tantalizing to speculate that reduced glycolysis and MHCI expression are linked by changes in the lipid content of raptor deficient cDC1s.

The type of lipid species that build up intracellularly seems important as the accumulation of peroxidised lipid bodies in DCs prevents the translocation of MHCI:peptide complexes to the cell surface [92, 93]. In line with this, inhibition of the cystine/glutamate antiporter (system xc-), which is involved in positively regulating levels of the antioxidant glutathione (GSH) and therefore the cells redox state, lowered the cellular ratio of GSH and its oxidized disulphide form (i.e. GSSG) in moDCs and lowered surface expression of MHCI independent of antigen uptake and co-stimulatory molecules expression. This resulted in a blockade of antigen presentation to CD8 T cells and to a lesser degree to CD4 T cells, which was recapitulated in murine splenic DCs [94]. It is also the only or one of the few demonstrations of how amino acid metabolism in DCs affects their canonical function of T cell priming, separately from the effects of amino acid deprivation during in vitro differentiation or prolonged ex vivo culture [95-97].

Taken together, DCs have intracellular stores of nutrients that in some instances helps them with the acute demands of activation and in other cases might prepare them for the needs of T cell interaction. Intracellular lipid bodies seem to be particularly important for cDC1s because they have more of them, generate more of them in response to DAMPs, and lipid quantity and quality is associated with antigen presentation in the context of MHCI. Whether the use of intracellular glycogen stores by DCs extends beyond that of T cell activating capacity and the production of the Th1/CTL associated cytokine IL-12, remains unknown. Moreover, it is currently unclear if specific classes of lipids (e.g. fatty acyls, sphingolipids and sterols) are important for different effector functions of DCs or whether the balance of normal versus oxidized lipids is more important. Finally, mouse models of inflammation with distinct local and lymph node functions for respectively infDCs and cDCs, such as during HSV infection [98], or where titration of antigen can change how a DC participates in the immune response, such as during house dust mite allergy [99], might provide another layer of complexity that can help to elucidate the contribution of intracellular nutrient stores in different situations.

Lymph fluid metabolome

In comparison to the presence of metabolites in whole blood, serum and plasma, little is known about the metabolome of lymph fluid. In this context, the content of lipid-transporting lipoproteins in mesenteric lymph fluid (MLF) has been studied best, because of the essential role for lipoproteins in the absorption of dietary fats and fat-soluble vitamins (reviewed in [100]). In line with the abundance of lipoproteins in MLF, lipids were the predominant class of metabolites found to change in MLF during the transition from a fasted to fed state. However, changes in glucose and essential amino acids such as tryptophan were also apparent [101].

One of the lipids that was clearly increased after eating is the short chain fatty acid butyric acid (also known as butyrate). In **Chapter 5** we showed how exposure of moDCs to butyrate conditions them for Treg priming, which was associated with suppression of LPS induced anaerobic glycolytic remodelling and in addition to a concomitant suppression of oxidative metabolism. Such a profile of overall suppressed metabolism also occurs after exposure to the tolerogenic compound dexamethasone (Pelgrom, *unpublished*), although others have reported that exposure of monocytes to vitamin D or retinoic acid while they are differentiating into moDCs leads to a more a anaerobic glycolytic moDC phenotype [102-104]. Butyrate is produced by gut microbiota and is consumed locally by colonocytes [105], although some will be transported from the gut into the blood circulation (reviewed in [106]) and lymphatics [101]. The observed fluctuations of butyrate levels [101] suggests that a one-time

exposure to butyrate as was done in **Chapter 5** of this thesis, is more in line with physiological exposure to butyrate, although the absolute concentrations of butyrate cannot be inferred from that study, as absolute concentrations of nutrients in MLF are rarely reported. However, concentrations of glucose and amino acids might be inferred from blood, as absolute levels of glucose and amino acids are comparable between lymph fluids and blood in certain steady state conditions [107, 108]. The metabolome of lymph fluid during inflammation remains elusive, as to our knowledge, only 2 studies investigated the change in the metabolome of lymph fluid during inflammation.

The first study focused on the lipid content of purified triglyceride-rich lipoproteins in MLF and found that puncture of the intestine and the subsequent release of faecal matter into the abdomen, resulted in the acute loss of lipid species in IMF lipoproteins. In contrast, reduced blood flow (i.e. ischaemia) induced by ligation of the mesenteric artery had no effect on lipid species [109]. It is tempting to speculate on the possibility of a preference for lipid consumption in response to the exposure to (commensal) bacteria versus the preferred consumption of non-lipid fuels as can be expected during hypoxia, which induces the transcription of glycolytic genes through the transcription factor HIF1α (reviewed in [110]). However, it should be noted that the duration of the first intervention was significantly longer and might have affected the absorption of dietary fats in the intestine through disruption of organ structural integrity [109].

The second study found that glutamine is one of three significantly modulated metabolites in brain lymph fluid (BLF) of rats after infection of the forelimb with a rabies pseudo virus [111]. Activated T cells consume large amounts of glutamine [20], so one might expect the concentration of glutamine to decrease during the initiation of anti-viral immune responses. However, instead, levels of glutamine were found to be increased in BLF after rabies pseudo virus infection [111]. The significance of improved glutamine availability was not investigated, but it is tempting to speculate on an intentional reorganization of glutamine metabolism to support the generation of glutamine-dependent anti-viral NK cells (reviewed in [57]) and anti-viral CD8+ cytotoxic T lymphocytes (CTLs; reviewed in [112]). Such a system has been described in the context of muscle repair, where intramuscular macrophages, endowed by high glutamine synthetase activity and low glutamate dehydrogenase activity, have the capacity to secrete glutamine and support the glutamine-dependent proliferation of muscle cells [113]. With respect to the brain, astroyctes are well known for their capacity to secrete glutamine to support the synthesis of glutamine-derived neurotransmitters by neurons (reviewed in [114]) and our brain releases measurable amounts of glutamine [115]. However, whether astrocytes use this capacity to support anti-viral immune responses remains to be elucidated.

The two other metabolites that were significantly modulated: dimethylglycine (DMG) - a product of choline metabolism - and dimethylamine were both decreased. Little is known about the interaction between DMG and immune cells, but a recent study suggested a potential mechanistic role for increased serum levels of DMG in the robust induction of IFNγ producing CD8 T cells in the intestine, which was dependent on intestinal CD103+ cDC1s and major histocompatibility class I (MHCI) expression, Moreover, this was associated with enhanced host resistance to infection with the intracellular bacterium *L. monocytegenes* [116]. However, this is the first publication linking DMG with immune cells, so, whether DMG can directly stimulate immune cells and if so, which immune cells, remains to be determined. Nevertheless, the intestine seems a relevant location for interaction between immune cells and DMG, as the abundance of its precursor choline is increased in IMF after feeding [101]. In addition, the consumption of DMG after rabies pseudo virus seems relevant, as CD103+ cDC1s are the subset best known for its capacity to cross-present antigens to CD8+ CTLS (reviewed in [117]).

Taken together, very little is known about changes in specific metabolites during lymph node inflammation except for glutamine and DMG in response to rabies pseudo virus.

The road ahead: development of new tools and platforms to study systems immunometabolism

As discussed in one of the previous sections, it takes time for a DC to migrate from a peripheral tissue to a draining lymph node and again more time to migrate from the outer SCS to the T cell area. Similarly, in the spleen, although the distances are considerably smaller, DCs still need to migrate from the marginal zone to the T cell area. This temporal aspect of DC function seems to be built into their biology. For example, blocking glycolysis was found to impair initial DC activation [54], which in vivo would most likely take place in the periphery, while blocking glycolysis was found to enhance later T cell priming capacity [21], that in vivo would most likely take place in T cell areas. Moreover, that there is also a spatial aspect to DC function is demonstrated in the most extreme manner by the evidence that two adjacent DCs can have completely opposite on and off states of the nutrient sensing kinase mTORC1 [21].

There is a clear need for new tools that can resolve these spatiotemporal aspects of DC metabolism and of immune cell metabolism in general [44]. However, the integration of single cell cytometry data with single cell RNA sequencing data might be sufficient to move the field of immunometabolism into the new era, while spatial mass spectrometry (MS) are still in their infancy [44].

One of the advantages of conventional fluorochrome based flow cytometry over mass cytometry by time of flight (CyTOF), is that it allows for the addition of other fluorescent tools such fluorescent nutrient analogues in addition to fluorescent antibodies [44]. The example given was the use of the fluorescent glucose analogue 2-NBDG [118], of which the uptake was said to be directly related to expression of the glucose transporter 1 [44]. However, another paper, that was published around the same time, has cast doubts on the reliability of 2-NBDG as a tool for the assessment of cellular glucose transporter capacity [119]. Herein, 2-NBDG was compared to the golden standard of stable isotope labelling (SIL) and it was found that in murine T cells, 2-NBDG uptake was not blocked by competitive substrates or inhibition of glucose transporters, while conversely, 2-NBDG could not competitively block the uptake of glucose [119]. Although the exact mechanism for the large discordance between 2-NBDG labelling and glucose transporter capacity was not investigated, the author has suggested that the attachment of such a bulky fluorophore [i.e. N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino] to 2-deoxyglucose probably interferes with the interaction between 2-deoxyglucose and its native transporters (personal communication with Linda Sinclair).

One possible solution to this problem is to simply stick to antibodies that recognize the nutrient transporter of interest and use the labelling intensity as a correlate for nutrient flux in or out of the cell. However, while this will probably be appropriate for most cases, there are instances where a reconfiguration of transporter localization is responsible for changes in nutrient flux before changes in transporter expression and therefore the labelling intensity that can be picked up. For example, flux through the glutamine transporter SLC1A5 (also known as ASCT2) is fully responsible for the acute increase in glutamine uptake that occurs 30 minutes after engagement of CD3 and CD28 on T cells in vitro, which manifests together with a rapid aggregation and colocalization of ASCT2 with the T cell receptor complex in the absence of altered glutamine transporter expression [49]. Moreover, the chances of an amino acid to be imported depends on the extracellular and intracellular abundance of that amino acid relative to other amino acids present (reviewed in [120]).

Taken together, the field of immunometabolism is quickly becoming a systems level science benefitting from technological improvements in single cell transcriptomics, proteomics and cytometry, which are simultaneously accompanied by a continuing refinement in antibody selection and computational approaches. Immunometabolism may also benefit from the development of more minimally modified nutrient analogues that are suitable for single cell cytometry and spatial microscopy.

Summary & conclusion

DCs are the canonical antigen presenting cell and therefore play a pivotal role in the establishment of successful antigen specific T cell responses. To facilitate their interaction with T cells, their biology undergoes drastic changes while migrating from peripheral tissues or spleen marginal zones all the way deep into the T cell areas of secondary lymphoid organs. These changes include necessary alterations in their metabolism as is illustrated in this thesis. In summary, the key novel findings presented in this thesis are:

- DCs can store glycogen to support early stages of their activation, which is especially important in environments with low levels of glucose (**Chapter 4**).
- Glycogen-derived carbons are preferentially used by DCs to support citrate metabolism (**Chapter 4**).
- Human DCs are conditioned by the short-chain fatty acid butyrate for Treg priming through production of retinoic acid (**Chapter 5**).
- Butyrate, propionate, and acetate – the three most common short-chain fatty acids - all suppress aerobic glycolysis and oxidative phosphorylation in human DCs (**Chapter 5**).
- DCs exposed to parasites or allergens are uniquely dependent on O-GlcNAcylation for Th2 priming, which is a post-translational modification of proteins with the metabolite N-acetylglucosamine (**Chapter 7**).
- Type 2 cDCs but not type 1 cDCs or pDCs in the blood of volunteers infected with hookworm show increased levels of protein O-GlcNAcylation (**Chapter 7**).
- The LKB1-AMPK signalling pathway – a master regulator of catabolic metabolism - limits DC immunogenicity in vivo **(Chapter 8**).
- Mice with LKB1-deficient DCs show enhanced output of thymic regulatory T cells that dominate the immunological phenotype of these mice (**Chapter 8**).
- The mTORC1 signalling pathway – a master regulator of anabolic metabolism – promotes DC immunogenicity in vivo (**Chapter 9**), in contrast to what was reported by in vitro bone marrow-derived DC models.
- Skin DCs are more severely affected by mTORC1 loss than splenic DCs (**Chapter 9**).
- mTORC1 limits skin Langerhans cells immunogenicity in vivo (**Chapter 9**).

Despite these novel insights, we are only beginning to delineate how temporal and qualitative changes in nutrient exposure control DC metabolism and biology in situ. Recently, elegant models that have allowed the study of type 1 conventional DCs have elucidated the presence of DC-immune cell clusters along this timeline of DC-mediated T cell priming, which include at least a NK cell-DC cluster, a migratory DC-CD8 T cell-plasmacytoid DC cluster and a CD8 T cell-resident type 1 conventional DC-CD4 T cell cluster. It is likely that

the metabolism of DCs keep adapting to these microenvironments that are transitioned between, but we at minimum know that DCs can display distinct high glycolysis and glucose starved/mTORC1 signalling off states that are required for their initial activation versus their prolonged activation respectively. Moreover, adjacent DCs can also display distinct glucose starved/mTORC1 signalling off and glucose satiated/mTORC1 signalling on states in relation to the cells they are interacting with while being in proximity of each other. Although direct evidence for DC metabolism adaptations to their described immune cell partners in vivo is absent, many studies provide suggestions for how these adaptations might be regulated. CD8 T cells can enforce a glucose starved/mTORC1 signalling off state in DCs that enhances and prolongs their capacity for T cell priming. Moreover, the cytokines that were reported necessary for the formation of the described immune cell clusters, including prototypical cytokines such as IFNα and IFNγ, have well described metabolism altering properties in other contexts. Little is known how DCs prepare their metabolic machineries to match those of their described immune cells partners, but some evidence exists for cross priming cDC1s relying heavily on lipids for their cross presentation, which would make sense in relation to the high glucose demands of NK cells and T cells, at least what is known from in vitro studies. Finally, even less is known about changes in the metabolome of lymph fluid during inflammation, although glutamine levels have been described to shift in response to rabies virus infection. The development of metabolic probes that are more minimally modified than the currently often used fluorescent nutrient analogues such as 2-NBDG, would facilitate in delineating these described spatiotemporal aspects of DC metabolism and of immune metabolisms in general.

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