extinguishing metaflammation: mechanisms and therapeutic opportunities for immunological control of metabolic dysfunctions

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CHAPTER 9

Summarizing discussion and future perspectives

Summarizing discussion

The work presented in this thesis aimed to contribute to a better understanding of the mechanisms responsible for the immunological control of metabolic homeostasis. In addition, the potency and underlying mechanisms of (helminth-derived) immunomodulatory molecules for alleviating obesity-induced metaflammation, insulin resistance and metabolic dysfunctions were investigated. This chapter summarizes the main findings and discusses these results in a broader perspective.

What was known about immunological control of metabolic homeostasis?

A plethora of evidence currently supports that chronic low-grade inflammation in insulin target tissues, *i.e.* adipose tissues, cardiac and skeletal muscle, liver and pancreas, contributes to the development of insulin resistance and type 2 diabetes (1, 2). Such control of tissue and whole-body metabolism by the immune system is one of the central themes in the emerging field of immunometabolism.

Increased expression of the inflammatory cytokine tumor necrosis factor (TNF) in obese white adipose tissue (WAT), which was found to induce insulin resistance by inhibiting canonical insulin signaling, was one of the landmark discoveries that fueled the interest in immunometabolism (3, 4). Additionally, macrophages were found to accumulate in obese WAT and account for the majority of TNF production, along with other proinflammatory mediators such as inducible nitric oxide synthase (iNOS) and interleukin (IL)-6 (5, 6). These adipose tissue macrophages (ATMs) were shown to express increased levels of the integrin CD11c and to predominantly localize around necrotic adipocytes in so-called crown-like structures (7-10), likely protecting their environment from lipotoxicity through lysosomal exocytosis and digestion of apoptotic/necrotic adipocytes (11). Conceivably, the flip side of this protective mechanism is inflammatory activation of ATMs (12), contributing to increased cytokine and chemokine production, immune cell recruitment, and the generation of a vicious circle that exacerbates inflammation and insulin resistance. Indeed, genetic manipulation to inhibit the monocyte chemoattractant protein 1 (MCP-1)-CCR2-axis, which coordinates circulating monocyte recruitment into tissues, alleviates inflammation and insulin resistance (13, 14). In the liver, activation of the liver-resident macrophages (Kupffer cells; KCs) and recruitment of monocytes that develop into proinflammatory monocyte-derived KCs (MoKCs) have also been demonstrated to drive the pathogenesis of non-alcoholic fatty liver disease (NAFLD) and progression towards non-alcoholic steatohepatitis (NASH) (15-17). Although the contribution of macrophages to the etiology

and pathogenesis of insulin resistance, NAFLD/NASH and type 2 diabetes has been well-established, other immune cells were also shown to accumulate in adipose tissue and liver during obesity (2). The underlying mechanisms responsible for deterioration of metabolic homeostasis by both macrophages and other immune cells are still incompletely resolved, and dissecting such mechanisms may provide novel therapeutic leads.

How did our studies advance the field?

Elucidating some of the processes involved in the regulation of proinflammatory macrophage activation may provide therapeutic leads to combat obesity-induced metabolic disorders. Immune cell function is increasingly recognized to be dictated by cellular metabolism (18). The cytosolic enzyme ATP citrate lyase (Acly) was recently shown to link cellular metabolism to inflammatory responses in LPS-activated macrophages (19). By converting mitochondrial-exported citrate resulting from increased glycolytic flux into oxaloacetate and acetyl-CoA, Acly provides metabolic intermediates allowing biosynthesis of fatty acids and cholesterol, as well as histone acetylation to regulate gene expression (19-21). Acly-deficient BMDMs were previously shown to exhibit enhanced LPS-induced cytokine production and surface marker expression, indicative of proinflammatory activation (22). Hence, in **chapter 2**, we studied the consequence of myeloid Acly-deficiency in the context of inflammatory disorders, including metaflammation. We confirmed that LPS treatment of Acly-deficient BMDMs indeed promoted a proinflammatory transcriptomic signature when compared to control BMDMs. Surprisingly, neither acute LPS-induced peritonitis, experimental autoimmune encephalomyelitis nor obesity-induced metabolic dysfunctions and metaflammation were significantly affected by myeloid Acly deficiency, indicating that the proinflammatory transcriptomic signature observed *in vitro* did not translate into worsening of inflammatory disorders *in vivo* (**Figure 1A**).

Acly was previously shown to link metabolism to inflammatory responses in LPS-stimulated macrophages by supporting histone acetylation and proinflammatory gene transcription (19, 21). These studies utilized small interfering RNAs for Acly knockdown *in vitro* or small molecule Acly inhibitors to study the role of Acly in macrophage biology, which may generate different outcomes when compared to constitutive genetic deletion of Acly. For example, Acly-deficient BMDMs may rewire cellular metabolism to rescue defects in cholesterol biosynthesis and lipogenesis (22). These processes are fueled by acetyl-CoA generated by Acly, and Acly-deficient BMDMs indeed displayed deregulated cholesterol metabolism. However, total cholesterol content was unchanged, indicating Acly-deficient macrophages employ strategies to compensate for the loss of Acly-derived acetyl-CoA. In

support of this, differentially regulated genes in Acly-deficient BMDMs compared with control BMDMs indicated an upregulation of genes involved in cholesterol biosynthesis and import, while cholesterol efflux genes were downregulated. In addition, acyl-coenzyme A synthetase short-chain family member 2 (*Acss2*), converting acetate into acetyl-CoA, was upregulated in Acly-deficient BMDMs, suggesting that this pathway may contribute to maintenance of acetyl-CoA levels in the absence of Acly (22). Hence, constitutive deletion of Acly may rewire cells to rescue the metabolic perturbation, which may explain the discrepancies between using small molecule Acly inhibitors and genetic deletion of Acly on macrophage inflammation *in vivo*. Of note, we found that *Acly* expression was upregulated in adipose tissue macrophages from obese mice, leading to speculate that Acly may play a role in obesity-induced proinflammatory activation of adipose tissue macrophages. Future studies could benefit from the use of inducible, macrophage-specific knockout models, such as the tamoxifen-inducible *Lyz2*$^{\text{Cre-ERT2}}$, or the *Cx3cr1*$^{\text{Cre-ERT2-IRES-YFP}}$ mouse model used in **chapter 7**.

Macrophages express a plethora of cell surface receptors that sense perturbations in the microenvironment, enabling their maintenance of homeostasis (23, 24). One of these receptors, the mannose receptor (MR/CD206), is a C-type lectin receptor that recognizes molecular patterns for internalization, processing and cross-presentation of antigens (25, 26). Interestingly, the MR can be proteolytically cleaved from the membrane and released as a soluble form (27, 28), which was recently shown to correlate with the pathogenesis of diverse inflammatory diseases (29-33). The effects of the MR and its soluble form (sMR) on proinflammatory macrophage activation in the context of metaflammation was studied in **chapter 3**. Here, we demonstrated that sMR reprogrammed mouse BMDMs and human monocyte-derived macrophages towards a proinflammatory phenotype *in vitro*. By binding to and inhibiting the phosphatase and pan-leukocyte marker CD45, sMR initiated a novel Src-Akt-NF-κB-mediated signaling pathway that resulted in proinflammatory cytokine production. sMR serum levels were increased in obese mice and humans, and correlated with adiposity. Strikingly, whole-body MR-deficient mice were completely protected against high fat diet (HFD)-induced hepatic steatosis, insulin resistance and glucose intolerance, which was associated with decreased CD11c-expressing obesity-associated macrophages that correlated with insulin resistance. Lastly, we found that treatment of lean, MR-sufficient mice with sMR increased insulin resistance and promoted proinflammatory activation of adipose tissue macrophages, unequivocally demonstrating a role for sMR in proinflammatory macrophage activation in the context of metaflammation (**Figure 1B**). These results and additional roles of the MR and other C-type lectins in regulating inflammation were reviewed in-depth in a broader context in **chapter 4**.

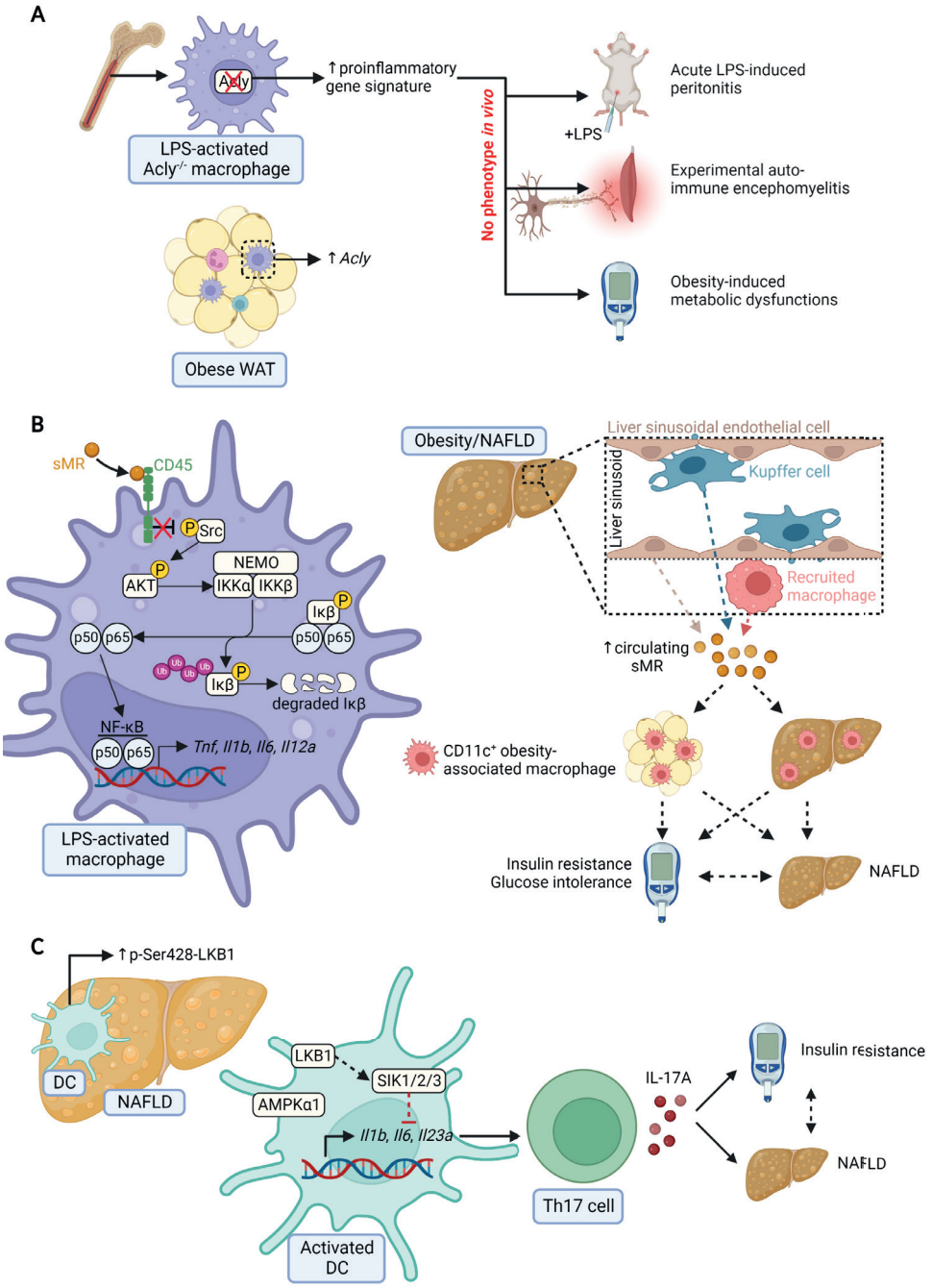

Figure 1. Mechanisms of immunological control of metabolic homeostasis. (**A**) Graphical summary of chapter 2. LPS-activated Acly-deficient bone marrow-derived macrophages (BMDMs)

Figure 1. Continued

display a proinflammatory gene signature compared to WT control BMDMs. However, myeloid Acly deletion did not impact acute LPS-induced peritonitis, experimental autoimmune encephalomyelitis and obesity-associated metabolic dysfunctions, although Acly was upregulated in adipose tissue macrophages of obese WT mice. (**B**) Graphical summary of chapter 3. Soluble mannose receptor (sMR) interacts with CD45 on the surface of macrophages, inhibiting its phosphatase activity. This enables Src-AKT-NF-κB-mediated signaling to promote proinflammatory macrophage activation and production of TNF, IL-1β, IL-6 and IL-12. Obesity increases circulating sMR levels, associated with increased CD11c-expressing obesity-associated macrophages in WAT and liver, which correlated with insulin resistance and glucose intolerance. Liver sinusoidal endothelial cells represent the majority of MR-expressing cells, while obesity increased MR-expressing macrophages in the liver. (**C**) Graphical summary of chapter 5. Obesity increased LKB1 phosphorylation at Serine428 in hepatic dendritic cells (DCs). LKB1 limits Th17 polarizing cytokine expression in DCs, potentially through SIK. In addition, LKB1 limits IL-17A+ Th17 cells in vivo, thereby controlling insulin resistance and NAFLD. Created with BioRender.com.

The MR is expressed by macrophages, DCs and endothelial cells (34). We show that liver sinusoidal endothelial cells constitute the majority of cells expressing the MR in metabolic tissues, yet MR-expressing macrophage numbers were increased particularly in livers of obese mice. Hence, one of the hypotheses is that obesity-induced liver macrophages are the main source of increased serum sMR in obesity, yet this remains to be verified. Unfortunately, no conditional knockout model for MR is available to date. Future studies could, however, rely on adoptive transfer of MR-deficient bone marrow to irradiated, MR-sufficient acceptor mice to address whether hematopoietic cells are the source of increased sMR in obesity.

Local increased expression and shedding of sMR might act in an autocrine fashion to promote proinflammatory macrophage activation and deteriorate insulin resistance. MR shedding is regulated by currently unidentified metalloproteases, and appears to occur constitutively as sMR levels in supernatant positively correlate with MR expression of cells in culture (27, 28). In this regard, it is worth noting that MR expression is regulated by PPAR-γ (35), a transcription factor that regulates expression of genes involved in glucose and lipid metabolism, and has been shown to be upregulated in both lipid-associated hepatic and WAT macrophages (36, 37). Strikingly, a recent study demonstrated that a novel subset of MR-expressing KCs is increased in steatotic livers of HFD-fed mice (38). These KCs display transcriptomic features of lipid metabolism and contribute to NASH pathogenesis at least partly through the fatty acid transporter CD36. As such, one may speculate that obesity could result in PPAR-γ-mediated upregulation of MR expression and shedding by lipid-associated macrophages in metabolic tissues, contributing to insulin resistance. Yet, given the beneficial effects of PPAR-γ agonists on whole-body insulin sensitivity (39), and the contribution of PPAR-γ to alternative activation of macrophages (40), this is a paradoxical

and challenging hypothesis that requires follow-up research. Alternatively, obesity was demonstrated to impact the expression of metalloproteinases in metabolic tissues (41), which may also contribute to increased MR shedding and warrants further studies.

Although we provide evidence for a role of sMR in proinflammatory macrophage activation and metaflammation through both loss-of-function experiments and exogenous administration of sMR, its *in vivo* cellular and molecular mechanisms are yet to be established. We demonstrated that sMR-mediated inflammatory reprogramming of macrophages is dependent on the interaction of sMR with CD45, which is expressed by macrophages but also other immune cells. Interfering with this interaction may provide clues to underlying mechanisms. For this, antibody-mediated neutralization or targeted mutagenesis of the region of CD45 interacting with sMR could be envisaged, although this first requires in-depth characterization of the sMR-CD45 molecular synapse. In addition, development of a CD45 conditional knockout model to delete CD45 from macrophages or other immune cells is of interest. Of note, after publication of our manuscript, an independent study demonstrated that MR-expressing adipose tissue macrophages in humans positively correlated with markers of metabolic dysfunctions, *i.e.* HbA1c, fasting blood glucose and criteria for metabolic syndrome (42). Moreover, these MR-expressing macrophages were enriched in visceral adipose tissue of obese, type 2 diabetics compared to both lean and obese non-diabetic humans, supporting a role for MR-expressing macrophages in contributing to metaflammation also in humans. Altogether, we propose that inhibiting sMR release, neutralizing sMR or interfering with the sMR-CD45 molecular synapse may hold promise to alleviate metaflammation and other inflammatory diseases.

Besides a well-established role for macrophages in the etiology of metaflammation, DCs also accumulate in metabolic tissues and contribute to insulin resistance (43-45). DCs are specialized antigen presenting cells that govern T cell responses depending on the inflammatory and metabolic microenvironment (46, 47). Indeed, T cell subset abundances were reported to change in metabolic tissues during obesity (48), indicative of altered DC function. The nutrient sensor liver kinase B1 (LKB1) was recently shown to control DC-mediated immune homeostasis and T cell priming in the context of allergic asthma and tumor development (49-51). In **chapter 5**, we report that Ser428-LKB1 phosphorylation is increased in hepatic DCs from obese mice, and that deletion of LKB1 from DCs increased HFD-induced hepatic steatosis, insulin resistance and glucose intolerance in obese mice. These metabolic perturbations were associated with increased regulatory T cells (Tregs) and T helper 17 (Th17) cells particularly in the liver, and were rescued through antibody-mediated neutralization of the canonical Th17 cytokine IL-17A. Indeed, LKB1-deficient DCs displayed increased expression of the Th17-polarizing cytokines IL-6, IL-1β and IL-23,

suggesting increased Th17 priming by LKB1-deficient DCs in line with previous work (51). Taken together, we identified LKB1 as a repressor of pathogenic Th17 cell priming in the liver, thereby controlling whole-body metabolic homeostasis (**Figure 1C**).

The tumor suppressor LKB1 is a serine/threonine kinase that controls cell polarity, growth and metabolism (52) by phosphorylating and activating AMP-activated protein kinase (AMPK) and 12 other AMPK-related kinases (53, 54). LKB1 activates AMPK in low-nutrient conditions, and its effects on cellular metabolism has been studied extensively in multiple *in vitro* and *in vivo* models (55). However, although reduced phosphorylation of the AMPK target acetyl-CoA carboxylase (ACC) was previously shown in LKB1-deficient splenic DCs (50), we show that increased Treg and Th17 priming as well as aggravated metabolic dysfunctions in LKB1-deficient obese mice was independent of AMPK. Instead, using pharmacological inhibitors in bone marrow DCs, we provide evidence for involvement of the LKB1 downstream salt-inducible kinase (SIK) family in repressing expression of the Th17-polarizing cytokines IL-6, IL-1β and IL-23. The SIK family consists of three isoforms (SIK1-3) and is involved in regulating gluconeogenesis, lipid metabolism and tumorigenesis (56). Interestingly, activation of SIKs retains class IIa histone deacetylases (HDACs) and cAMP-regulated transcriptional coactivators (CRTCs) in the cytoplasm, thereby either promoting or inhibiting transcription, respectively (57). CRTCs are coactivators of the transcription factor cAMP response element-binding protein (CREB) (58). The promotors of *Il6*, *Il1b* and *Il23a* genes all contain CREB binding sites (59-61), which leads to speculate that the absence of SIK activation in LKB1-deficient DCs may promote nuclear translocation of CRTCs and enhanced CREB-dependent *Il6*, *Il1b* and *Il23a* expression. Supporting this, SIK1 and SIK3 deficiencies were both reported to increase IL-6 production in tumor cells (62), and IL-6 and IL-1β production in immortalized Raw264.7 macrophages (63). However, SIK inhibition was also reported to inhibit TLR-induced proinflammatory cytokine production in macrophages and DCs (64, 65). As such, investigating whether SIK inhibition indeed increases nuclear translocation of CRTCs, thereby promoting Th17 polarization, would definitely be of interest. In addition, future transgenic studies are also required to identify the SIK isoforms involved in regulating Th17-polarizing cytokine expression in DCs. However, deleting individual SIK isoforms revealed that SIK family members display functional redundancy in some settings (57). Identification of the isoform involved may thus require the development of a DC-specific, inducible triple knockout (SIK1/2/3) model, which is currently not available and would be difficult to achieve. Finally, it is necessary to confirm that increased Th17-polarizing cytokine expression after SIK inhibition or deletion indeed results in polarization of Th17 cells *in vivo*, for instance via adoptive transfer of DCs that were *ex vivo* pulsed with a SIK inhibitor.

The composition of the cytokine milieu in which Th17 differentiation takes place was shown to determine pathogenicity of the effector Th17 cells, where the presence of IL-6, IL-1β and IL-23

promoted the development proinflammatory Th17 cells (66, 67). As we found increased expression of these cytokines in LKB1-deficient DCs, the increased Th17 cells in the livers of these mice are likely pathogenic in the context of obesity. However, the phenotype of these Th17 cells and underlying mechanisms for promoting hepatic steatosis and insulin resistance are still unclear. Since antibody-mediated neutralization of IL-17A in $CD11c^{\Delta LKB1}$ mice rescued metabolic perturbations, the Th17 effector cytokine IL-17A likely plays a role. Indeed, Th17 cells and IL-17A signaling have previously been shown to impair whole-body insulin sensitivity and drive hepatic steatosis (68-71). Mechanistically, IL-17A was suggested to either exert its effects on hepatocytes directly (68, 69), or signal through the IL-17RA on myeloid cells (71) to increase insulin resistance and hepatic steatosis. Recent single-cell transcriptomic analysis of Th17 cells in the liver identified an obesity-induced inflammatory hepatic Th17 (ihTh17) cell subset with increased expression the surface receptor CXCR3 and co-expression of the inflammatory cytokines IL-17A, interferon (IFN)γ and TNF (72). Here, the ihTh17 cells exacerbated NAFLD pathogenesis, which was at least partly dependent on their IFNγ-expression and increased glycolysis. Although in our settings the Th17 cells induced by LKB1-deficient DCs did not co-express IFNγ (data not shown), it would be interesting to investigate similarities with the ihTh17 phenotype by mapping the transcriptomic signature of ihTh17 cells onto transcriptomic data of hepatic Th17 cells of $CD11c^{\Delta LKB1}$ mice (73). This could be done by either performing single cell RNA sequencing of total liver leukocytes or bulk RNA sequencing on sorted Th17 cells from livers of $CD11c^{\Delta LKB1}$ mice.

Obesity increased Ser428-LKB1 phosphorylation in hepatic DCs. Although many posttranslational modifications of LKB1 have been identified, how these modifications affect LKB1 activity is only beginning to be resolved (74). LKB1 phosphorylation at Ser428 (in mice) or Ser431 (in humans) is dependent on the upstream kinases p90 Ribosomal S6 Kinase (p90RSK), protein kinase A and protein kinase C (PKC)ζ, and has been reported to increase LKB1 nucleocytoplasmic translocation and phosphorylation of downstream AMPK-related kinases (75-78). However, the role of this phosphorylation site remains controversial, as AMPK-related kinase phosphorylation by LKB1 has been reported to be normal in multiple tissues from homozygous knockin mice in which Ser431 is mutated to alanine (79). We currently cannot explain why and how Ser428-LKB1 phosphorylation is increased in hepatic DCs from obese mice, and what the exact functional consequence is, *i.e.* whether kinase activity and/or subcellular localization is altered. Still, we may hypothesize that this posttranslational modification constitutes a compensatory mechanism to limit pathogenic Th17 cell priming. While Ser428-LKB1 phosphorylation was increased in hepatic DCs from obese mice, it was unaltered in splenic and adipose tissue DCs, suggesting obesity-induced changes in the hepatic microenvironment that may alter LKB1 activation and DC effector functions. Obesity compromises the intestinal barrier function, resulting in increased gut permeability and altered serum metabolome (80). As

a result, increased transport of gut-derived bacterial products through the mesenteric and portal veins first targets the liver through a gut-liver axis. Indeed, LPS injection was previously reported to increase Ser428-LKB1 phosphorylation in whole lung and liver lysates, and in Raw264.7 macrophages (81). In addition, sodium butyrate, an indigestible fiber that is metabolized by the gut microbiome, was also demonstrated to increase Ser428-LKB1 phosphorylation in an *in vitro* model of hepatocytes (82), supporting that gut-derived metabolites may increase pLKB1 in hepatic resident cells. Obesity-induced gut permeability and/or metabolic endotoxemia might thus explain increased pLKB1 selectively in hepatic DCs. Future studies are required to elucidate the upstream molecular mechanisms that increase Ser428-LKB1 phosphorylation in hepatic DCs from obese mice. Interestingly, although obesity increased pLKB1 in hepatic DCs, phosphorylation of the AMPK downstream target ACC was unchanged, supporting AMPK-independent effects of LKB1 in DCs. Whether this increase in pLKB1 indeed results in downstream SIK-mediated repression of Th17-polarizing cytokines is currently unknown. In sum, we identified LKB1 as a regulator of DC function that empowers DC-mediated control of metabolic homeostasis. Targeting the LKB1-SIK axis in DCs may thus constitute a novel therapeutic approach for alleviating obesity-induced metabolic dysfunctions.

Altogether, our work identified novel mechanisms of myeloid cell-mediated control of whole-body metabolic homeostasis, that may present new therapeutic targets for treating metaflammation.

Box: Summary of main findings

- Myeloid Acly expression controls proinflammatory macrophage activation *in vitro*, without affecting acute peritonitis, chronic encephalomyelitis and metaflammation models *in vivo* (**chapter 2**)
- A soluble form of the mannose receptor (sMR) reprograms macrophages towards a proinflammatory phenotype by interacting with CD45 and a novel Src/Akt/NK-κB-mediated signaling pathway (**chapter 3**)
- MR-deficient mice are protected against HFD-induced hepatic steatosis, insulin resistance and glucose intolerance, associated with reduced proinflammatory macrophages in metabolic tissues (**chapter 3**)
- Serum sMR levels correlate with adiposity in both mice and humans, and sMR promotes metaflammation as well as obesity-induced metabolic dysfunctions (**chapter 3**)
- Mice with LKB1-deficient DCs develop worse insulin resistance, glucose intolerance and hepatic steatosis upon HFD feeding, which is dependent on the canonical Th17 cytokine IL-17A (**chapter 5**)
- LKB1 limits LPS-induced expression of Th17-polarizing cytokines IL-6, IL-1β and IL-23 in bone marrow DCs, potentially through its downstream target SIK (**chapter 5**)

What was known about immunomodulatory molecules and obesity-induced metabolic dysfunctions?

Obesity-induced metaflammation could be seen as a protective mechanism of physiologic inflammation that aims to prevent tissue damage and/or restore homeostasis (83), but ultimately fails and promotes chronic low-grade inflammation. Indeed, apart from its role in defense against pathogens, the immune system is increasingly recognized to support tissue function and control homeostasis (83, 84). Lean, insulin-sensitive adipose tissue is populated by type 2 innate lymphoid cells (ILC2s), T helper 2 (Th2) cells, eosinophils and alternatively activated macrophages (AAMs) (2). These cells, belonging to type 2 immunity, display a self-maintaining network through production of the type 2 cytokines IL-4, IL-5 and IL-13 that culminates in the alternative activation of macrophages (85-87). These AAMs are thus considered the effector type 2 immune cells in lean adipose tissue that control insulin sensitivity (2), although the underlying molecular mechanisms are not fully understood. In the liver, IL-4 and IL-13-mediated signaling, engaging their downstream transcription factors STAT6 and/or STAT3, have also been shown to increase glucose oxidation, decrease gluconeogenesis and reduce hepatic steatosis (88-90). Although the homeostatic type 2 immune network in liver of lean individuals is ill-defined, these findings suggest that type 2 immunity plays a significant role in the control of metabolic homeostasis both in adipose tissue and the liver. During obesity, these type 2 immune cells are lost, thus it is tempting to speculate that restoring type 2 immunity in obese metabolic tissues may reinstall tissue homeostasis and improve insulin sensitivity. Parasitic helminths are the strongest natural inducers of type 2 immunity, characterized by tissue eosinophilia, production of type 2 cytokines, Th2 cells and alternative activation of macrophages (91). As such, helminths and their immunomodulatory molecules have gained considerable interest as a potential resource to manipulate the immune system and combat insulin resistance (92). Indeed, cross-sectional studies conducted in helminth-endemic areas demonstrate an inverse correlation between helminth infection and metabolic dysfunctions (93-95). Experimental infection of obese mice has allowed for investigating isolated effects of helminth infection on metabolic homeostasis, showing that different helminth species induced type 2 immunity in metabolic tissues and alleviated metabolic dysfunctions in obese mice (85, 96-99). Importantly, our group and others have also shown that treatment of obese mice with helminth-expressed immunomodulatory molecules in a pathogen-free setting recapitulated these immunometabolic effects (97, 98, 100). However, hitherto there was little evidence for a causal role of helminth-induced type 2 immunity to increased insulin action. Altogether, harnessing immunomodulation to improve whole-body metabolic homeostasis is a promising and exciting area of research that may build on lessons learned from helminths (101).

How did our studies advance the field?

Helminth immunomodulatory molecules

As sentinels of homeostasis (23, 24), AAMs are believed to control insulin sensitivity in metabolic tissues (2). These AAMs are maintained through a type 2 immune axis involving ILC2s, Th2 cells and eosinophils (85-87). Obesity induces chronic low-grade inflammation in metabolic tissues, where these type 2 immune cells are lost and proinflammatory macrophages accumulate that contribute to insulin resistance (2). Exploiting the type 2 immunity-inducing properties of parasitic helminths and their immunomodulatory molecules (91) to improve obesity-associated metabolic complications has sparked an interesting line of research. **Chapter 6** provided an overview of the literature concerning the regulation of metabolic homeostasis by helminths and their molecules. Here, we discussed cross-sectional studies conducted in helminth-endemic areas showing an inverse correlation between helminth infection and metabolic dysfunctions (93-95, 102). In support of this correlation, deworming helminth-infected individuals using antihelminthic drugs increased proxies of systemic insulin resistance (102-104). Furthermore, we discussed that experimental infection of obese mice with different types of helminths improved whole-body metabolic homeostasis (85, 96-99, 105-107). In fact, our group has previously shown that both infection of obese mice with the helminth *Schistosoma mansoni*, but also treatment with the Th2-inducing soluble egg antigens (SEA) of *S. mansoni*, induced type 2 immunity in adipose tissue and liver, and improved whole-body glucose tolerance and insulin sensitivity (97). However, only few studies have described a dependency of metabolic effects to helminth-induced immunomodulation (105, 108). In **chapter 7**, we investigated the contribution of type 2 immunity to the metabolic effects of SEA using mice deficient for STAT6, a key transcription factor transducing canonical type 2 cytokines IL-4 and IL-13 signaling (109, 110). As expected, in obese *Stat6*$^{-/-}$ mice, SEA failed to induce the Th2-eosinophil-AAM axis in WAT that was observed in wildtype mice. Strikingly, the beneficial effect of SEA on whole-body glucose tolerance was lost in *Stat6*$^{-/-}$ mice, indicating that induction of type 2 immunity is required for the metabolic effects of SEA (**Figure 2A**). While this reinforces the paradigm that AAMs are the effector cells of a type 2 immune cascade that maintains insulin sensitivity, these data were obtained using a whole-body *Stat6*$^{-/-}$ mouse model and do not demonstrate a causal role for AAMs in the metabolic effects of SEA.

Infection of obese mice with the gastrointestinal helminth *Heligmosomoides polygyrus* was also shown to increase markers of type 2 immunity and improve whole-body glucose tolerance (99, 105). Interestingly, adoptive transfer of *H. polygyrus*-induced AAMs to uninfected mice via tail vein injection blunted HFD-induced adiposity and glucose intolerance

(105), highlighting a role for AAMs in promoting insulin sensitivity. Adoptive transfer of these macrophages was associated with increased expression of uncoupling protein 1 (UCP-1) in adipose tissues, indicative of brown adipose tissue (BAT) activation or WAT beiging. These are physiological responses to cold exposure to induce non-shivering thermogenesis, producing heat at the expense of ATP production by uncoupling mitochondrial oxidative phosphorylation, thereby combusting large amounts of glucose and lipids and significantly increasing energy expenditure (111-113). As such, BAT activation and beiging have gained considerable attention as therapeutic goals for combating metabolic disorders (114). Although AAMs were previously suggested to promote beiging through the release of catecholamines (115, 116), this concept was later refuted by an elegant study showing that AAMs are incapable of producing catecholamines and do not contribute to beiging (117). In our studies, we have not observed effects of helminth infection or SEA/ω1 treatment on white adipose tissue beiging or BAT activation (**chapter 7** and unpublished results), while AAMs were increased in adipose tissue in all settings. The transferred macrophages from *H. polygyrus*-infected mice are thus unlikely to improve metabolic homeostasis through beiging. In addition, these macrophages were not selected for AAM markers and derived from spleen and peritoneal cavity, rather than AAMs from metabolic tissues. Moreover, the fate of these macrophages after transfer and mechanisms for improving whole-body glucose tolerance were not investigated. Together, this hinders the interpretation of the data showing that *H. polygyrus*-induced AAMs promote metabolic homeostasis, and more work is undoubtedly required to elucidate mechanisms by which helminth-induced AAMs may govern insulin sensitivity.

In addition to *S. mansoni* SEA, we also investigated the effects and underlying mechanisms of recombinantly produced ω1, one of the major immunomodulatory molecules present in SEA (118), on whole-body metabolic homeostasis in **chapter 7**. SEA and other helminth worm or egg antigen mixtures are crude, heterogeneous preparations that display batch variability. This impedes detailed, batch-transcending molecular and functional characterization, which leaves room for potential off-target effects. Hence, dissecting underlying mechanisms of type 2 immunity induction and improvement of metabolic homeostasis by single molecules expressed by helminths may aid in identifying therapeutic targets. SEA-induced type 2 immunity, through dendritic cell (DC)-mediated T helper 2 (Th2) polarization, is at least partly dependent on glycosylated antigens present in SEA (101, 119). Among these antigens is the T2 RNase glycoprotein ω1 that licenses DCs to polarize Th2 cells dependent on glycan-mediated uptake and its enzymatic activity (118, 120). Interestingly, treatment of obese mice with ω1, that was recombinantly produced using human embryonic kidney 293 (HEK293) cells, acutely reduced body weight and improved whole-body glucose tolerance (108).

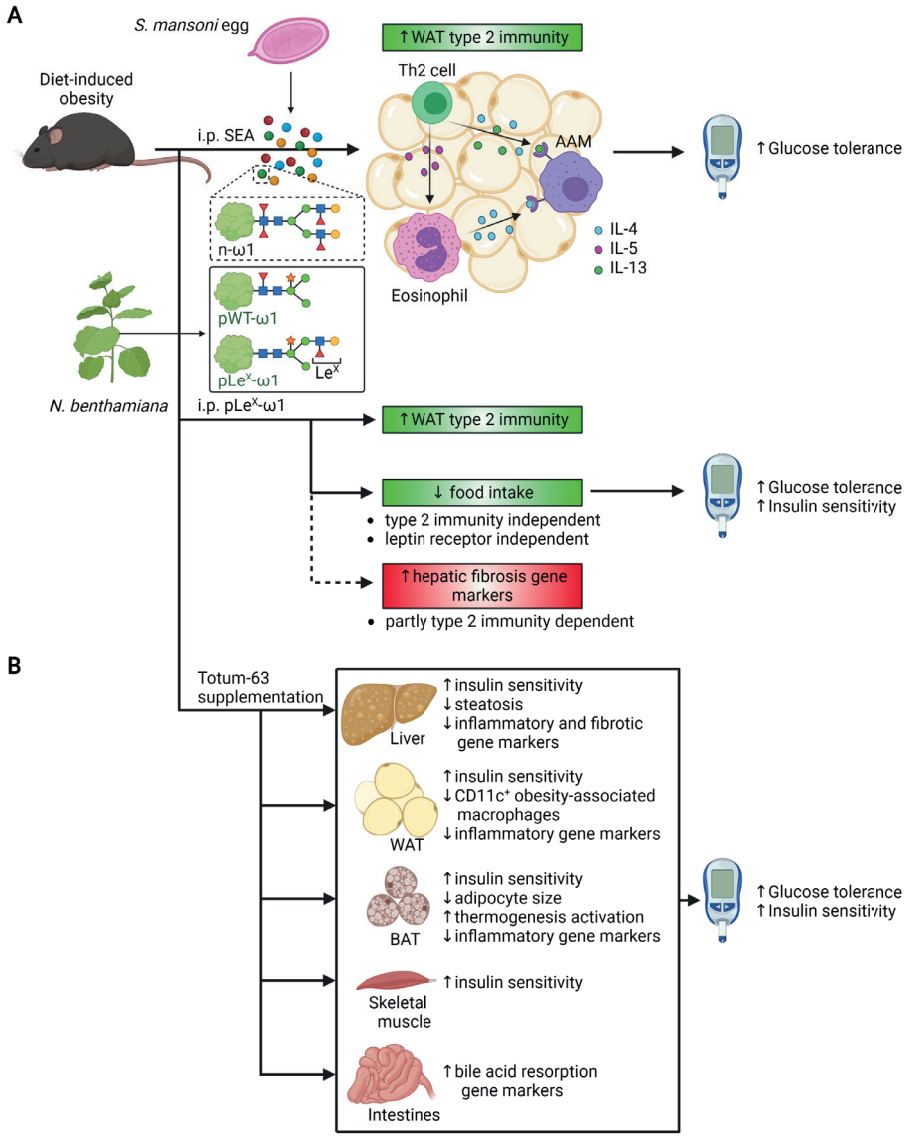

Figure 2: Mechanisms employed by immunomodulatory (helminth) molecules to alleviate obesity-induced metabolic dysfunctions. (**A**) Graphical summary of chapter 7. *Schistosoma mansoni* soluble egg antigens (SEA) improved whole-body glucose tolerance in obese mice through STAT6-mediated type 2 immunity. *Nicotiana benthamiana*-produced glycosylation variants of ω1 also induced WAT type 2 immunity, but improved whole-body glucose tolerance and insulin sensitivity by reducing food intake, which was independent of type 2 immunity and leptin receptor signaling. Furthermore, pLe^X-ω1 upregulated hepatic fibrosis gene markers, which was partly dependent on type 2 immunity. (**B**) Graphical summary of chapter 8. Totum-63 supplementation improved whole-body metabolic homeostasis through pleiotropic effects on various metabolic organs. Created with BioRender.com.

However, glycan structures of HEK293-produced ω1 differ from the *S. mansoni* native molecule, specifically lacking immunogenic Lewis-X (Le^X) motifs on glycan termini (120, 121). Glycans on protein may profoundly affect protein function, *e.g.* by affecting protein folding, receptor binding and biodistribution (122), and play important roles in controlling immune responses (123). By exploiting the flexible N-glycosylation machinery of *Nicotiana benthamiana* plants (124), we investigated the immunometabolic effects of two ω1 glycosylation variants, either carrying the Le^X motif on one of its glycan branches or not, in obese mice (**Figure 2A**).

Both of these plant-produced, glyco-engineered ω1 molecules induced type 2 immunity in metabolic tissues, which was associated with reduced fat mass and improvements in both tissue-specific and whole-body insulin sensitivity. In stark contrast to SEA, ω1 glycovariants significantly improved whole-body metabolic homeostasis in obese *Stat6*$^{-/-}$ mice in the absence of type 2 immunity. The ω1 glycovariants rather inhibited food intake, without affecting locomotor activity, lean mass, or behavior of the mice, indicating that discomfort is unlikely to explain decreased appetite. Nonetheless, reduced food intake explained most of the beneficial metabolic effects of at least the Le^X-glycoengineered ω1 (pLe^X-ω1), which occurred independent of leptin receptor signaling, a central hormone involved in regulation of energy intake (**Figure 2A**; 125).

The regulation of food intake by plant-produced ω1 glycovariants was surprising, as HEK293-produced ω1 was previously not suggested to affect feeding behavior (108), although this was not assessed in detail. Interestingly, deworming helminth-infected school children was found to be associated with increased appetite and growth (126), leading to speculate that helminth-expressed molecules may regulate satiety. We showed that plant-produced ω1 does not accumulate in the brain, indicating its effect is likely mediated by peripheral rather than central mechanisms. Bidirectional communication between the gastrointestinal tract and the central nervous system, the so-called gut-brain axis, has gained interest in the context of metabolic disorders, where it has been shown to be involved in regulation of energy intake and energy expenditure (127). Interestingly, several mechanisms for sensing gastrointestinal helminths to induce mucosal type 2 immunity and expel the worms have also recently been identified. For instance, the gastrointestinal helminth *Nippostrongylus brasiliensis* activates an intestinal tuft cell-ILC2 program that results in epithelial remodeling and increased mucus production (128-130). Tuft cells are rare, secretory epithelial cells that closely interact with enteroendocrine cells and enteric neurons (131). Although underlying mechanisms are still largely elusive, tuft cells and other chemosensory cells are hypothesized to relay nutritional signals to brain regions that control food intake, and are thereby potentially involved in the regulation of appetite (132, 133). In

addition, *N. brasiliensis* is sensed by intestinal neurons, initiating type 2 immunity through production of neuromedin U (NmU) and activating NmU receptor-expressing ILC2s (134, 135). Both intracerebroventricular (136) as well as peripheral administration of NmU (137) or a NmU receptor-selective agonist (138) have been demonstrated to suppress food intake. Mechanistically, peripherally administered NmU was suggested to inhibit food intake by signaling to brain regions that regulate satiety through the vagal nerve (137). Although speculative, whether plant-produced ω1 glycovariants alter energy intake, through potential changes in tuft cell activation or intestinal neuroimmune interactions that affect the gut-brain axis, would be an interesting new angle to explore in future studies.

In addition to its beneficial effects on whole-body glucose tolerance and insulin sensitivity, ω1 also increased fibrotic gene marker expression in the liver and alanine aminotransferase (ALAT) levels in serum, indicative of liver injury. During *S. mansoni* infection, adult worms reside in hepatic veins where they release eggs that lodge in the liver (139). This triggers granuloma formation through IL-4 and IL-13, and consequently hepatic fibrosis surrounding egg granulomas via IL-13 (140). Indeed, also in the absence of *S. mansoni* infection, IL-13 was shown to be pro-fibrotic in the liver (141). Given that we found increased hepatic IL-13-expressing Th2 cells in ω1-treated mice, it is likely that this effect may underlie increased fibrotic gene marker expression in the liver. In line with this, we found that fibrotic gene expression in the liver was at least partly dependent on STAT6 (**Figure 2A**). Interestingly, *S. mansoni* eggs in which ω1 has been knocked down also generated smaller granulomas *in vivo* (142), further supporting a role for ω1 in driving hepatic fibrosis. Using radioactively-labelled pLe^X-ω1, we found that pLe^X-ω1 distributes throughout abdominal organs after intraperitoneal injection, while single-photon emission computerized tomography (SPECT) identified apparent accumulation of pLe^X-ω1 in the liver 24 hours post injection. Although both glycovariants increased leukocyte numbers in WAT and liver, these effects were more pronounced in WAT for pWT-ω1 and liver for pLe^X-ω1, respectively, suggesting that the glycans present on the molecule may affect its biodistribution. Congruent with this, *in vivo* administration of different glycoconjugates have been shown to display glycan-dependent, specific distribution kinetics [as reviewed in (122, 143)]. Specific tissue and/or cell targeting approaches using glycans are currently conducted. For example, triantennary N-acetyl galactosamine improved targeting of antisense oligonucleotides to the liver through interacting with the hepatocyte-specific asialoglycoprotein receptor (ASGPR) (144), and glucan-encapsulated particles containing siRNAs specifically target phagocytic cells expressing Dectin-1 or other β-glucan recognizing receptors (145). This would suggest that manipulating ω1 glycosylation may have potential to bypass the liver and specifically target adipose tissue DCs to elicit type 2 immunity and

improve whole-body metabolic homeostasis, although the glycan structures and valency to achieve this are yet to be identified.

To conclude, our work has provided evidence for involvement of SEA-induced type 2 immunity in improvement of metabolic homeostasis in obese mice. In addition, we found that the *S. mansoni*-expressed Th2-inducing molecule ω1 unexpectedly regulates food intake through a peripheral mechanism independent of its Th2-inducing capacity. One of our hypotheses is that ω1 may regulate the gut-brain axis involved in the control of food intake. As bidirectional gut-brain communication is mediated by the vagal nerve (146), future studies may benefit from vagotomy as recently used in other studies assessing the gut-brain axis in the context of obesity (147, 148). In addition, follow-up studies should address whether helminth-induced AAMs contribute to improvements in insulin sensitivity and elucidate underlying mechanisms, which may be facilitated through the development of inducible mouse models with defective alternative activation of macrophages.

The polyphenol-rich plant extract Totum-63 and metabolic homeostasis

The types of food we eat may impact inflammatory conditions (149) and nutritional supplements have thus gained attention for modulating immune responses (150). Using dietary supplements for weight management and improving metabolic health is not novel, yet provides an interesting, non-invasive method for prevention or amelioration of obesity-induced metabolic disorders (151-153). Given the immunomodulatory properties of some of these nutraceuticals, they may function as a double-edged sword: both ameliorating systemic metaflammation as well as improving insulin sensitivity and/or glucose homeostasis directly in metabolic organs. In **chapter 8**, the effects and underlying mechanisms of Totum-63, a recently developed dietary supplement consisting of a blend of polyphenol-rich plant extracts with potential immunomodulatory effects, on metabolic homeostasis in obese, insulin resistant mice was investigated. In-depth metabolic phenotyping revealed that Totum-63 reduced body weight, completely attributable to a decrease in fat mass, and improved whole-body insulin sensitivity and glucose tolerance independent of body weight changes. Totum-63 improved metabolic and immunological parameters in various metabolic tissues, including intestines, liver, skeletal muscle, visceral and subcutaneous WAT, and BAT, indicating the principle promotes metabolic homeostasis through pleiotropic effects, likely owing to its chemical composition containing a variety of bioactive molecules (**Figure 2B**).

We demonstrated that Totum-63 reduced CD11c-expressing obesity-associated macrophages in visceral WAT and reduced inflammatory gene markers in subcutaneous WAT, BAT and liver. As hepatic steatosis was almost completely reversed in Totum-63-

supplemented mice, reduced inflammation in BAT and liver probably results from reduced ectopic lipid deposition and lipotoxicity, potentially through insulin-mediated inhibition of WAT lipolysis. However, Totum-63 is rich in polyphenols, and several of these micronutrients possess intrinsic immunomodulatory properties, *e.g.* by inhibiting NF-κB-mediated proinflammatory cytokine production (154, 155). Hence, we cannot completely exclude potential immunomodulatory effects of polyphenols or other components (*i.e.* saponins, alkaloids and fibers) of Totum-63 that may impact metaflammation and thereby promote tissue-specific and whole-body insulin sensitivity.

Although whole-body energy expenditure was unchanged, Totum-63 increased BAT activation, as illustrated by decreased BAT mass and increased expression of UCP-1 and other thermogenic gene markers. Some polyphenols were reported to increase BAT activation (153, 156), yet we also found increased ileal expression of bile acid transporters, indicative of increased bile acid resorption. Bile acids are increasingly recognized as signaling molecules that impact whole-body metabolism, immunity and also BAT activation (157, 158). Interestingly, polyphenols have been demonstrated to regulate bile acid bioavailability (159). Whether Totum-63 increased systemic bile acid levels and whether bile acids may contribute to the pleiotropic effects of Totum-63, for instance through immunomodulation and/or direct effects on metabolic organs, remains to be determined. Future studies supplementing mice that are deficient for bile acid receptors, such as FXR or TGR5, could be considered for answering such questions.

Totum-63 was developed for the treatment of pre-diabetes and to reduce the risk of developing type 2 diabetes. As such, the bioactive principle was shown to protect against obesity-induced metabolic dysfunction in a progression model, when lean mice received HFD supplemented with Totum-63 (160). Our work indicates that Totum-63 may also hold potential in treating established type 2 diabetes by exerting pleiotropic effects on multiple metabolic organs. Importantly, safety and tolerability of Totum-63 were also recently demonstrated in pre-diabetic men, where 6 months supplementation induced body weight loss, reduced fasting blood glucose and improved glucose tolerance (160, 161). Together, our work has illuminated the potential mechanistic underpinnings of Totum-63-mediated improvements in metabolic homeostasis. Increased bile acid bioavailability and immunomodulation by Totum-63 may contribute to the immunometabolic effects of Totum-63, yet this remains to be investigated.

Box: Summary of main findings

- The beneficial metabolic effects of *S. mansoni* SEA on whole-body glucose tolerance in obese mice are dependent on SEA-induced type 2 immunity (**chapter 7**)
- Glyco-engineered, plant-produced ω1 improves whole-body metabolic homeostasis in obese mice through leptin receptor-independent inhibition of food intake; not through its type 2 immunity-inducing properties (**chapter 7**)
- The polyphenol-rich plant extract Totum-63 improves whole-body metabolic homeostasis in obese mice through pleiotropic effects on multiple metabolic organs, including a reduction in proinflammatory macrophages in adipose tissue (**chapter 8**)

Future perspectives and concluding remarks

This thesis deepens our understanding of how immune cells control whole-body metabolic homeostasis. Developments in immunology and immunometabolism research have provided new tools and perspectives to propel the field forward, of which several will be highlighted below.

Single-cell and spatial transcriptomics

Analyses of immune cells in metabolic tissues have long relied on conventional flow cytometry, with a limitation in the number of parameters to be measured based on the number of detectors that are assigned to a single fluorophore. Historically, based on the expression of a selected set of markers, macrophages in lean WAT were considered to resemble *in vitro* IL-4-polarized M2 macrophages, whereas proinflammatory macrophages in obese WAT were thought to be similar to *in vitro* LPS + IFNγ-polarized M1 macrophages. While this dichotomy was at the time already reported to be an oversimplification (162), recent technological advances have confirmed that metabolic tissue macrophage phenotypes *in vivo* are indeed much more complex. The development of single-cell transcriptomics during the last decade now allows an unbiased approach to obtain unprecedented insights into the cellular heterogeneity of complex samples (163). Single-cell RNA sequencing (scRNAseq) has recently been extensively employed to investigate the immune cell composition of adipose tissue (36, 164) and liver (37, 38, 165) isolated from lean and obese mice and humans. This has provided novel insights into phenotypes and mechanisms underlying immune-mediated control of metaflammation. Among the key findings is the identification of an evolutionary conserved lipid-associated macrophage phenotype expressing CD9, Trem2 and/or CD36 in both obese WAT and fatty liver, which contributes to obesity and NASH pathogenesis (36-38).

Of note, whereas hepatocytes and other parenchymal cells in the liver can readily be identified using scRNAseq, mature adipocytes are too fragile to survive the procedure.

Isolating nuclei from snap-frozen adipose tissue samples and performing single-nuclei RNAseq was shown to be a valuable strategy to overcome this problem and revealed a previously unrecognized heterogeneity in adipocyte subsets during obesity (164). Although nuclei carry fewer RNA which may hinder resolution, single-nuclei RNAseq uncouples sample acquisition from processing and may represent a goldmine allowing the analysis of previously biobanked tissue samples.

scRNAseq enables detailed characterization of the cellular composition of metabolic organs, yet it does not resolve cellular microenvironmental niches and cell-cell interactions. As a spectacular recent example, a spatial proteogenomic atlas was generated for healthy and obese mouse and human livers by integrating single-cell proteomic and transcriptomic information with spatial transcriptomics (166). This provided clues regarding the development and function of cell subsets based on their microanatomical niche, and strategies for identifying and further studying specific hepatic cell subsets. Similar endeavors to provide spatial resolution of cell subsets identified in snRNAseq of adipose tissue may deepen our understanding of immunological control of obesity-induced metabolic dysfunctions.

In the timespan during which our studies took place, the development of these tools and their applications in immunometabolism research have contributed a wealth of new knowledge to the field. Unfortunately, at the time, we could not implement these cutting-edge new tools in our work and have thus mostly relied on a selected set of markers by conventional flow cytometry for identifying macrophages in adipose tissue and liver (i.e. CD11b, CD64 and F4/80), as well as to predict their function (e.g. YM1 for AAMs and CD11c for proinflammatory obesity-associated macrophages). As such, one of the limitations of our work is that this approach did not allow to fully capture the heterogeneity of the macrophage pool in metabolic tissues. Consequently, during the course of our own studies we were not able to assess in depth some of the new macrophage subsets identified by others using single cell transcriptomic and proteomic technologies (36-38, 164-166). For example, obese adipose tissue accommodates a broad spectrum of macrophages, such as lipid-associated macrophages (LAMs) (36), vascular-associated macrophages (167) and sympathetic neuron-associated macrophages (168) that display phenotypical and functional diversity at least partly based on their localization within adipose tissue. Likewise, in the liver, $CD64^{+}CD11c^{+}CLEC4F^{-}$ monocyte-derived KCs were shown to be more inflammatory as compared to $CLEC4F^{+}TIM4^{+}$ resident KCs, which are lost during obesity (37, 169). Furthermore, osteopontin-expressing monocyte-derived macrophages in the liver were also shown to resemble WAT LAMs and to be enriched in fibrotic liver (37). Our studies could have benefited from mapping the transcriptional signatures defining these diverse macrophage subsets onto the myeloid cells-of-interest in our studies. This may have provided

more detailed and broad insights into the effects of given genetic or pharmacological interventions on the leukocyte pool in metabolic tissues, and likely left fewer questions unanswered. Finally, how (helminth-induced) AAMs contribute to insulin sensitivity is still poorly understood. Further studies will likely benefit from in-depth characterization of these cells by scRNAseq and establishing their microenvironmental niche.

Neuroimmunometabolism and the gut-brain axis

Besides bidirectional communication with metabolic cells, immune cells can also interact with neurons. Such interactions are particularly apparent at mucosal areas, *e.g.* the intestines and the lungs, where immune cells interact with dense neuronal networks to preserve tissue homeostasis and assist in establishing immune responses (170). For example, several recent studies demonstrated that the neuropeptide NmU promotes ILC2 responses in the intestines and the lung (134, 135, 171). Strikingly, the excretory/secretory products of the gastrointestinal helminth *N. brasiliensis* were found to be sensed by neuronal organoids, resulting in increased NmU expression, thereby likely contributing to increased worm expulsion through ILC2-mediated type 2 immunity (134). Such neuro-immune circuits have not only been reported for enteric ILC2s, but also for muscularis macrophages in the gut wall. These macrophages were shown to protect enteric neurons from infection-induced cell death (172), and by this way, to preserve the self-sustaining crosstalk between muscularis macrophages and enteric neurons that regulates gastrointestinal motility (173). Altogether, this may lead one to speculate that at least some of the metabolic effects of helminth molecules and the nutritional supplement Totum-63 may be secondary to altered neuro-immune circuits in the intestines, as a result of sensing of these molecules either by enteric neurons or muscularis macrophages. As vagal afferent nerves are known to relay intestinal sensory information to regulate food intake (147, 174), such interactions could potentially also contribute to the regulation of satiety by ω1.

The findings that subsets of macrophages co-localize and interact with sympathetic neurons in BAT and WAT has sparked the research topic of neuroimmunometabolism (175). Sympathetic innervation promotes lipolysis in BAT and WAT, and regulates adaptive thermogenesis (112, 176). BAT macrophages were found to control tissue innervation, which increased HFD-induced adiposity upon disruption (177). In WAT, obesity promotes the accrual of sympathetic neuron-associated macrophages that scavenges norepinephrine to reduce its extracellular bioavailability and WAT lipolysis (168). Removing the norepinephrine importer Slc6a2 from myeloid cells increased lipolysis and limited weight gain upon HFD feeding. WAT sympathetic neurons also indirectly interact with ILC2s through mesenchymal cells (178). Neuronal-derived norepinephrine stimulates glial-derived neurotrophic factor

(GDFN) release by mesenchymal cells, which binds to its receptor tyrosine kinase RET on ILC2s to increase cytokine production. Loss of RET on ILC2s promoted HFD-induced metabolic dysfunctions, whereas gain of function had an opposite effect, presumably by controlling WAT beiging (178). Whether (helminth-induced) AAMs also interact with sympathetic neurons in their microenvironmental niche, and whether such neuro-immune interactions could contribute to regulation of tissue insulin sensitivity, is a novel perspective that warrants further study.

Immunomodulatory helminth molecules and controlled human infections

Experimental infection of obese, insulin-resistant mice has unequivocally demonstrated that different helminth species alleviate whole-body metabolic dysfunctions in mice. In **chapter 6** we provided an overview of immune regulation of metabolic homeostasis by helminths and their molecules. We described in **chapter 7** that both *S. mansoni* SEA as well as the Th2-inducing molecule ω1, one of the molecules present in SEA, improve whole-body glucose tolerance. SEA is a crude mixture containing many (glyco)proteins with potential Th2-inducing properties, such as currently unidentified Dectin-1/2 ligands (179). Identification of such molecules, either expressed by *S. mansoni* or other helminths, and assessment of their potency to improve whole-body metabolic homeostasis remains a promising undertaking.

Cross-sectional studies in helminth-endemic areas indicate that an inverse correlation between helminth infection and insulin sensitivity also exists in humans (**chapter 6**). However, whether therapeutic helminth infection holds promise for patients with type 2 diabetes remains to be studied. Given the risks associated with experimental infection, such studies require thorough ethical consideration. Still, controlled human infection trials have provided a wealth of scientific insights and contributed to the development of vaccines and drugs for infectious diseases, such as infection with rhinovirus, influenza and the malaria parasite *Plasmodium falciparum* (180). Interestingly, controlled infection of humans with the soil-transmitted helminth *Necator americanus* has been reported to be well tolerated (181, 182). Moreover, a recent landmark study described experimental infection of volunteers with *S. mansoni*, albeit with only male cercariae - the infectious larval form of *S. mansoni* - to prevent egg deposition and associated pathogenesis (183). Even though these trials are currently focused on drug and vaccine development, they may potentially pave the way for applying controlled experimental helminth infection in the context of obesity-associated metabolic dysfunctions or other inflammatory disorders.

To conclude, our works describes novel mechanisms by which immune cells control whole-body metabolic homeostasis, and that (helminth) immunomodulatory molecules are potent candidates for alleviating metaflammation. We provide new insights, but our

studies also raised new questions that remain to be addressed (see Outstanding questions box). While one should not overlook the efficacy of lifestyle modifications, harnessing immunomodulation through helminths, their expressed molecules or other sources presents a potent means for improving obesity-associated metabolic dysfunctions that warrants follow-up.

Box: Outstanding questions

- What is the source of increased sMR serum levels in obesity, and how is increased sMR shedding regulated?
- What are the effector cells and molecular mechanism(s) of sMR-induced metaflammation?
- What is the role of SIKs in controlling Th17 polarization by DCs?
- What is the contribution of helminth-induced AAMs to the control of whole-body metabolic homeostasis, and what are their phenotypes and underlying mechanisms?
- Does the gut-brain axis mediate the inhibitory effects of ω1 on food intake?
- What is the contribution of neuroimmunometabolism and the gut-brain axis to the metabolic effects of helminth molecules and the polyphenol-rich nutritional supplement Totum-63?
- Does controlled human helminth infection hold promise as a translational model to explore the impact of helminth-induced immunomodulation on metabolic homeostasis ?

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