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Review

Immunometabolic control of trained immunity

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ABSTRACT

Innate immune cells can adopt long-term inflammatory phenotypes following brief encounters with exogenous (microbial) or endogenous stimuli. This phenomenon is named trained immunity and can improve host defense against (recurrent) infections. In contrast, trained immunity can also be maladaptive in the context of chronic inflammatory disorders, such as atherosclerosis. Key to future therapeutic exploitation of this mechanism is thorough knowledge of the mechanisms driving trained immunity, which can be used as pharmacological targets. These mechanisms include profound changes in intracellular metabolism, which are closely intertwined with epigenetic reprogramming at the level of histone modifications. Glycolysis, glutamine replenishment of the tricarboxylic acid cycle with accumulation of fumarate, and the mevalonate pathway have all been identified as critical pathways for trained immunity in monocytes and macrophages. In this review, we provide a state-of-the-art overview of how these metabolic pathways interact with epigenetic programs to develop trained immunity.

1. Introduction

Pharmacological modulation of the immune system has taken central stage in the prevention and treatment of a wide variety of diseases. Vaccination strategies exploit the immunological memory of the adaptive immune system to prevent infectious diseases. In patients with severe infections, host directed therapy aimed at modulating the immune system is a promising emerging approach (Kaufmann et al., 2018). Immunosuppressive drugs are the cornerstone of the treatment of patients with auto-immune or auto-inflammatory diseases, such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease. Also in patients with atherosclerotic cardiovascular diseases, which are characterized by a chronic low-grade inflammation of the arterial wall, immunomodulating drugs such as canakinumab and colchicine are effective in reducing future cardiovascular events (Ridker et al., 2017; Tardif et al., 2019).

Modulating immune system function is not trivial and is often associated with unwanted side effects, such as an increased risk of infections in the case of immunosuppressive drugs. To allow the development of more specific immunomodulatory drugs with less side effects, a deeper understanding of immune cell function in physiological and pathophysiological conditions is warranted. In the past ten years, it has been discovered that innate immune cells, such as monocytes, macrophages, and natural killer cells, can also build an immunological memory, in a process which has been termed trained immunity (Netea et al., 2020). Key intracellular processes that regulate trained immunity include remodeling of intracellular metabolic pathways as well as epigenetic reprogramming. Accumulating evidence points to an important role for trained immunity in a broad spectrum of (patho)physiological conditions (Netea et al., 2020). Therefore, it has recently been proposed that specific pathways that drive trained immunity offer exciting novel pharmacological targets to modulate immune cell function (Mulder et al., 2019).

The aim of the present review is to provide a state-of-the-art overview of the role of immunometabolism in trained immunity. We will first describe the triggers and functional effects of trained immunity in the context of various clinical situations. Subsequently, we will describe in detail how changes in glycolysis, the tricarboxylic acid cycle (TCA cycle) and glutaminolysis, oxidative phosphorylation, fatty acid synthesis, and cholesterol biosynthesis contribute to innate immune memory and how these pathways interact with epigenetic programs. Finally, we will describe how we can use the knowledge of these mechanisms to ultimately exploit trained immunity to improve patient care.

2. Trained immunity

In the traditional immunological dogma, immune memory is restricted to the adaptive immune system in which T-lymphocytes can

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develop a life-long specific immunological memory by somatic rearrangement of gene elements. This is challenged however by the observations that also plants and non-vertebrate species, which lack adaptive immunity, are able to mount resistance to recurrent infections (Netea, Quintin, and van der Meer, 2011). Only in the last decade, our lab and others have shown that indeed monocytes are also able to build a long-term pro-inflammatory phenotype after primary stimulation, i.e. a *de facto* immune memory (Kleinnijenhuis et al., 2012; Quintin et al., 2012).

In the classical experimental design to investigate trained immunity in vitro, isolated human primary monocytes are exposed for 24 h to a microorganism or a microbial ligand; subsequently the cells are washed and rested in culture medium for 6 days, after which they are restimulated for 24 h with unrelated stimuli, such as Toll-like receptor agonists: the boosting of cytokine production compared to cells not exposed to the training agent is calculated as a marker of trained immunity (Bekkering et al., 2016). It appeared that cytokine production capacity is profoundly augmented after 24 h exposure to various micro-organisms or microbial products, including Bacille Calmette-Guérin (BCG), *Candida albicans*, and its cell wall component β-glucan. This observation could be corroborated in humans in vivo by showing enhanced cytokine production capacity of isolated monocytes up to one year after BCG-vaccination of healthy individuals (Kleinnijenhuis et al., 2014). The remarkable evolutionary conservation of trained immunity is probably related to its strong protective effect in the setting of recurrent infections, which has been demonstrated in multiple animal models. In T/B cell-defective *Rag1*-deficient mice, a nonlethal dose of live *C. albicans* improves survival rate following subsequent exposure to systemic candidiasis (Quintin et al., 2012). Also, in severe combined immunodeficiency mice that lack T and B cells, BCG vaccination completely prevents mortality by an otherwise lethal *C. albicans* infection (Kleinnijenhuis et al., 2012). That trained immunity is able to protect the host against future infections is also suggested in human in vivo infection models: first, viremic load following yellow fever vaccination (which is a live attenuated virus) was significantly reduced by BCG vaccination 28 days earlier in healthy subjects (Arts et al., 2018); secondly, BCG vaccination alters the clinical and immunological response to experimental malaria infection in healthy subjects (Walk et al., 2019). Given these experimental findings, trained immunity is a plausible explanation for the clinical observation that in newborns, BCG vaccination potently protects against a wide range of severe infections, not only against tuberculosis (Benn et al., 2013). Studies on the use of the BCG vaccine with the sole aim to exploit these beneficial heterologous effects of trained immunity in the clinical setting have been greatly accelerated by the recent pandemic caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2): several randomized clinical trials are currently being performed aiming to prevent coronavirus disease 2019 (COVID-19) in subjects with increased risk, such as healthcare workers or elderly (Netea et al., 2020; Curtis et al., 2020).

Contrasting the benefits of trained immunity in the setting of infectious threats, it might actually be detrimental in non-infectious chronic inflammatory diseases in which innate immune cells themselves contribute to tissue injury, e.g. rheumatoid arthritis or atherosclerosis (Bekkering et al., 2013). Also, trained immunity might be one of the mechanisms to explain the well-known association between infectious diseases and cardiovascular disease (Leentjens et al., 2018). In vitro, various endogenous compounds that are known to accelerate the process of atherosclerosis, induce trained immunity in isolated human monocytes. These factors include oxidized low-density lipoprotein (oxLDL) (Bekkering et al., 2014), lipoprotein (a) (van der Valk et al., 2016), and the adrenal hormones aldosterone (van der Heijden et al., 2020) and adrenaline and noradrenaline (van der Heijden et al., 2020). Feeding atherosclerosis prone *LDL-receptor* deficient mice a Western type diet for a period of four weeks induces profound inflammatory reprogramming of circulating innate immune cells, which persists despite switching diet back to chow diet (Christ et al., 2018). The relevance of these experimental findings is highlighted by the observation that isolated monocytes from patients with established severe coronary atherosclerosis have a trained immune phenotype, in terms of an augmented cytokine production capacity ex vivo (Bekkering, van den Munckhof et al., 2016). This also holds true for patients with an increased risk for atherosclerotic cardiovascular disease due to elevated LDL-cholesterol levels (patients with familial hypercholesterolemia), in whom the augmented cytokine production capacity could not be restored by three months of LDL-cholesterol lowering with statin treatment (Bekkering et al., 2019). Similar findings have been obtained in patients with elevated lipoprotein (a) levels, and patients with pheochromocytoma, who are exposed to repetitive bouts of adrenal catecholamine release (van der Valk et al., 2016; van der Heijden et al., 2020). In these latter patients, the increased cytokine production capacity persisted for one month following surgical removal of the catecholamine-producing tumor, emphasizing the memory aspect of this phenotype.

The observation that the enhanced cytokine production capacity that defines trained immunity persists for at least several months in humans in vivo – i.e. much longer than the half-life of circulating monocytes – prompted researchers to focus on the myeloid progenitor cells in the bone marrow niche. Indeed, in murine models, stimuli that induce trained immunity in circulating monocytes, including Western type diet feeding, BCG vaccination, or administration of β-glucan, were found to trigger a persistent inflammatory reprogramming of myeloid progenitor cells in the bone marrow (Christ et al., 2018; Kaufmann et al., 2018; Mitroulis et al., 2018). In healthy subjects, BCG vaccination also induced an inflammatory functional and transcriptional reprogramming of hematopoietic stem and progenitor cells, measured 90 days after vaccination (Cirovic et al., 2020).

To allow optimal future exploitation of the mechanism of trained immunity as pharmacological target, it is critical to be fully informed about the pathways that drive, maintain, and modulate innate immune memory. The two major processes that regulate this memory are epigenetic and metabolic reprogramming. Although these processes are regulated by separate sets of epigenetic and metabolic enzymes, there are close bilateral interactions connecting changes in metabolic pathways to epigenetic alterations and vice versa (Fig. 1). We will first briefly touch upon the essential epigenetic alterations in trained cells, followed by an extensive overview of the various metabolic changes and how these two processes interact.

3. Epigenetic reprogramming in trained immunity

Gene transcription is regulated by the binding of transcription factors and RNA polymerase at gene promoters that are proximal to the transcription start site and distal regulatory elements called enhancers. Accessibility of the DNA to transcription factors and other transcriptional machinery at these regulatory regions is essential for gene expression. Three processes can regulate gene transcription without alterations of the DNA sequence, including DNA methylation, modifications of histone proteins, and the effects of non-coding RNAs. Several research papers have now described an essential regulatory role of histone modifications in innate immune memory (van der Heijden et al., 2018). Also, recently a role for a specific long non-coding RNA molecule UMLILO in trained immunity was reported (Fanucchi et al., 2019), while the role of DNA methylation has only been suggested in one study in BCG-vaccinated subjects (Verma et al., 2017).

Histone lysine residues can be enzymatically modified by the addition of methyl groups or acetyl groups which regulate the accessibility of the promoter and enhancer regions for the transcriptional machinery. Some histone modifications, such as trimethylation of lysine 4 at histone 3 (H3K4me3), mark active promoters, while H3K4me1 marks distal regulatory elements (enhancers). Histone acetyl modifications are generally associated with an open active chromatin, e.g. H3K27ac, which marks active promoters and enhancers (Saeed et al., 2014). Importantly, these histone modifications are written and erased by

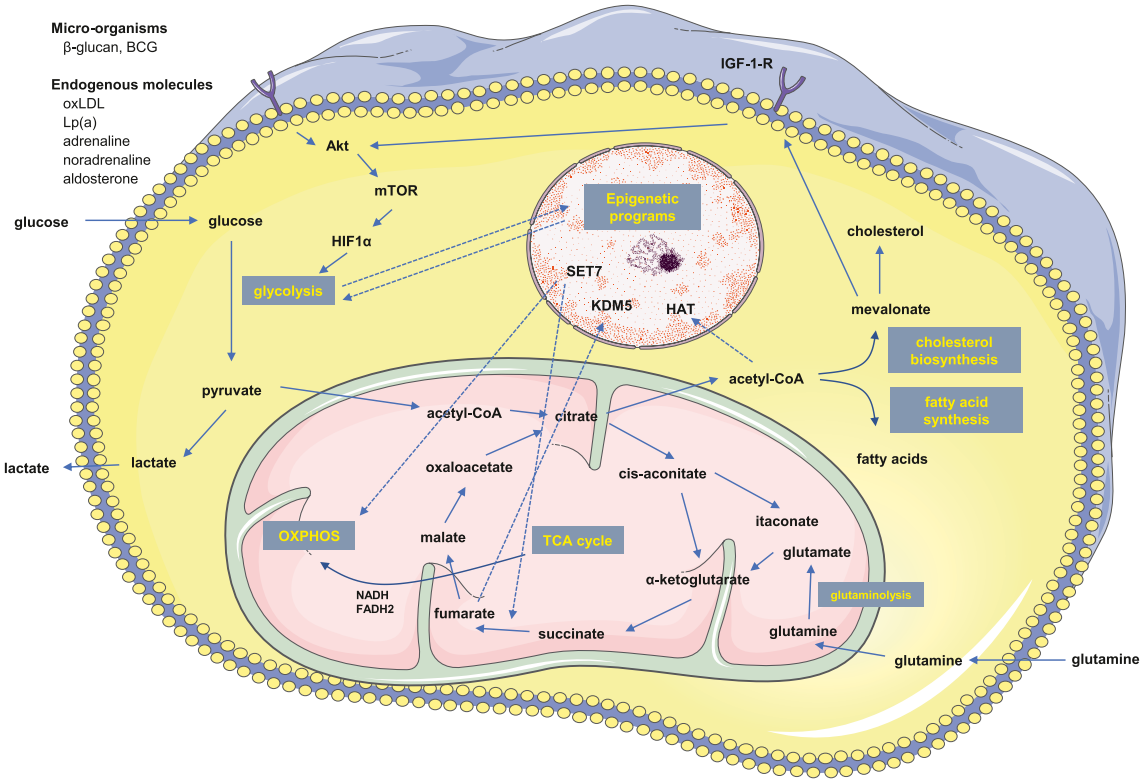

Fig. 1. Schematic overview of the intracellular mechanisms operating in monocytes/macrophages to build trained immunity. Depicted are the extracellular exogenous and endogenous triggers, the various metabolic pathways that are activated with their most important metabolites, and the bidirectional interaction with epigenetic enzymes in the nucleus.

specific epigenetic enzymes (including histone methyltransferases and demethylases, histone acetylases and deacetylases), which makes them potentially modifiable by drugs targeting these enzymes.

Stimulation of innate immune cells is accompanied by the rapid deposition of these chromatin marks and changes in the DNA methylation status, which leads to the unfolding of the chromatin and facilitates transcription and expression of proinflammatory factors. Importantly, in the context of trained immunity, these histone modifications are only partially removed after cessation of the training stimulus, resulting in a persistent enrichment of promoters of these cytokines with H3K4me3 and of enhancers with H3K4me1. This allows quicker and enhanced recruitment of transcription factors and gene expression after secondary challenge with another stimulus. For detailed information about the dynamics and regulation of this epigenetic reprogramming, we refer to previous papers by our group (Saeed et al., 2014; Netea et al., 2020; van der Heijden et al., 2018). Thus far, only two specific epigenetic enzymes have been characterized that regulate the epigenetic remodeling of training. First, the KDM5 family of histone demethylases, which are responsible for demethylation of H3K4 appears critical for trained immunity: β-glucan training of monocytes resulted in decreased biological activity of KDM5 demethylases on day 6 after training, which was regulated by changes in intracellular fumarate, which will be discussed later (Arts et al., 2016). More recently, the lysine methyltransferase Set7, which writes H3K4me1 marks, was identified as key enzyme necessary for β-glucan-induced trained immunity in vitro and in vivo (Keating, Groh, van der Heijden, et al., 2020).

4. Reprogramming of metabolic pathways in trained immunity

Just like all other cells, innate immune cells require several metabolic pathways to execute their diverse roles and respond to changing micro-environments; the most important pathways include glycolysis, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS), the pentose phosphate pathway, fatty acid oxidation, fatty acid synthesis and amino acid metabolism. These pathways provide energy in the form of ATP, but they also regulate biosynthesis and proliferation by providing essential macromolecular building blocks, and they regulate redox signaling and intercellular signaling. By doing so, they not only follow metabolic demands of the cells and nutrient availability, but are able to dictate cellular function themselves, which is recently discussed in detail in excellent review papers (O'Neill et al., 2016). In particular, metabolic pathways can regulate epigenetic programs by providing intermediate metabolites that can either serve as substrates for epigenetic enzymes (e.g. acetylCoA is the major substrate for histone acetyltransferases) or as co-activators or co-repressors of epigenetic writers or erasers (e.g. the $[NAD^+]/[NADH]$ ratio as activator of the SIRT1 histone deacetylase, fumarate as inhibitor of the KDM5 family of histone demethylases, and α-ketoglutarate as cofactor for the histone demethylase JMJD3, reviewed in (van der Heijden et al., 2018)).

Many of these pathways also serve important functions in the development of innate immune memory, which we will discuss in detail in the next sections. To elucidate which metabolic pathways are differentially expressed in trained immunity, Arts et al. recently performed RNA-sequencing and full intracellular metabolome assessment at different time points in β-glucan trained primary human isolated monocytes (Arts et al., 2016). After 24 h of exposure to β-glucan, the RNA expression patterns of metabolic enzymes differed from control monocytes, with the largest difference observed after 6 days. Similarly, metabolome data showed that at early time points (4 h and 24 h after stimulation), only small differences existed between the trained and control cells, whereas at day 6, the intracellular metabolome of β-glucan-trained cells was clearly different. Altogether, these data indicate that major transcriptional changes associated with metabolic pathways occur at an early timepoint after β-glucan exposure, which precede the metabolic phenotype observed in fully differentiated β-glucan-trained macrophages on day 6 (Arts et al., 2016). Integration of the

transcriptome and metabolome data revealed an upregulation of several metabolic pathways in β-glucan-trained macrophages, including glycolysis, the pentose phosphate pathway, glutamine metabolism, fatty acid synthesis, and cholesterol metabolism. In addition, several intermediary metabolites of the TCA cycle and glutaminolysis were enriched.

4.1. Glycolysis

Glycolysis is one of the major metabolic pathways in all cell types to provide energy and building blocks for essential biosynthetic pathways. It involves the conversion of glucose to pyruvate in the cytosolic compartment, which can either by shuttled into the TCA cycle or fermented to lactate. The latter reaction commonly occurs in hypoxic situations in which OXPHOS is switched off, but can also occur in normoxia, which is called aerobic glycolysis. Although glycolysis is much less efficient in ATP production than OXPHOS, it is the major pathway for energy production in proliferating cells, e.g. tumor cells, which was already described by Otto Warburg, (1956). Hence, the aerobic glycolysis that is typical of tumor cells has been named the Warburg effect. The same glycolytic preference holds true for activated immune cells, including LPS-activated M1-macrophages and dendritic cells, activated effector T cells, and activated natural killer cells. The benefits of switching preferentially to glycolysis are explained by 1) the possibility for rapid induction of glycolytic enzymes in situations of acute need of rapid energy production, such as inflammation 2) the conversion of NAD^+ to NADH, which is an important co-factor for many enzymes, and 3) the diversion of intermediate products into biosynthetic pathways (O'Neill et al., 2016). In the latter regard, glucose-6-phosphate fuels the pentose phosphate pathway for the production of ribose for nucleotides, 3-phosphoglycerol into the serine biosynthetic pathway, and pyruvate into the TCA cycle to provide citrate.

Several lines of evidence point towards a key role for glycolytic metabolism in the development of trained immunity in response to the exposure to β-glucan and BCG (Arts et al., 2016; Cheng et al., 2014): first, there is an upregulation of mRNA expression of glycolytic enzymes six days after exposure to the training stimulus (Cheng et al., 2014). Second, trained macrophages show increased lactate production, a higher glucose consumption, and an increased NAD + to NADH ratio, both during the first 24 h of exposure as well as on day 6 in the trained macrophage (Arts et al., 2016). Third, glucose flux analyses with nuclear magnetic resonance (NMR) with radiolabeled ^{13}C-glucose indeed showed increased incorporation of 13C into lactate during the 24 h of restimulation with LPS. In addition, incorporation of the 13C-labels was also increased in ribosyl-1 which is suggestive of an induction of the pentose phosphate pathway (Arts et al., 2016). For both β-glucan and BCG, upregulation of glycolysis is secondary to activation of the Akt – mTOR – HIF1α pathway. Pharmacological inhibition of this pathway, or of glycolysis with 2-deoxyglucose indeed abolishes the augmentation of cytokine production capacity (Arts et al., 2016; Cheng et al., 2014). Finally, additional proof for a role of glycolysis in trained immunity stems from the observations that in cohorts of healthy subjects, single nucleotide polymorphisms (SNPs) in key glycolytic enzymes are associated with the induction of cytokine production capacity by training of the cells ex vivo; this was reported for SNPs in hexokinase 2 (HK2) and phosphofructokinase, platelet (PFKP) for training with BCG (Arts et al., 2016). In vivo, BCG vaccination induced a trained immune phenotype in isolated peripheral blood mononuclear cells, which was associated with a higher expression of HK2 and PFKP.

Also for training with oxLDL, an increased glycolytic activity is essential for the development of the trained immune phenotype: oxLDL-trained macrophages are characterized by an increased extracellular acidification rate using Seahorse technology, and pharmacological inhibition of glycolysis (either by 2-DG or by inhibition of the inducible PFK-2/FBPase isozyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3)) prevents trained immunity (Keating et al., 2020). In addition, genetic variation in genes encoding PFKFB3 and PFKP were associated with the potentiation of TNF-α and IL-6 production upon training with oxLDL ex vivo (Keating et al., 2020).

We recently reported that endogenous adrenal products that are associated with hypertension and atherosclerotic cardiovascular disease, including aldosterone and adrenaline/noradrenaline also induce innate immune memory in human isolated primary monocytes (van der Heijden et al., 2020; van der Heijden et al., 2020). Remarkable, aldosterone-induced trained immunity was not associated with changes in ECAR and OCR using Seahorse technology. For the catecholamines, however, adaptations in these metabolic pathways strongly associated with the effects on cytokine production capacity: the immediate immunosuppressive effect of 24 h exposure to adrenaline/noradrenaline was associated with reductions in ECAR and OCR, whereas the augmented cytokine production capacity that developed six days later was associated with increased glycolysis and OXPHOS (van der Heijden et al., 2020).

There is a close bilateral relationship between the upregulation of glycolytic metabolism and epigenetic reprogramming. Glycolysis is essential for the induction of cytokine production capacity which is mediated by enrichment of the promoters of these genes by activating histone modifications, including H3K4me3 (Keating et al., 2020) (Arts et al., 2016; Cheng et al., 2014). One mechanism that links glycolytic activation to epigenetic reprogramming is the dependency of the sirtuin family of histone deacytelases (HDACs) on the intracellular NAD + concentration (Anderson et al., 2017). Indeed, the NAD+/NADP ratio is increased in trained macrophages (Cheng et al., 2014) and the induction of cytokine production capacity by β-glucan is blunted by co-administration of the HDAC inhibitor resveratrol (Cheng et al., 2014). Conversely, the increased expression of glycolytic enzymes is mediated by activating histone modifications itself, and co-administration of epigenetic inhibitors, such as the nonspecific methyltransferase inhibitor methylthioadenosine or the Set7 methyltransferase inhibitor cyproheptadine, prevents the upregulation of glycolysis by β-glucan on day 6, whereas it does not affect the lactate production in the first 24 h of exposure to β-glucan (Cheng et al., 2014; Keating, Groh, van der Heijden, et al., 2020). Interestingly, oxLDL-trained macrophages are characterized by an enrichment of H3K4me3 on the promoters of PFKFB3 and HK2, which is attenuated by pharmacological inhibition of glycolysis, pointing to a self-perpetuating epigenetic activation of glycolysis (Keating et al., 2020).

4.2. Pentose phosphate pathway

As mentioned before, one of the benefits of glycolytic activation for proliferating or inflammatory cells is that it feeds into the pentose phosphate pathway, which is important for the production of amino acid precursors and nucleotides, necessary for cellular growth and proliferation. In addition, activation of the PPP generates NADPH, which is needed for the production of reactive oxygen species important to combat micro-organisms (O'Neill et al., 2016). Both transcriptomic analyses as well as flux studies with ^{13}C-labeled glucose point to an activation of the oxidative branch of the PPP in macrophages trained with BCG or β-glucan (Arts et al., 2016; Arts et al., 2018). However, pharmacological blockade of this branch of the PPP did not interfere with trained immunity development in vitro showing that the PPP is dispensable for trained immunity (Arts et al., 2016; Arts et al., 2016). It is logical to assume though that the PPP is essential for persistence of trained immunity in vivo by its involvement in the proliferation of myeloid progenitor cells, but this has not been investigated.

4.3. Tricarboxylic acid cycle and oxidative phosphorylation

In resting or anti-inflammatory immune cells, such as the prototypical anti-inflammatory IL-4/IL-13 polarized M2-macrophages and Treg cells, the TCA cycle (also known as citric acid cycle or Krebs cycle) and

subsequent OXPHOS act as the single most important and highly efficient pathway for ATP synthesis (O'Neill et al., 2016). The TCA cycle and OXPHOS provide a highly efficient mechanism in which glycolysis-derived pyruvate or fatty acids are used for the generation of the high-transfer-potential electron carriers nicotinamide dinucleotide (NADH) and flavin adenine dinucleotide (FADH2). These redox metabolites subsequently feed electrons into the Electron Transport Chain (ETC) to support OXPHOS finally resulting in the generation of ATP by ATP synthase (complex V of the ETC).

Importantly, when needed, the TCA cycle can serve other goals for proper cell function other than energy production. When innate immune cells, such as monocytes, are stimulated by growth signals or inflammatory stimuli, e.g. lipopolysaccharide, the TCA cycle shifts function to provide intermediary metabolites for biosynthetic pathways, including the production of amino acids and lipids, which is termed cataplerosis. The efflux of intermediates for biosynthesis requires replenishment of the TCA cycle by nutrients in a process called anaplerosis. In the setting of inflammatory stimulation of macrophages (e.g. by LPS), this functional shift involves accumulation of citrate and succinate by the transcriptional repression of isocitrate dehydrogenase (IDH), and inhibition of succinate dehydrogenase (SDH) function, respectively (Ryan and O'Neill, 2020). In this situation, citrate is transported from the mitochondrial matrix into the cytoplasm and converted into acetyl-CoA, which can be used for acetylation by acetyltransferases, or fuels synthesis of fatty acids and cholesterol, which are important for the formation of prostaglandins and lipid rafts. Succinate further fuels inflammatory activation by various mechanisms including interleuking-1β synthesis by stabilization of hypoxia-inducible factor (HIF)-1α (Ryan and O'Neill, 2020).

Several recent papers have investigated the role of TCA cycle and OXPHOS in trained immunity. In general, these studies revealed that the TCA cycle remains function for ATP production by OXPHOS, while at the same time it provides intermediate metabolites that modulate inflammatory function by regulating activity of epigenetic enzymes.

Analyses of cellular oxygen consumption of in vitro trained human primary macrophages, either with a low dose of β-glucan (of 1 μg/ml), BCG, oxLDL, or the catecholamines adrenaline and noradrenaline, all revealed an augmented basal and maximum oxygen consumption rate on day 6 before restimulation of the cells (Arts et al., 2016; van der Heijden et al., 2020; Keating, Groh, van der Heijden, et al., 2020; Keating et al., 2020). In contrast, a high concentration of β-glucan of 10 μg/ml, which also potently induce a trained immune response in terms of augmentation of cytokine production capacity, induced a classical Warburg effect with an increased glycolysis but repression of OXPHOS (Cheng et al., 2014). The mechanism of this dose-dependency requires further investigation. An additional argument suggesting a role for OXPHOS in trained immunity is the finding that in a cohort of healthy subjects, genetic variation in NADH:ubiquinone oxidoreductase (complex I of mitochondrial electron transport chain) is associated with augmentation of IL-6 production in ex vivo β-glucan trained cells (Keating, Groh, van der Heijden, et al., 2020). Similar associations were described for genetic variation in *IDH* and *SDH*, the genes previously described to mediate the repurposing of the TCA cycle in LPS-stimulated macrophages (Keating, Groh, van der Heijden, et al., 2020). Finally, co-administration of the ATP synthase inhibitor oligomycin during LPS-restimulation in β-glucan trained cells prevents the increased cytokine production (Keating, Groh, van der Heijden, et al., 2020), suggesting that OXPHOS-derived energy production is required for the trained immune phenotype. Inhibition of OXPHOS with oligomycin during training with BCG, however, did not interfere with the induction of cytokine production capacity (Arts et al., 2016). Co-administration of metformin with β-glucan or with oxLDL prevents the development of a trained macrophage phenotype. Metformin inhibits complex I of the respiratory chain which suppresses ATP production (El-Mir et al., 2000). However, the effect of metformin on trained immunity can also be explained by its inhibition of mTOR through activation of adenosine triphosphate dependent protein kinase (AMPK) (Cheng et al., 2014).

Full metabolic assessment of β-glucan-trained macrophages revealed an upregulation of the TCA cycle metabolites succinate, fumarate, and malate, and also of 2-hydroxy-glutarate, which is derived from glutaminolysis (Arts et al., 2016). The observation that pharmacological inhibition of the conversion of glutamine into glutamate prevented trained immunity, confirmed that replenishment of the TCA cycle by glutaminolysis is essential for trained immunity. Interestingly, exposing monocytes to fumarate, but not succinate or malate, for 24 h recapitulated the trained macrophage phenotype in terms of augmented cytokine production capacity. This was accompanied by an enrichment of H3K4me3 on the promoters of the cytokine genes, due to a direct inhibitory effect of fumarate on the KDM5 family of histone demethylases, which are responsible for demethylation of H3K4 (Arts et al., 2016). This effect could partly be restored by the addition of α-ketoglutarate.

4.4. Itaconate

Another metabolite from the TCA cycle that appears to be important in modulating trained immunity is itaconate. Itaconate is synthesized from the decarboxylation of cis-aconitate in the TCA cycle by the enzyme immune-responsive gene 1 (IRG1) (Michelucci et al., 2013). Itaconate has long been known to have strong antimicrobial properties, and more recently has been described as a natural endogenous break on inflammation in innate immune cells, which contributes to the resolution of inflammation (Hooftman and O'Neill, 2019). Briefly, during LPS stimulation in macrophages, itaconate inhibits inflammatory responses, notably IL-1β production, by various independent mechanisms, including inhibition of SDB, and activation of the master antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (NFE2L2 or NRF2) (Mills et al., 2018). In addition, itaconate can increase the expression of activating transcription factor 3 (ATF3), which is a negative regulator of NF-κB zeta (IκBζ), a transcription factor that regulates secondary transcriptional responses to TLR activation, including the release of IL-6 (Bambouskova et al., 2018).

In a recent paper, Domínguez-Andrés et al. investigated the role of itaconate in innate immune tolerance and training (Dominguez-Andres et al., 2019). They underscored the potent anti-inflammatory effects of itaconate in macrophages by showing that LPS exposure, which induces immune cell tolerance, leads to a rapid increase of itaconate by upregulation of the expression of IRG1. Itaconate subsequently increases succinate levels by the inhibition of SDH. Importantly, co-administration of β-glucan and LPS prevented this upregulation of IRG1 expression, and training with β-glucan was associated with a long-lasting inhibition of IRG1 expression as secondary exposure to LPS only minimally increased IRG1 expression. These results fit the previous observation that β-glucan prevents and reverts LPS-induced tolerance (Novakovic et al., 2016). Itaconate itself also modulates trained immunity by its inhibitory effect on SDH: in the context of β-glucan-induced trained immunity, the addition of exogenous itaconate (dimethylitaconate) prevents the development of the hyperresponsive trained immune phenotype by preventing the accumulation of fumarate.

In summary, while itaconate inhibits the induction of trained immunity, β-glucan-induced trained immunity can revert LPS-induced immunoparalysis by inhibiting activation of the IRG1-itaconate-SDH axis.

So, TCA cycle intermediates can impact on cell function by modulating epigenetic enzymes. At the same time, epigenetic processes can regulate metabolic functions of monocytes and macrophages. Keating et al. recently described a critical role for the methyltransferase Set7 in the adaptations of the TCA cycle function and OXPHOS that occur in β-glucan trained cells (Keating, Groh, van der Heijden, et al., 2020). They validated that in β-glucan trained macrophages, on day 6 the intracellular concentrations of the TCA cycle metabolites succinate, fumarate, malate, oxaloacetate, and citrate were higher than in

non-trained control cells (Keating, Groh, van der Heijden, et al., 2020). This increase was prevented for succinate, fumarate, and malate by co-incubation of β-glucan with the Set7 inhibitor cyproheptadine (CPH). This co-incited with an augmented expression of the enzymes Succinate-CoA Ligase GDP/ADP-Forming Subunit Alpha (SUCLG1), the B subunit of succinate dehydrogenase (SDHB), fumarate hydratase (FH), malate dehydrogenase 2 (MDH2) and citrate synthase (CS) mRNA expression, which was prevented by CPH for SDHB, FH, and MDH2. This enhanced expression was regulated by Set7 dependent enrichment of H3K4me1 at distal enhancers that form regulatory domains with *MDH2* and *SDHB* (Keating, Groh, van der Heijden, et al., 2020). *Setd7* deficient mice were used to validate the effect of β-glucan training on TCA cycle enzymes in bone marrow progenitor cells and it was shown that mRNA expression of MDH2 and SDHB, but not FH1 or Suclg2 was increased by β-glucan in wild-type mice, but not in the *Setd7* deficient mice (Keating, Groh, van der Heijden, et al., 2020).

4.5. Lipid synthesis pathway

In the cytosol, citric acid-derived acetyl-CoA can enter the cholesterol biosynthesis pathway, which is controlled mainly by the enzyme 3-hydroxy-3-methylglutaryl CoA reductase. This pathway is important for the inflammatory function of immune cells for various reasons. First, cholesterol determines the structural integrity and fluidity of the cellular membranes and compartmentalization of the membrane in lipid rafts, which are important for inflammatory signal transduction pathways. In addition, isoprenoid intermediates of the cholesterol synthesis pathway can modify the function of multiple proteins by a process called prenylation which can modify the function of these proteins, many of which are involved in inflammatory processes. In the context of atherosclerosis, it has been established that cholesterol accumulation in myeloid progenitor cells in the bone marrow promotes myelopoiesis and leads to an acceleration of atherosclerosis (Yvan-Charvet et al. 2008, 2010; Tall and Yvan-Charvet, 2015). Analysis of the transcriptome and metabolome of β-glucan trained macrophages revealed, in addition to glycolysis, the PPP, and glutamine metabolism, also an upregulation of cholesterol and fatty acid synthesis pathways (Arts et al., 2016; Bekkering et al., 2018). Pharmacological inhibition of 3-hydroxy-3-methylglutaryl CoA reductase with statins prevented trained immunity by β-glucan and oxLDL in vitro and also in an in vivo model of β-glucan-induced trained immunity in mice. Importantly, pharmacological inhibition of 5-fluoromevalonate, which catalyzes the conversion of mevalonate-5-PP into isopentyl-PP augmented cytokine production capacity in the in vitro trained immunity model (Bekkering et al., 2018). This finding excludes a role for protein prenylation as well as cholesterol synthesis in trained immunity and points to a role for intracellular accumulation of mevalonate. Indeed, exposure of human monocytes for 24 h to mevalonate induced a similar trained immune phenotype with enhanced cytokine production, accompanied by an activation of glycolysis and an enrichment of H3K4me3 on cytokine gene promoters. Additional pharmacological experiments revealed that mevalonate induced trained immunity by activation of the insulin-like growth factor-1 receptor pathway and subsequent activation of mTOR and glycolysis (Bekkering et al., 2018).

The importance of the mevalonate synthesis pathway for trained immunity is further highlighted by the finding that RNA sequencing of hematopoietic stem cells from mice seven days after β-glucan administration showed an enrichment of the cholesterol biosynthesis pathway and especially the mevalonate pathway (Mitroulis et al., 2018).

In addition to the cholesterol synthesis pathway, citrate-derived acetyl-CoA can also fuel fatty acid synthesis, which positively regulates the generation and function of pro-inflammatory immune cells of both the innate and adaptive immune systems (O'Neill et al., 2016). Several studies have reported that inflammatory stimuli such as LPS trigger an increase in fatty acid synthesis in macrophages. The relevance of this pathway for the development of atherosclerosis is illustrated by the observation that in atherosclerosis-prone *ApoE* $^{-/-}$ mice, macrophage-targeted deletion of *Fasn* (the gene that encodes fatty acid synthase) reduces atherosclerotic plaque formation and foam cell formation (Schneider et al., 2010). β-Glucan trained macrophages show an upregulation of the fatty acid synthase pathway, but pharmacological inhibition of this pathway during the 24 h exposure to β-glucan did not interfere with trained immunity (Arts et al., 2016). Fatty acid synthesis also appears to be essential for trained immunity induced by aldosterone (van der Heijden et al., 2020). As mentioned before, brief exposure of human monocytes to aldosterone also enhances cytokine production capacity after restimulation with LPS and the TLR2 ligand Pam3Cys, and this was not associated with activation of glycolysis or oxidative phosphorylation. Four hours after restimulation with Pam3Cys however, the aldosterone-trained cells showed an upregulation of various genes that are central to the fatty acid synthesis pathway, fatty acid elongation, and the formation of very long chain fatty acids, compared to vehicle-exposed cells. Furthermore, these genes showed enrichment of H3K4me3 on their respective promoters. Preincubation of the aldosterone-trained cells with the fatty acid synthesis inhibitor cerulenin before the restimulation with Pam3Cys completely abolished the augmentation of cytokine production capacity (van der Heijden et al., 2020).

The fatty acid oxidation (FAO) pathways occurs in the mitochondrial matrix and allows for the conversion of fatty acids, after transport from the cytosol via carnitine palmitoyl transferase 1, into acetyl-CoA, NADH, and FADH2, which are further used in the TCA cycle for energy production. FAO also importantly regulate immune cell function, being the primary source of energy production used by anti-inflammatory IL-4/IL-13 activated ('M2') macrophages (Van den Bossche, O'Neill, and Menon, 2017; Huang et al., 2014). LPS/IFNγ activated ('M1') macrophages on the other hand down-regulate FAO, favoring glycolytic metabolism for their energy demands. FAO has not yet been studied in the context of trained immunity.

5. Clinical implications and future directions

The studies summarized above have provided a detailed mechanistic framework on how monocytes and macrophages can build an immunological memory and how trained monocytes can persist for months in vivo despite their short circulating half-life. Within the cells, activation of various metabolic pathways and repurposing of intermediate metabolites strongly interact with epigenetic enzymes to mold a chromatin landscape that facilitates persistent hyperresponsiveness to a subsequent stimulation. Similar processes occur in myeloid progenitors in the bone marrow ensuring persistence of circulating trained monocytes for months after a transient trigger for training, such as an infection.

Since accumulating evidence points to an important role for trained immunity in the host defense against infectious threats as well as in the pathophysiology of non-infectious chronic inflammatory diseases, the intracellular machinery that regulates training offers exciting novel pharmacological targets. The potential clinical relevance of trained immunity has been expanded further by recent observations that 1) other cell types are also able to build trained immunity-like memory (Hamada et al., 2018; Netea et al., 2020) and 2) trained immunity might also contribute to additional clinical conditions (Netea et al., 2020). With regard to the first series of observations, trained immunity has also been shown to occur in natural killer cells (NK cells) (Kleinnijenhuis et al., 2014; Gamliel et al., 2018), and dendritic cells (Hole et al., 2019). Interestingly, memory responses resembling innate immune memory, and making use of overlapping epigenetic and metabolic mechanisms, can also occur in non-immune cells, including stromal cells and epithelial cells. As such, endothelial cells and vascular smooth muscle cells also show hyperinflammatory responses following brief exposure to glucose or oxLDL (El-Osta et al., 2008; Schnack et al., 2019). Epithelial stem cells also maintain chromosomal accessibility at key stress response genes that are activated by a primary danger stimulus (Naik

et al., 2017). Similarly, cerebral microglia cells can build a long-term pro-inflammatory phenotype by epigenetic programs that can last for at least six months after peripherally applied inflammatory stimuli (Wendeln et al., 2018). This identification of multiple cell types in the literature that are able to build inflammatory memory has gone hand in hand with an expansion of the clinical scenarios in which trained immunity might be involved, including atherosclerosis (Riksen, 2019), neurogenerative diseases including cerebral small vessel disease (Wendeln et al., 2018; Noz et al., 2018), and organ transplant rejection (Braza et al., 2018).

As such, the metabolic and epigenetic machinery that regulates the immunological memory in the various cell types offers an exciting and extensive array of novel targets for pharmacological strategies, which have recently been described by Mulder et al., (2019). Boosting trained immunity, e.g. by BCG vaccination, can improve protection against infections in susceptible populations. In addition, boosting trained immunity might reverse immune paralysis in patients with severe infections; indeed, in isolated human monocytes, β-glucan can reverse the LPS-induced tolerance and ex vivo β-glucan treatment of monocytes from volunteers with experimental endotoxemia re-instates their capacity for cytokine production (Novakovic et al., 2016). Finally, promoting trained immunity could be developed as an additional strategy in malignant diseases (Fig. 2). In contrast, drugs that interfere with trained immunity could be beneficial in patients with chronic inflammatory diseases, including atherosclerosis, auto-immune disorders, and organ rejection after transplantation.

Several existing drugs target one of the metabolic pathways that drive trained immunity. Statins inhibit 3-hydroxy-3-methylglutaryl CoA reductase and prevent trained immunity induction by β-glucan and oxLDL in vitro (Bekkering et al., 2018). In contrast, statins are not able to revert the trained immune phenotype in monocytes once this has been established: monocytes isolated from patients with an increased cardiovascular risk due to hypercholesterolemia have an augmented cytokine production capacity that is driven by epigenetic reprogramming and this trained phenotype persisted despite cholesterol lowering with statins during three months (Bekkering et al., 2019). The anti-hyperglycemic drug metformin inhibits the mTOR pathway by activating adenosine monophosphate activated protein kinase (AMPK). Given the key role for mTOR in the glycolytic activation necessary for training, it is not surprising that metformin can prevent trained immunity, both by β-glucan as well as by oxLDL (Keating et al., 2020). This mechanism could theoretically contribute to the anti-atherosclerotic effects of metformin in clinical trials.

Interfering with the metabolic processes that are involved in the regulation of trained immunity is challenging, mainly because these processes are general processes that are used by all cell types for survival and function. Specificity for trained immune processes can be increased by pharmacokinetic strategies that improve the delivery of the pharmaceutical compound to the specific cell type in which trained immunity contributes to disease pathophysiology, e.g. the macrophages in atherosclerotic plaques. This is possible with the use of nanobiologicals, e.g. recombinant high-density lipoprotein (HDL) particles which specifically target macrophages (Mulder et al., 2019). Previous studies have shown that these HDL particles loaded with drugs that interfere with trained immunity, including statins can suppress atherosclerotic plaque inflammation (Tang et al., 2015) (Duivenvoorden et al., 2014). Also, in a mouse model of transplantation, a short-term mTOR-specific HDL-nanobiological treatment averted macrophage aerobic glycolysis and the epigenetic modifications underlying inflammatory cytokine production and improved graft survival (Braza et al., 2018). Yet another way to prevent side effects and toxicity is to only partly inhibit metabolic pathways that are upregulated in trained immunity. In isolated human monocytes inhibition of the inducible glycolytic enzyme PFKFB3 (6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 3) with the small-molecule 3PO (3-[3-pyridinyl]-1-[4-pyridinyl]-2-propen-1-one) prevents oxLDL-induced trained immunity in vitro (Keating et al., 2020). Interestingly, 3-PO only partially inhibits glycolysis and systemic treatment of atherosclerosis prone mice with 3-PO significantly reduces atherosclerotic lesion development (Perrotta et al., 2020). This is due to the fact that inhibiting endothelial cell glycolysis limits intraplaque neovascularization, but it cannot be excluded that this treatment strategy also prevented trained immunity in innate immune cells. A final strategy that can potentially limit side effects of interfering with trained immunity metabolic pathways is to exploit the memory aspect trained immunity. A pharmacological intervention that prevents trained immunity needs to be given during only the short period that the trained immunity is switched on, e.g. during acute infections, immediately after myocardial infarction, or after organ transplantation), which does not interfere with future plasticity of the innate immune system.

In conclusion, we foresee that the rapidly expanding knowledge about the intracellular metabolic and epigenetic processes that modulate trained immunity will stimulate the development of novel pharmacological compounds for patients in various clinical settings. Targeted delivery of these compounds to the specific trained cells in a time- and site-specific manner is needed to limit side effects and increase the therapeutic index.

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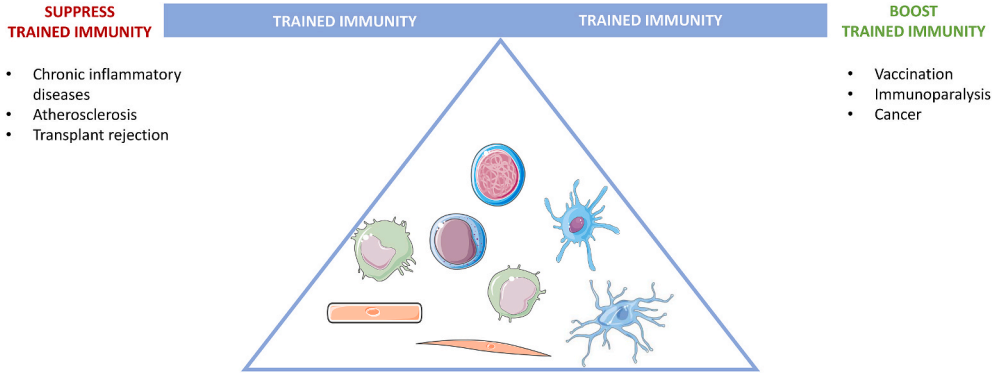

Fig. 2. Schematic representation of the various cell types that are able to build trained immunity or comparable inflammatory memory characteristics, including monocytes, macrophages, myeloid progenitor cells, NK cells, dendritic cells, microglia, endothelial cells, and vascular smooth muscle cells. Trained immunity can either improve host defense against infections or cancer, or be maladaptive in situations of chronic inflammation. Therefore, suppression of trained immunity might be beneficial in situations of chronic inflammatory diseases, such as atherosclerosis, or transplant rejection. On the other hand, boosting trained immunity might improve host defense against infections and might be beneficial in patients with cancer.

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