Partial Inhibition of the 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase-3 (PFKFB3) Enzyme in Myeloid Cells Does Not Affect Atherosclerosis

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Background: The protein 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) is a key stimulator of glycolytic flux. Systemic, partial PFKFB3 inhibition previously decreased total plaque burden and increased plaque stability. However, it is unclear which cell type conferred these positive effects. Myeloid cells play an important role in atherogenesis, and mainly rely on glycolysis for energy supply. Thus, we studied whether myeloid inhibition of PFKFB3-mediated glycolysis in Ldlr−/−/−LysMCre+/−/−Pfkfb3fl/fl (Pfkfb3fl/fl) mice confers beneficial effects on plaque stability and alleviates cardiovascular disease burden compared to Ldlr−/−/−LysMCre+/−/−Pfkfb3wt/wt control mice (Pfkfb3wt/wt).

Methods and Results: Analysis of atherosclerotic human and murine single-cell populations confirmed PFKFB3/Pfkfb3 expression in myeloid cells, but also in lymphocytes, endothelial cells, fibroblasts and smooth muscle cells. Pfkfb3wt/wt and Pfkfb3fl/fl mice were fed a 0.25% cholesterol diet for 12 weeks. Pfkfb3fl/fl bone marrow-derived macrophages (BMDMs) showed 50% knockdown of Pfkfb3 mRNA. As expected based on partial glycolysis inhibition, extracellular acidification rate as a measure of glycolysis was partially reduced in Pfkfb3fl/fl BMDMs. Unexpectedly, plaque and necrotic core size, as well as macrophage (MAC3), neutrophil (Ly6G) and collagen (Sirius Red) content were unchanged in advanced Pfkfb3fl/fl lesions. Similarly, early lesion plaque and necrotic core size and total plaque burden were unaffected.
Conclusion: Partial myeloid knockdown of PFKFB3 did not affect atherosclerosis development in advanced or early lesions. Previously reported positive effects of systemic, partial PFKFB3 inhibition on lesion stabilization, do not seem conferred by monocytes, macrophages or neutrophils. Instead, other Pfkfb3-expressing cells in atherosclerosis might be responsible, such as DCs, smooth muscle cells or fibroblasts.

Keywords: myeloid cells, PFKFB3, macrophage, dendritic cell, glycolysis, atherosclerosis, neutrophil, glycolysis inhibition

INTRODUCTION

Myeloid cells [i.e., monocytes, macrophages, neutrophils and dendritic cells (DCs)] play an active role in atherosclerosis. Early pathogenesis of atherosclerotic plaques is characterized by activation of intimal endothelial cells (ECs) in arteries, followed by extravasation of low-density lipoprotein (LDL) cholesterol (Tabas et al., 2007). In the subendothelial space, LDL is oxidized (oxLDL) by reactive oxygen species (ROS) and enzymes (Tabas et al., 2007). This results in a pro-inflammatory response that triggers myeloid cell recruitment (Moore and Tabas, 2011; Silvestre-Roig et al., 2020). Recruited myeloid cells act in parallel to stimulate inflammation through cytokine secretion and other mechanisms. Recruited, activated neutrophils further stimulate monocyte recruitment and macrophage activation. Furthermore, neutrophils contribute to the pro-inflammatory environment by secretion of ROS and neutrophil extracellular traps (NETs), and to LDL oxidation by secreting myeloperoxidase (Silvestre-Roig et al., 2020). DCs modulate T cell responses in atherosclerosis. Additionally, recruited monocytes can differentiate into macrophages or monocyte-derived DCs (moDCs), which ingest oxLDL and become lipid-laden foam cells (Moore and Tabas, 2011; Subramanian and Tabas, 2014; Zernecke, 2015). Excess uptake of oxLDL can result in leukocyte apoptosis. In advanced disease stages, accumulation of apoptotic leukocytes in combination with decreased phagocytic clearance contributes to formation of a detrimental necrotic core (Moore and Tabas, 2011). Moreover, during atherogenesis, smooth muscle cells (SMCs) migrate into the plaque and synthesize collagen, forming a stabilizing fibrous cap. Secretion of matrix metalloproteinases, serine proteases and NETs by macrophages and neutrophils can cause fibrous cap thinning (Moore and Tabas, 2011; Silvestre-Roig et al., 2020). This increases the risk of plaque rupture, which can have detrimental consequences.

Activated neutrophils, DCs and pro-inflammatory macrophages highly depend on glycolysis for their energy production and function (Galván-Peña and O’Neill, 2014; Kumar and Dikshit, 2019; Wculek et al., 2019). During glycolysis, glucose is metabolized to pyruvate, yielding ATP and NADH (Lunt and Heiden, 2011). A rate-limiting step of glycolysis is the conversion of fructose-6-phosphate into fructose-1,6-bisphosphate, catalyzed by phosphofructokinase-1 (PFK-1). Another enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3), catalyzes the conversion of fructose-6-phosphate into fructose-2,6-bisphosphate, which is an allosteric activator of PFK-1. Thus, PFKFB3 is a potent stimulator of glycolytic rate (Lunt and Heiden, 2011), and possibly an attractive target to interfere with myeloid cell function in atherogenesis.

A few studies have indeed assessed the effect of systemic administration of 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propan-1-one (3PO) or derivatives to partially inhibit PFKFB3 in atherosclerosis. These studies reported decreased total plaque burden (Perrotta et al., 2020) and increased plaque stabilization, respectively (Beldman et al., 2019; Poels et al., 2020). However, as these studies entailed systemic pharmacological PFKFB3 inhibition, it is unclear which cell type confers these positive effects. Although Pfkfb3 expression in atherosclerotic DCs and neutrophils remains to be assessed, Tawakol et al. (2015) reported increased Pfkfb3 expression in macrophages incubated with atherosclerosis-relevant stimuli in vitro. This effect was exacerbated by hypoxia. Still, the in vivo effect of partial inhibition of PFKFB3-mediated glycolysis, specifically in myeloid cells, on atherogenesis has not been studied. Thus, we studied the hypothesis that myeloid inhibition of PFKFB3-mediated glycolysis in Ldrl−/−LysMCre+/−Pfkfb3fl/fl (Pfkfb3fl/fl) mice confers beneficial effects on plaque stability and alleviates cardiovascular disease burden compared to Ldrl−/−LysMCre+/−Pfkfb3wt/wt control mice (Pfkfb3wt/wt).

MATERIALS AND METHODS

Single-Cell Gene Expression Analysis

Single-cell RNA-sequencing (scRNA-seq) datasets from atherosclerotic plaques were collected from Gene Expression Omnibus (GEO) database or requested to corresponding authors: Wirka et al., 2019 (4 human specimens, GSE131780), Zernecke et al., 2020 (meta-analysis from 9 mouse datasets), and van Kuijk et al., 2021 (11 pooled Ldrl−/−LysMCre+/− mice, GSE150089). Seurat R package (v3.0.1) was used as toolbox for analysis (Stuart et al., 2019) in R (v3.6.1). Single-cell
gene expression was normalized by library size, multiplied by a scaling factor of 10,000 and log-transformed. Original cell cluster annotations were used for analysis. HIF1α/HIF3α (hypoxia-inducible factor 1-alpha) transcription factor (TF) activity was estimated using DoRothEA1 (Garcia-Alonso et al., 2019), using the TF regulons of A, B, and C confidence classes as previously described (Holland et al., 2020). For 2-group comparison between cells undergoing and not undergoing hypoxia response, cells were stratified by the third quartile (Q3) of HIF1A/HIF3α TF activity within each cell cluster (high > Q3, low ≤ Q3). Differential PFKFB3/Pfkfb3 expression was performed using Wilcoxon Rank Sum test. No test was performed when the sample size of any condition was lower than 5 observations. P-values were adjusted for multiple testing using the Benjamini and Hochberg method. R effect sizes from each cluster with individual observations (each cell) as data points, 50th percentile of the distribution as a horizontal line, and sample sizes (number of cells) at the bottom. For 2-group comparisons, violin plots were split by hypoxia response stratification, with Wilcoxon test statistics of FDR-adjusted p-values and r effect sizes at the top. Analysis code is available at https://github.com/saezlab/Myeloid_PFKFB3_atherosclerosis.

Experimental Animals
Mouse experiments were approved by regulatory authorities of Maastricht University Medical Centre and performed in compliance with Dutch governmental guidelines and European Parliament Directive 2010/63/EU on protection of animals used for scientific purposes. Mice with a loxP-flanked Pfkfb3 gene (Pfkfb3lox/+lox) (De Bock et al., 2013) were crossed to mice with both a LDL receptor knockout (Ldlr−/−) to ensure atherosclerosis susceptibility, and hemizygous Cre-recombinase expression under control of the LysM gene promoter (LysMCre+/−). Lys2 is highly expressed in macrophages, monocytes and neutrophils, and to a lower extent in DCs (Supplementary Figure 1A: Faust et al., 2000). Thus, myeloid-specific Cre-mediated excision of the Pfkfb3 gene could be ensured. Resulting mice (Ldlr−/−LysMCre+/−Pfkfb3fl/fl) are referred to as Pfkfb3fl/fl. Ldlr−/−LysMCre+/−Pfkfb3wt/wt mice were used as controls (Pfkfb3wt/wt). Mice were housed in the Maastricht University laboratory animal facility under standard conditions, in individually ventilated cages (GMS00, Techniplast) with up to 5 animals per cage, with bedding (corncob, Technilab-BMI) and cage enrichment. Cages were changed weekly, reducing handling of mice during non-intervention periods.

Induction of Atherosclerosis and Tissue Collection
To induce atherosclerosis, 11-week-old male Pfkfb3wt/wt and Pfkfb3fl/fl mice were fed a high cholesterol diet (HCD) for 12 weeks ad libitum, containing 0.25% cholesterol (824171, Special Diet Services). Mice were euthanized by intraperitoneal pentobarbital injection (100 mg/kg). Blood was withdrawn from the right ventricle and centrifuged (2,100 rpm, 10 min, 4°C). Plasma aliquots were stored at −80°C. Brachiocephalic arteries (BCAs) and hearts were dissected, fixed in 1% PFA overnight and paraffin-embedded.

Plasma Cholesterol and Triglyceride Levels
Plasma cholesterol (Cholesterol FS Ecoline, 11309990314; DiaSys Diagnostic Systems GmbH) and triglyceride (FFS’ Ecoline, 15760990314; DiaSys Diagnostic Systems GmbH) levels were assessed by standard enzymatic techniques, automated on the Cobas Fara centrifugal analyzer (Roche).

Histology and Immunohistochemistry
Paraffin-embedded BCA and aortic root (AR) were serially sectioned (4 μm) and stained with hematoxylin and eosin (H&E) to quantify plaque size and necrotic core content. For ARs, five consecutive H&E sections with 20 μm intervals were blinded and analyzed using computerized morphometry (Leica QWin V3, Cambridge, United Kingdom). The sum of plaque within three valves was averaged per mouse. Total plaque burden was quantified in BCA (Σ total plaque length/Σ total vessel length). Furthermore, AR atherosclerotic plaques were analyzed for macrophage content (MAC3+ area/plaque area, 553322, BD), collagen content (Sirius Red+ area/plaque area, 09400, Polyscience) and neutrophil content (Ly6G+ cells/plaque area, 551459, BD). Antigen retrieval was performed with pepsin digestion (Ly6G) or at pH 6 (MAC3, Target Retrieval Solution, S2031, DAKO). Stainings were analyzed using Leica Qwin software (V3, Cambridge United Kingdom) or QuPath V0.2.3 (Bankhead et al., 2017).

Isolation and Differentiation of Bone Marrow Cells
Femur and tibia of Pfkfb3wt/wt and Pfkfb3fl/fl mice on standard laboratory diet were dissected. Bones were flushed with PBS and cells passed through a 70 μm cell strainer.

To obtain bone marrow-derived macrophages, bone marrow cells were cultured in RPMI 1640 medium (72400047, Gibco), with 15% L929-conditioned medium, 10% fetal calf serum (FCS, FBS-12A, Capricorn Scientific) and 1% penicillin-streptomycin (15070-063, Gibco). After 7-day differentiation, BMDMs were detached with lidocaine and plated for downstream assays. For pro-inflammatory polarization of BMDMs, cells were incubated with LPS (10 ng/ml, L2880, Sigma) and IFN-γ (100 units/ml, HC1020, Hycult Biotech) for 24 h after overnight attachment.

To obtain bone marrow-derived DCs, bone marrow cells were cultured in IMDM medium (21980032, Thermo Fisher Scientific), with 5% FCS, 0.029 mM 2-mercaptoethanol, 150

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1https://saezlab.github.io/dorothea/
ng/ml Flt3 ligand (472-FL, R&D Systems) and 1% penicillin-
streptomycin for 8 days. After differentiation, DCs were
detached by rinsing.

**Quantitative PCR**

RNA was isolated with TRIzol reagent (15596026, Thermo
Fisher Scientific) according to manufacturer’s protocol. RNA
centrations were determined by NanoDrop 2000 (Thermo
Fisher Scientific) according to manufacturer’s protocol. RNA
was isolated with TRIzol reagent (15596026, Thermo
Fisher Scientific) and reverse transcription performed following

**Lactate and Glucose Levels**

Lactate and glucose levels in Pfkfb3+/wt and Pfkfb3fl/fl BMDM
cell culture medium were assessed after 26 h of conditioning,
using a GEM Premier 4000 Analyzer and the manufacturer’s
protocol (Instrumentation Laboratory).

**Seahorse**

BMDMs were plated onto XF96 tissue culture microplates.
Growth medium was replaced with glucose-free assay medium
(RPMI-1640 (R1383, Sigma), 143 mM NaCl, 3 mg/L Phenol
Red, 2 mM L-glutamine, in dH2O, pH 7.35) and cells were
incubated in a non-CO2 incubator for 1 h. Thereafter, the assay
was performed according to manufacturer’s protocol (103020-
100, Agilent), using a 10 mM glucose stimulus, with a Seahorse
XF96 Analyzer (Agilent).

**Statistical Analyses**

Data are represented as mean ± SEM. For results besides
single-cell analysis, ROUT outlier analysis was performed and
subsequently, normality (Shapiro-Wilk) and equal variances (F-
test) analysis and corresponding parametric or non-parametric
testing were performed for two-independent groups. *p < 0.05,
**p < 0.01, and ***p < 0.001.

**RESULTS**

**Expression of PFKFB3/Pfkfb3 in Human and Murine Plaques in Both Immune and Stromal Cells**

We first sought to assess PFKFB3/Pfkfb3 expression patterns
in human and murine atherosclerotic plaques. The scRNA-seq
dataset from human atherosclerotic coronary arteries by Wirka
et al. (2019) showed PFKFB3 expression mainly in macrophages,
but also in ECs, fibroblasts and other leukocytes as T
cells (Figure 1A). This confirms PFKFB3 expression in human
atherosclerosis, and particularly in macrophages.

Next, we analyzed murine Pfkfb3 expression in myeloid cells
specifically, from the scRNA-seq meta-analysis by Zernecke
et al. (2020) including data from 9 atherosclerosis studies of
murine aorta. Traditionally, macrophages were classified into
pro-inflammatory M1 and anti-inflammatory M2 macrophages
(Mantovani et al., 2009). However, the rise of single-cell

**Decreased Glycolysis and Pro-inflammatory Profile in Pfkfb3fl/fl Macrophages**

To study if myeloid cells were indeed responsible for the
observed effects of systemic PFKFB3 inhibition, we generated
Ldlr−/− LysMCre+/− Pfkfb3fl/fl (Pfkfb3fl/fl) mice, using Ldlr−/−
LysMCre+/− Pfkfb3wt/wt mice as controls (Pfkfb3wt/wt). Partial
myeloid Pfkfb3 knockdown was confirmed in Pfkfb3fl/fl versus
Pfkfb3wt/wt BMDMs (50%, Figure 2A). As available antibodies
are non-specific, confirmation of PFKFB3 knockdown on a
protein level was prevented. Therefore, we further sought
to obtain functional confirmation of Pfkfb3 knockdown. The
(near-)complete inhibition of glycolysis (≥ 80%) induces cell
FIGURE 1 | Expression pattern of PFKFB3/Pfkfb3 in human and murine atherosclerosis. (A) Violin plot of PFKFB3 expression in single-cell populations of human atherosclerotic coronary arteries (Wirka et al., 2019). (B) Violin plot of Pfkfb3 expression in single-cell populations of murine atherosclerotic myeloid cells (Zernecke et al., 2020). (C) Violin plot of Pfkfb3 expression in single-cell populations of murine Ldr−/−/LysMCer+− aortic arch lesions (van Kuijk et al., 2021). (D) Split violin plot of PFKFB3 expression in cells with high versus low HIF1α signature from human atherosclerotic coronary arteries (Wirka et al., 2019). (E) Split violin plot of Pfkfb3 expression in murine atherosclerotic myeloid cells, with high versus low Hif1α signature (Zernecke et al., 2020). In (D,E), Wilcoxon test statistics of FDR-adjusted p-values and r effect sizes are indicated at the top. Sample sizes per cell type indicated under (split) violin plots. CD, cluster of differentiation; cDC, conventional dendritic cell; EC, endothelial cell; HIF1α, hypoxia-inducible factor 1-alpha; IFN, interferon-inducible; Mac, macrophage; moDC, monocyte-derived dendritic cell; NK cell, natural killer cell; pDC, plasmacytoid DC; SMC, smooth muscle cell; TREM2, triggering receptor expressed on myeloid cells 2. ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001.
death (Schoors et al., 2014). Thus, partial glycolysis inhibition is desirable to affect cell function, without compromising cell viability. Seahorse analysis after glucose dosing revealed decreased basal extracellular acidification rate (ECAR) in Pfkfb3\(^{-}\) BMDMs compared to controls (Figure 2B), indicating partially decreased glycolytic rates. During glycolysis, glucose is metabolized into pyruvate. Pyruvate can either be utilized partially decreased glycolytic rates. During glycolysis, glucose is metabolized into pyruvate. Pyruvate can either be utilized for ATP, or metabolized into organic acids such as lactate (Lunt and Heiden, 2011). As expected based on glycolysis disruption, residual glucose levels were increased, whereas lactate levels were decreased in Pfkfb3\(^{-}\) BMDM-conditioned medium (Figures 2C,D).

We previously mentioned that pro-inflammatory macrophages rely on glycolysis for their energy supply (Galván-Peña and O’Neill, 2014). As PFKFB3 silencing using siRNA previously reduced glycolysis and pro-inflammatory activation of human macrophages (Tawakol et al., 2015), we studied the effect of Pfkfb3 knockdown on BMDM cytokine gene expression. Indeed, already in unstimulated Pfkfb3\(^{-}\) BMDMs, we observed increased expression of anti-inflammatory Il10 (Figure 2E). Moreover, we stimulated Pfkfb3\(^{-}\) BMDMs with LPS and IFN-γ to mimic the plaque pro-inflammatory phenotype of these cells and showed that partial Pfkfb3 knockdown was maintained (60%, Figure 2F). Moreover, pro-inflammatory Il6 and Il12b expression were decreased in Pfkfb3\(^{-}\) versus Pfkfb3\(^{\text{wt}}\) BMDMs after pro-inflammatory stimulation (Figures 2G,H). These results indicate a decreased pro-inflammatory profile in Pfkfb3\(^{-}\) macrophages, and thus confirm a role of Pfkfb3 in pro-inflammatory macrophage polarization.

In our mouse model, Cre-recombinase expression is under control of the Lys2 promoter. Compared to macrophages, monocytes and neutrophils, Lys2 gene expression is low in DCs (Supplementary Figure 1A). Nevertheless, we assessed if DCs were targeted by our model, as Pfkfb3 expression was abundant in this cell type (Figures 1B,C). We differentiated DCs from bone marrow cells and confirmed protein expression of the DC marker CD11c by flow cytometry (Supplementary Figure 1B). As expected based on lower Lys2 gene expression, DCs were not targeted in our model, as Pfkfb3 expression was unchanged between Pfkfb3\(^{-}\) and Pfkfb3\(^{\text{wt}}\) DCs (Figure 2I).

### No Effect of Partial Myeloid Pfkfb3 Disruption on Atherosclerosis

After confirming partial Pfkfb3 knockdown, functional disruption of glycolysis and a decreased pro-inflammatory profile in macrophages in vitro, we studied the effects of myeloid Pfkfb3 disruption on atherosclerosis. Therefore, Pfkfb3\(^{-}\) and Pfkfb3\(^{\text{wt}}\) mice were fed a HCD for 12 weeks. We observed advanced atherosclerotic plaques in ARs with a necrotic core and fibrous cap (Figure 3A). Body weight and plasma cholesterol and triglyceride levels were similar between Pfkfb3\(^{-}\) and Pfkfb3\(^{\text{wt}}\) mice after HCD (Figures 3B–D).

Unexpectedly, plaque and necrotic core size, as well as plaque macrophage and collagen content were unaffected in Pfkfb3\(^{-}\) advanced AR lesions compared to controls (Figures 3E–G). Moreover, Ly6G+ neutrophil content was also unchanged between Pfkfb3\(^{\text{wt}}\) and Pfkfb3\(^{-}\) AR lesions (Figure 3H). Similarly, no changes in plaque or necrotic core size were observed in early lesions without or with very little necrosis in BCA (Supplementary Figure 2A). Besides plaque size, total plaque burden, as measured by plaque index (Perrotta et al., 2020), was also unaffected in Pfkfb3\(^{-}\) BCA (Supplementary Figure 2B).

### Pfkfb Isoenzyme Expression in Plaque Myeloid Cells

To study potential genetic compensation by other Pfkfb isoenzymes keeping glycolytic rate above a certain threshold, we assessed expression of these isoenzymes in murine plaque myeloid cells. Expression of Pfkfb1 and Pfkfb2 was minimal in myeloid cells of the meta-analysis (Figures 4A,B) and Ldrl\(^{-}/-\) LysMcru\(^{-}\) datasets (Supplementary Figures 3A,B). Similarly to Pfkfb3, Pfkfb4 was expressed in macrophage and DC subsets, monocytes and neutrophils, albeit in a small proportion of cells (Figure 4C and Supplementary Figure 3C). To assess possible genetic compensation, we determined expression of Pfkfb1, Pfkfb2, and Pfkfb4 in Pfkfb3\(^{\text{wt}}\) versus Pfkfb3\(^{\text{wt}}\) BMDMs, which was unaffected (Figure 4D). Thus, genetic compensation by other Pfkfb isoenzymes seems absent in BMDMs.

### DISCUSSION

The current study assessed the effect of partial myeloid Pfkfb3 disruption on atherosclerosis in vivo, after 12 weeks of HCD. Collectively, our findings suggest that although myeloid Pfkfb3 disruption decreases the pro-inflammatory macrophage profile in vitro, it does not affect atherosclerosis development in vivo, neither in advanced, nor early lesions. No effects on circulating lipids, plaque size and composition, or total plaque burden were observed.

A few studies have looked into partial pharmacological inhibition of glycolysis in atherosclerosis by targeting PFKFB3, using 3PO(-derivatives). Similar to the current study, no effect on plaque size was reported (Beldman et al., 2019; Perrotta et al., 2020; Poels et al., 2020). Although plaque size was unchanged, total plaque burden over the aorta length was reduced in 3PO-treated ApoE\(^{-}/-\) and ApoE\(^{-}/-\) Fbn1\(^{1039G\rightarrow E}\) mice (Perrotta et al., 2020). This decreased plaque occurrence was independent of changes in plaque composition, such as macrophage content, necrosis, fibrosis or angiogenesis.

In contrast, other studies did report effects of 3PO treatment on plaque composition. Plaque stability was increased, as indicated by decreased necrotic core area and a thicker fibrous cap, in Ldrl\(^{-}/-\) mice treated with 3PO-derivative PFK158 (Poels et al., 2020). While Perrotta et al. (2020), hypothesized that decreased plaque burden after 3PO treatment was linked to decreased expression of EC adhesion molecules during early lesion development, no changes in EC adhesion molecules were observed in PFK158-treated Ldrl\(^{-}/-\) mice (Poels et al., 2020). It was suggested that glycolysis inhibition
in macrophages and monocytes could be responsible for the observed plaque stabilization.

On the contrary, here, we show that partially decreased PFKFB3-mediated glycolysis in monocytes, macrophages and granulocytes does not affect atherogenesis. Possibly, opposing effects of Pfkfb3 knockdown within myeloid cells and subsets, result in an absence of net effect. However, we did not observe changes in neutrophil and macrophage numbers. Thus, positive effects reported after systemic 3PO treatment are likely conferred by other myeloid or stromal cell types, that are affected by inhibition of PFKFB3-mediated glycolysis and are important in atherogenesis, such as DCs, SMCs and fibroblasts. Indeed, we show that our model does not induce Pfkfb3 knockdown in DCs. However, Pfkfb3 expression is high in atherosclerotic DCs, and DCs play a fundamental role in atherogenesis by contributing to activation of adaptive immunity, foam cell formation and pro-inflammatory cytokine secretion (Zhao et al., 2021). Next to DCs and other myeloid cells, through analysis of scRNA-seq datasets, we showed that fibroblasts and SMCs, but also ECs and lymphocytes express PFKFB3/Pfkfb3 in human and murine atherosclerosis. Importantly, increased αSMA+ cells, i.e., SMCs and fibroblasts, were observed upon PFK158-treatment in Ldlr−/− and upon 3PO-treatment in ApoE−/− mice (Beldman et al., 2019; Poels et al., 2020). Additionally, EC activation and dysfunction are at the center of atherogenesis, while ECs also highly depend on glycolysis (De Bock et al., 2013; Eelen et al., 2015). Both specific PFKFB3/Pfkfb3 knockdown in ECs and 3PO treatment reduced EC sprouting in vivo and in vitro, by affecting EC migration and proliferation (De Bock et al., 2013; Schoors et al., 2014). Moreover, 3PO decreased EC activation...
and increased endothelial barrier stability in vitro. However, while increasing plaque stability, 3PO treatment in ApoE−/− mice did not affect plaque endothelial barrier function (Beldman et al., 2019). Except for myeloid-specific Pfkfb3 knockdown in the current study, effects of other cell-specific Pfkfb3 knockdowns in atherosclerosis have not been studied yet. This could shine additional light on cell-specific effects of disrupted PFKFB3-mediated glycolysis on atherogenesis.

Another factor that might explain the lack of effect on atherosclerosis compared to studies utilizing 3PO treatment,
is recent evidence that 3PO inhibits glycolysis through intracellular acidification, rather than specific PFKFB3 inhibition (Burmistrova et al., 2019; Emini Veseli et al., 2020). Thus, one should take possible unintended off-target effects of intracellular acidification into consideration when using 3PO-derivatives. Small molecule AZ67 does bind to PFKFB3 specifically (Boyd et al., 2015; Emini Veseli et al., 2020) and might be an interesting pharmacological inhibitor for future in vivo atherosclerosis studies, while keeping in mind that effects are likely not mediated by monocytes, macrophages or neutrophils.

In addition to greater relevance of PFKFB3-mediated glycolysis in other cell types in atherosclerosis, or off-target effects of reported inhibitors, other factors may explain the observed lack of effect of myeloid Pfkfb3 inhibition on atherosclerosis. Firstly, Pfkfb3 knockdown in Pfkfb3<sup>fl/fl</sup> BMDMs is only partial (≈50–60%). The LysMCre-loxP system often results in >70% deletion efficiency in myeloid cells (Clausen et al., 1999). Efficiency of the Cre-lox system in our model could be complicated by Pfkfb3 gene locus (Murray and Wynn, 2011). Moreover, it should be noted that although we report expression of Pfkfb3 in atherosclerotic myeloid cells, the percentage of monocytes, neutrophils and macrophages that express Pfkfb3 is low (≈10–20%, Supplementary Figure 4A). Furthermore, as PFKFB3 is merely one of several stimulators of glycolytic flux (Mor et al., 2011), Pfkfb3 inhibition reduces glycolysis only partially, in line with previous studies that targeted PFKFB3-mediated glycolysis (De Bock et al., 2013; Schoors et al., 2014; Yetkin-Arik et al., 2019). Nevertheless, glycolysis inhibition by 3PO treatment in vivo is also partial (Schoors et al., 2014; Poels et al., 2020), and a similar, partial approach was very successful to change EC function in vivo (De Bock et al., 2013; Beldman et al., 2019).

Indeed, we focus only on PFKFB3-mediated glycolysis in the current study. Atherosclerotic plaques are associated with increased glycolytic activity (Ali et al., 2018). As glycolysis is controlled at different levels, other glycolytic regulators than PFKFB3 might be involved in this association, such as hexokinase...
Pfkfb1 that the future (Berg et al., 2002; Ali et al., 2018).

Another factor that could explain the lack of effect is the possible role of other PFKFB isoenzymes. Although we showed that Pfkfb1, Pfkfb2, and Pfkfb4 expression was unaffected in Pfkfb2lox/lox BMMDs, PFKFB isoenzyme activity could still be increased, independent of expression (Macut et al., 2019).

Finally, differences in experimental setup, gender, HCD length and composition, and vascular sites assessed may cause differences in observed effects between the current and previous studies (Supplementary Table 2; VanderLaan et al., 2004; Getz and Reardon, 2006; Man et al., 2020). Moreover, glycolysis inhibition using chronic gene silencing by LysMCre from embryonic stage versus acute pharmacological protein inhibition or siRNA silencing in adult mice may result in different functional outcomes and may also explain a lack of effect in the current study (Knight and Shokat, 2007). As mentioned, the selectivity of pharmacological agents is often not entirely clear.

In conclusion, we showed that partial myeloid knockdown of PFKFB3 does not affect atherosclerosis development. Positive effects of systemic, partial glycolysis inhibition on lesion stabilization or total plaque burden that were previously reported, might be conferred by other Pfkfb3-expressing cells such as DCs, fibroblasts, SMCs and lymphocytes. Possibly, more severe reduction of myeloid glycolysis may be needed.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

**ETHICS STATEMENT**

The animal study was reviewed and approved by the regulatory authority of the Maastricht University Medical Centre.

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**AUTHOR CONTRIBUTIONS**

JS, JDB, LT, and RT devised and planned the experiments. JDB, KVK, and RT carried out the experiments. JDB, KVK, MG, and RT performed the data analysis. JP-P, JS-R, YG, and KL were responsible for single-cell sequencing data analysis. PC kindly provided Pfkfb3lox/lox mice for the experiments and provided critical input to the manuscript. RT and JS wrote the manuscript. All authors reviewed and approved the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.695684/full#supplementary-material
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