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Bone marrow adipocytes drive the development of tissue invasive Ly6C^{high} monocytes during obesity

Running Title: Bone marrow fat linked to Ly6C^{high} monocytes in obesity

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Summary (150 Words, 150):

During obesity and high fat-diet (HFD) feeding in mice, sustained low-grade inflammation includes not only increased pro-inflammatory macrophages in the expanding adipose tissue, but also bone marrow (BM) production of invasive Ly6C^{high} monocytes. As BM adiposity also accrues with HFD, we explored the relationship between the gains in BM white adipocytes and invasive Ly6C^{high} monocytes *in vivo* and through *ex vivo* paradigms. We find a temporal and causal link between BM adipocyte whitening and the Ly6C^{high} monocyte surge, preceding the adipose tissue macrophage rise during HFD. Phenocopying this, *ex vivo* treatment of BM cells with conditioned media from BM adipocytes or from *bona fide* white adipocytes favoured Ly6C^{high} monocyte preponderance. Notably, Ly6C^{high} skewing was preceded by monocyte metabolic reprogramming towards glycolysis, reduced oxidative potential and increased mitochondrial fission. In sum, short-term HFD changes BM cellularity, resulting in local adipocyte whitening driving a gradual increase and activation of invasive Ly6C^{high} monocytes.

Keywords:

Obesity; Immunometabolism, Bone marrow monocytes, Bone marrow adipocytes, Tissue invasive monocytes, Ly6C^{high} monocytes, Glycolytic shift.

Introduction:

A continuum of chronic obesity leading to metabolic disorders such as insulin resistance and eventually type 2 diabetes is of paramount concern worldwide (Centers for Disease Control and Prevention, 2017; Hamann, 2017; WHO, 2016). An important contributor to the development and exacerbation of insulin resistance is a gain in macrophages in adipose tissue (AT) (Liu et al., 2019; Weisberg et al., 2003), muscle (Fink et al., 2014; Garvey et al., 2008) and liver (Lanthier et al., 2010) as well as their enhanced pro-inflammatory (Lumeng et al., 2007, 2008; De Souza et al., 2005) and glycolytic skewing (Sharma et al., 2020). This low-grade inflammation is partly explained by circulating monocyte infiltration into tissues where they differentiate to pro-inflammatory macrophages (Kohyama et al., 2016).

Ly6C^{high} monocytes in mice, and classical CD14⁺⁺ CD16⁻ monocytes in humans, constitute the predominant tissue-infiltrating monocyte subset during chronic inflammation. These tissue-invasive monocytes are classified by high surface abundance of the Ly6C protein, the chemokine receptor CCR2 and the adhesion molecule L-selectin, all of which allow for monocyte extravasation from the BM and infiltration into the tissues (Auffray et al., 2007; Boniakowski et al., 2018; Fink et al., 2014; Geissmann et al., 2003; Weisberg et al., 2006). With obesity, the development of Ly6C^{high} monocytes along with their precursor cells in the BM is boosted (Griffin et al., 2018; Manicone et al., 2016; Singer et al., 2014) leading to a rise in available circulating Ly6C^{high} monocytes for tissue-infiltration (Friedrich et al., 2019; Singer et al., 2014).

Various obesity-driven factors amalgamate for greater Ly6C^{high} monocyte infiltration into the AT including (a) increased AT-dependent chemoattraction (Kamei et al., 2006; Nagareddy et al., 2014; Nov et al., 2013; Renovato-Martins et al., 2017) and (b) enhanced BM myelopoiesis (Griffin et al., 2018; Manicone et al., 2016; Singer et al., 2014). However, it is not clear whether the obesity-induced BM myelopoiesis occurs prior to, concurrently, or after the elevation of AT macrophages. Since the BM is a highly-vascularized tissue, home to hematopoietic and mesenchymal lineages, and is dynamically reactive to environmental changes, we reasoned it might be an early responder to HFD-induced obesity, producing increased Ly6C^{high} monocytes prior to their accumulation in the AT. Further, since BM-resident adipocytes

undergo hyperplasia and hypertrophy during obesity (Boroumand and Klip, 2020; Doucette et al., 2015; Scheller et al., 2015; Tratwal et al., 2020) we contemplated that this process might be temporally and potentially causally related to the HFD-driven BM myelopoiesis and Ly6C^{high} monocytosis. Finally, we considered whether the response of the monocytic lineage to the BM environment during obesity includes a metabolic shift leading to the increase in Ly6C^{high} monocytes. In fact, and surprisingly, little is known about the metabolic orientation of the Ly6C^{high} monocytes in states of obesity, although there is mounting evidence of a link between a macrophage-intrinsic metabolic shift and their pro-inflammatory orientation in this condition (O'Neill et al., 2016; Sharma et al., 2020; Thapa and Lee, 2019; Verdeguer and Aouadi, 2017).

Hence, the overall hypothesis driving this study is that, with obesity, the increased production of Ly6C^{high} monocytes in the BM is accompanied and possibly caused by a glycolytic shift in these cells, driven by changes in bone marrow adipocytes (BMA). Accordingly, we investigated the temporality of enhanced BM myelopoiesis, BM Ly6C^{high} monocyte accumulation, and AT macrophage changes during high fat diet (HFD) feeding in mice. Next, we explored the potential causal relationship between the obesity-induced changes in BMA and increased Ly6C^{high} monocyte production. Using an *in vitro* platform, we deconstructed the effects of obesity-induced BM cellular changes on Ly6C^{high} monocyte expansion. We report that BM Ly6C^{high} monocytes accumulate in the BM prior to the gain in pro-inflammatory AT macrophages. The rise in BM monocytes temporally correlated with the elevation in BMA. Moreover, BMA whitening correlated with the timely increases in BM Ly6C^{high} monocytes, and most notably, isolated BMA from HFD-fed mice directly favoured Ly6C^{high} monocyte development *ex vivo*. This monocyte developmental change was also induced *ex vivo* by *bona fide* white adipocytes while *bona fide* brown adipocytes favoured Ly6C^{low} monocyte predominance. Notably, the conversion of Ly6C^{low} to Ly6C^{high} monocytes was preceded by a switch in their metabolism, from oxidative to glycolytic. Mechanistically, white adipocytes induced conversion of Ly6C^{low} to Ly6C^{high} monocytes, whereas brown adipocytes increased Ly6C^{low} monocyte proliferation. We propose that HFD-induced changes in BMA disrupts the normal balance of BM monocyte subsets leading to a higher output of tissue invasive Ly6C^{high} monocytes.

Results

Increased fasting blood glucose, peripheral and BM adiposity by 3 weeks of HFD

To study the temporal relationship of HFD-induced myeloid changes in the BM relative to that in epididymal white AT (EWAT), C57BL/6 mice were fed HFD or control low fat diet (LFD) for either 3, 8 or 18 weeks. HFD-fed mice had higher body weight and impaired fasting blood glucose by 3 weeks, compared to controls (**Figure 1A,B**). Body weight and hyperglycemia continued to rise with prolonged feeding. HFD-fed mice also displayed higher EWAT weight (**Figure 1C**) and inter-scapular brown AT (BAT) weight at 3 weeks (not shown). Cross-sectional tibia and femur bone histology visualized via H & E and Oil Red O staining revealed an increase in BMA size by 3 weeks and an increase in BMA number by 8 weeks of HFD (**Figure 1D-G**). This indicated that 3 weeks of HFD sufficed to induce whole body metabolic disturbance and augmented adiposity in peripheral tissues and in the BM.

BM monocytes increase prior to accumulation of EWAT macrophages and monocytes with HFD

Although total EWAT weight increased by 3 weeks, myeloid cells in the stromal vascular fraction (SVF) of AT did not increase until 8 weeks of HFD, as revealed by gene expression of the general myeloid marker *Itgam* (encoding CD11b) and monocyte markers Gr1⁻ CD115⁺ CD11b⁺ Ly6C⁺ (determined by flow cytometry) (**Figures 2A,D**). Similarly, AT macrophages, assessed by gene expression of the AT macrophage marker *Adgre1* (encoding F4/80) and by flow cytometry counting of CD11b⁺ Ly6C⁻ F4/80⁺ macrophages in the SVF were not elevated at 3 weeks of HFD but were at 8 weeks (**Figure 2B,E**). Further, gene expression of the pro-inflammatory myeloid marker *Itgax* (encoding CD11c) and surface detection of the pro-inflammatory markers CD11b⁺ Ly6C⁻ F4/80⁺ CD11c⁺ were also higher in the SVF by 8 weeks of HFD relative to control diet, but not at 3 weeks (**Figure 2C,F**). Of note, gene expression of the embryonically-conserved tissue-resident AT macrophage markers *Timd4* and *Marco* (Beattie et al., 2016; Gibbings et al., 2015) was not elevated at 3 or at 8 weeks and only rose at 18 weeks of HFD (**Figure 2G-H**). This indicates that the gain in AT macrophages at 8 weeks of HFD is unlikely due to proliferation of resident macrophages and instead likely results from monocyte recruitment from the circulation.

In the BM, total monocytes, gated via the Gr1⁻ CD115⁺ CD11b⁺ Ly6C⁺ markers, increased at 3 weeks and continued to rise by 8 and 18 weeks of HFD (**Figure 2I**). Using the surface abundance of the Ly6C protein as well as the CX3CR1 and CCR2 markers (Auffray et al., 2007, 2009; Kratochil et al., 2017), we subcategorized Ly6C^{low} and Ly6C^{high} monocyte subsets. At 3 weeks into the feeding study, Ly6C^{low} and Ly6C^{high} monocytes represented about 40% of the total BM monocyte population in the control diet group, whereas HFD promoted a higher proportion of Ly6C^{low} monocytes (**Figure 2J**). However, by 8 weeks of HFD there was a marked switch, whereby the relative proportion of Ly6C^{low} monocytes in the total monocyte population was *lower* and that of Ly6C^{high} monocytes was *higher* than under the control diet (**Figure 2J**). These differences were more prominent by 18 weeks of HFD feeding (**Figure 2J**). Collectively, these findings revealed that, in the BM, HFD favoured monocytosis at 3 weeks, with a subsequent and prominent skewing towards tissue invasive Ly6C^{high} monocytes at 8 weeks that continued into 18 weeks of HFD. Singer et al. also demonstrated an increase in BM monocytosis and elevated monocyte precursor cells with 18 weeks of HFD, that persisted after BM transplantation into chow-fed recipients (Singer et al., 2014). In the present study, while we clearly recorded an earlier and continuing rise in total monocytes in HFD-fed mice (**Figure 2I**), we did not observe statistically significant increases in monocyte precursor and stem cells at any time point analyzed (**Figure S1A-C**).

Glycolytic metabolic reprogramming in monocyte subsets with HFD

Recent discoveries have highlighted the importance of glycolytic metabolism in the activation of AT macrophages with HFD (Lee and Murphy, 2019), but the metabolic status of monocytes in obesity has not been reported, although some studies have investigated the metabolism of circulating human CD14⁺CD16⁻ monocytes (homologous to murine tissue invasive Ly6C^{high} monocytes) during aging (Lee and Murphy, 2019; Pence and Yarbrough, 2019). To assess whether HFD affects the metabolic status of BM monocyte subsets in obesity, we analyzed Ly6C^{low} and Ly6C^{high} from control and HFD mice at 3, 8 and 18 weeks of feeding. The mitochondrial activity-indicator dye MitoTracker Red CMXRos was incorporated into the monocyte flow cytometry panel (Table 1). MitoTracker Red CMXRos is recruited to the

mitochondrial membrane proportionally to the degree of mitochondrial membrane potential and is thus an indicator of mitochondrial activity in oxidative phosphorylation. The results revealed that Ly6C^{low} monocytes have a higher mitochondrial membrane potential than Ly6C^{high} monocytes in the control mice (**Figure 3A-C**). Notably, 3 weeks of HFD reduced the mitochondrial membrane potential in both monocyte subsets indicated by the left-ward shift in the MitoTracker Red CMXRos peak (**Figure 3A**). The HFD-induced effects were more pronounced by 8 and 18 weeks of HFD (**Figure 3B,C**). To further understand the effects of HFD on the developing BM monocytes, Ly6C^{low} and Ly6C^{high} monocytes from mice fed control or HFD for 3, 8 or 18 weeks were isolated via fluorescent activated cell sorting (FACS) and plated for metabolic analysis with a Seahorse XF Analyzer. The results revealed a gradual lowering in the oxygen consumption rate (OCR) of Ly6C^{low} monocytes with HFD (**Figure 3D-I**). Comparison of the area under the curve (AUC) revealed that at 18 weeks, the OCR of the Ly6C^{low} monocytes was significantly lowered with HFD (**Figure 3I**). Interestingly, at 8 and 18 weeks, the AUC of the OCR of the Ly6C^{low} monocytes was significantly higher than the AUC of the OCR of Ly6C^{high} monocytes in the control group (**Figure 3H-I**). Moreover, this difference was lost in the HFD group as the AUC of the OCR of Ly6C^{low} monocytes diminished (**Figure 3H-I**).

Comparison of the various sections of the OCR graph revealed that the changes in OCR observed with 3 weeks of HFD were not manifest at basal respiration nor at the ATP-dependent respiration, rather they were evident in the maximal respiratory capacity portion of the metabolic stress test (**Figure 3D**). A drop in basal respiration of both monocyte subsets emerged with 8 weeks of HFD and became more pronounced with 18 weeks of HFD (**Figure 3E-F**). This showcased the sustained effects of HFD on monocyte metabolic status. Conversely, the extracellular acidification rate (ECAR) of Ly6C^{low} monocytes was higher than that of Ly6C^{high} monocytes, consistent with a more active metabolism in the former (**Figure 3J**). However, this difference was lost with HFD, as ECAR dropped in the Ly6C^{low} monocytes and rose in the Ly6C^{high} monocytes relative to the control diet group (**Figure 3J**). Overall, these results suggest that, along with promoting the preponderance of Ly6C^{high} monocytes, HFD also lowers OCR in Ly6C^{low} monocytes.

Notably, an analysis of the BM revealed elevated lactate levels across all HFD time points (**Figure 3K**). This increase in lactate may be due to the glycolytic shift in BM monocytes described above, among other potential sources within the BM cellular composition. Further analysis of the BM soluble fraction failed to evince any changes between control and 18-week HFD-fed mice in pro-inflammatory cytokines $TNF\alpha$, $IL-1\beta$, $IL-6$, $CXCL1$ or $CCL2$ (**Figure S2A-C**). Similarly, there were no differences between diet groups in pro-inflammatory gene expression of *IL-6*, *IL-1 β* or *Itgax* in FACS-sorted $Ly6C^{low}$ and $Ly6C^{high}$ monocytes (Not Shown). However, *TNF α* gene expression was higher in FACS-sorted $Ly6C^{high}$ monocytes from 18-week HFD-fed mice compared to controls (**Figure S2D**). Collectively, these findings reveal that HFD alters the BM metabolic micro-environment with little effect on pro-inflammatory cytokine secretion (based on the panel tested).

Expression of mitochondrial fusion and fission genes are altered monocytes with HFD

The efficiency of mitochondrial function is largely reliant on its structure. Mitochondria are highly dynamic in structure, largely regulated by concentration of proteins that control fission and fusion (Gottlieb and Bernstein, 2017). Dynamic transitions are closely aligned to metabolic performance. To further assess the mitochondrial activity of monocytes, we enquired whether their expression of mitochondrial dynamic genes is altered with HFD compared to the control group.

The analyzed genes involved in mitochondrial fusion were *Mfn1*, *Mfn2*, *Opa1*, *Tomm20* and *Tomm40*, while those involved in mitochondrial fission were *Drp1*, *CypD* and *Fis1*. BM monocytes from mice fed HFD for 3 weeks did not present with differences in gene expression in the tested mitochondrial fusion genes compared to control diet counterparts (**Figure 4A**). BM monocytes isolated from mice fed HFD for 8 weeks showed downregulated *Mfn1* and *Mfn2* compared to the controls (**Figure 4B**). BM monocytes isolated from 18 week HFD-fed mice downregulated the mitochondrial fusion genes *Mfn2*, *Opa1*, and *Tomm20* (**Figure 4C**). Furthermore, from the tested mitochondrial fission genes, *Drp1* was upregulated in the BM monocytes of 3 weeks HFD-fed mice (**Figure 4D**). *Drp1* and *CypD* were also upregulated in BM monocytes of 8 weeks HFD-fed mice (**Figure 4E**). At 18 weeks, all three tested

mitochondrial fission genes, *Drp1*, *CypD* and *Fis1* were upregulated (**Figure 4F**). This gene expression pattern suggests that HFD skews the monocyte gene expression to favour downregulation of genes involved in mitochondrial fusion and upregulation of genes involved in fission.

Given that Ly6C^{low} and Ly6C^{high} monocytes presented with differences in metabolic preference (glycolytic and oxidative, respectively), and the known link of glycolytic metabolism with mitochondria fragmentation and conversely oxidative metabolism to mitochondrial fusion, we assessed whether the expression patterns of the genes governing mitochondrial fission and fusion would correlated distinctly with the BM monocyte subset abundance in control and HFD-fed mice. Indeed, and notably, the abundance of the Ly6C^{high} monocyte subset negatively correlated with *Mfn2* and positively correlated with *Drp1* levels (**Figure 4G-H**). In contrast, the abundance of BM Ly6C^{low} monocytes did not correlate with the expression patterns of either *Mfn2* or *Drp1* (**Figure 4G-H**). These data are consistent with our metabolic findings suggesting Ly6C^{high} monocytes are more glycolytic and adopt a gene expression pattern that would promote mitochondrial fragmentation.

A shift in BMA type at 8 weeks of HFD influences monocyte subset preference and mitochondrial dynamic gene expression

Hematopoietic myeloid cells reside in specific niches of the BM that determine their cellular interaction with other co-resident BM cells (Boroumand and Klip, 2020). BMA, while representing a unique type of adipocytes, respond to obesity with an increase their white adipocyte-like fat storing properties and a decrease in brown-like characteristics (Ambrosi et al., 2017; Doucette et al., 2015; Krings et al., 2012; Scheller et al., 2016). To explore the temporal relation of BMA whitening in relation to the development of monocyte subsets, we followed the gene expression of a suite of recently identified markers that are good differentiators of adipocyte types (Nascimento et al., 2014; Ussar et al., 2014) in isolated BMA from femur and tibia of control and HFD-fed mice. By 3 weeks into HFD, the white adipocyte marker *Asc1* (encoding the guanine nucleotide-binding protein subunit beta-like protein) was significantly higher and the brown

adipocyte marker *P2rx5* (encoding purinergic receptor P2X) trended to be lower albeit without reaching statistical significance, relative to control diet. White adipocyte markers *Lep* (leptin) and *AdipoQ* (adiponectin) as well as the brown adipocyte markers *Ucp1* (UCP1) and *Pat2* (Integrin alpha pat-2) did not change (**Figure 5A**). By 8 weeks of HFD, *Asc1* mRNA remained elevated and *Lep* and *AdipoQ* trended in the same direction (**Figure 5B**). Brown adipocyte markers *P2rx5* and *Ucp1* in the isolated BMA of HFD mice were markedly lower relative to those from control diet mice (**Figure 5B**). Thus, 8 weeks of HFD sufficed to shift BMA towards white adiposity. By 18 weeks of HFD, all white adipocyte markers genes showed were upregulated while brown adipocyte markers were downregulated compared to the control group (**Figure 5C**). Analyses of adipocyte differentiation and progenitor cell markers were conducted in the BM pelleted fraction. *Hoxc8*, a regulator of adipocyte progenitors (Nakagami, 2013), was significantly upregulated at 8 weeks along with *Hoxc9* at 18 weeks of HFD (**Figure S3B,C**). The beige adipocyte progenitor marker *Tmem26* was not significantly higher at any of the tested HFD durations (**Figure S3A-C**). Expression of the brown adipocyte determinant genes *Tbx1* and *Lhx8* was undetectable in the isolated BMA. Thus, during the course of HFD, while markers for white adipocyte progenitor cells were upregulated, precursors of beige/brown adipocytes were not affected.

The rise in BM Ly6C^{high} monocytes and the shift in BMA whitening both manifested by 8 weeks of HFD. Correlative analysis was performed for both Ly6C^{low} and Ly6C^{high} with the white adipocyte marker *Asc1* and the brown adipocyte marker *Ucp1*. The results revealed a significant positive correlation (P value of 0.0123) for Ly6C^{high} monocytes with *Asc1* gene expression (**Figure 5D**). Conversely, the surge in Ly6C^{high} monocytes presented a significant negative correlation (P value of 0.0415) relative to *Ucp1* gene expression (**Figure 5E**). Changes in Ly6C^{low} monocytes did not significantly correlate with *Asc1* (P value of 0.0768) or *Ucp1* (P value of 0.1299) (**Figure 5D-E**). Collectively, the above data suggest the possibility that the mere shift in BMA towards white adiposity may directly favour induction of BM Ly6C^{high} monocytes. To test this hypothesis, we generated conditioned media (CM) from BMA (BMA-CM) pooled from three control and three HFD-fed mice, collected 3, 8 and 18 weeks into the diets. In parallel, BM of control mice was used to generate monocytes through a 5-day *in vitro* differentiation protocol. These

monocytes were treated with BMA-CM from control and HFD-fed mice for 18 hours thereafter. Subsequently, the monocyte population was analyzed via flow cytometry. BMA-CM from 3 weeks HFD-fed mice had no effect on the monocyte subset proportions compared to BMA-CM from control diet-fed mice (**Figure 5F**). Strikingly, however, BMA-CM from 8-week HFD-fed mice applied to BM-derived monocytes from control mice resulted a significant production of Ly6C^{high} monocytes compared to the effect of BMA-CM from mice on the 8-week control diet (**Figure 5G**). This skewing was further enhanced when exposing the control BM cells to BMA-CM from 18-week HFD-fed mice (**Figure 5H**).

Lastly, we tested whether BMA-CM was capable to induce changes in gene expression of the mitochondrial fusion and fission genes in monocytes, as observed with monocytes isolated from HFD-fed mice. Accordingly, *in vitro-generated* monocytes were treated with BMA-CM from control and HFD-fed mice. As before, the analyzed genes involved in mitochondrial fusion were *Mfn1*, *Mfn2*, *Opal*, *Tomm20* and *Tomm40*, while the tested genes involved in mitochondrial fission were *Drp1*, *CypD* and *Fis1*. Monocytes treated with BMA-CM from HFD-fed mice for 18 weeks showed significant downregulated mitochondrial fusion genes *Mfn2*, *Tomm20* and *Tomm40* (**Figure 5I**). Conversely, the mitochondrial fission genes *Drp1* and *CypD* were significantly up-regulated in monocytes treated with HFD-derived BMA-CM (**Figure 5J**).

These results not only show that HFD-derived BMA-CM can skew monocyte subset cellularity but also can alter mitochondrial morphology via changes in mitochondrial dynamic gene expression. These changes can have consequential effects on the metabolic properties on the monocytes.

White adipocytes favour Ly6C^{high} monocytes and promote mitochondrial fragmentation

To assess whether the effects of BMA on monocyte subset skewing and metabolic differences can be attributed to BMA whitening, we designed a proof of concept experiment studying the influence of *bona fide* white or brown adipocytes on monocyte subset determination and metabolic changes *ex vivo*. Isolated adipocytes from EWAT and brown adipocyte tissue (BAT) of three mice were pooled to generate white AT adipocytes (WATA) and brown AT adipocytes (BATA), respectively. Confirming the phenotype of the

isolated cells, classical white adipocytes markers were solely expressed in the isolated WATA and classical brown adipocyte markers were exclusively expressed in the BATA (**Figure S3D-E**). The isolated WATA and BATA were cultured for 24 hours and the corresponding CM were collected for subsequent monocyte treatment. BM-derived monocytes from chow-fed mice were treated with WATA-CM, BATA-CM or left untreated. The proportion of Ly6C^{low} and Ly6C^{high} monocyte subsets was determined by flow cytometry analysis (**Figure S4A-C**). This *in vitro* paradigm allowed for direct mechanistic analysis of the influence of white or brown adipocytes on monocytes. These results indicate that WATA-CM favoured Ly6C^{high} monocyte predominance, while BATA-CM propitiated Ly6C^{low} monocyte abundance (**Figure 6A-B**), revealing a novel, differential influence of white and brown adipocytes on monocyte orientation preference. Moreover, CCR2 surface receptors also increased in the monocytes incubated with WATA-CM (Not Shown), validating the effects of white adipocytes in promoting an abundance of tissue invasive Ly6C^{high} monocytes.

Since segregated Ly6C^{low} and Ly6C^{high} monocyte subsets had different metabolic orientations, we investigated if the differential adipocyte influence on monocyte subset development corresponded to a change in their metabolic status. To this end, *in vitro*-generated monocytes were treated with WATA-CM, BATA-CM or left untreated for the last 18 hours of the monocyte differentiation protocol. The mitochondrial membrane potential was assessed using MitoTracker Red CMXRos and flow cytometry analysis. Remarkably, Ly6C^{low} monocytes incubated *in vitro* with WATA-CM experienced a left-ward shift in MitoTracker Red CMXRos peak revealing a reduction in mitochondrial membrane potential induced by the WATA-CM (**Figure 6C**), reproducing what was observed with monocytes from HFD-fed mice (see **Figure 3A-C**). Furthermore, the gene expression of mitochondrial fusion genes *Mfn1*, *Mfn2*, *Tomm20* and *Tomm40* were reduced in monocytes treated with WATA-CM (**Figure 6D**). The mitochondrial fission genes *Drp1*, *CypD* and *Fis1* were up-regulated in monocytes treated with WATA-CM (**Figure 6E**). Conversely, treating monocytes with BATA-CM downregulated the mitochondrial fission gene *CypD* (**Figure 6E**). To explore if the changes in mitochondrial fusion and fission gene expression correlated with the corresponding mitochondrial morphology, we utilized super-resolution microscopy to image the

mitochondrial protein TOM20 in *in vitro* monocytes adhered to coverslips. Compared to control-medium treated monocytes, WATA-CM induced a striking mitochondrial fragmentation while BATA-CM promoted a more complete mitochondrial elongation (**Figure 6F**). These observations suggest that white adipocytes, which favour Ly6C^{high} monocyte abundance, can alter the metabolic status of Ly6C^{low} monocytes by reducing their mitochondrial membrane potential and altering mitochondrial dynamic gene expression and morphology, thereby linking changes in BMA whitening to monocyte metabolic status.

White and brown adipocytes influence monocytes through distinct mechanisms

Finally, we investigated the mechanisms whereby monocytes undergo the change in subsets in response to *bona fide* white and brown adipocyte media. Although we had not detected HFD-induced changes in hematopoietic stem and monocyte precursor cells *in vivo*, we re-assessed the abundance of these populations upon exposing *in vitro*-generated monocytes to WATA-CM and BATA-CM. The abundance of hematopoietic stem and monocyte precursor cells was determined using appropriate surface markers and flow cytometry analysis (**Table 1, Figure S4D**). A gain in myeloid precursor cell (CMP) was observed in response to WATA-CM treatment (**Figure S5A-C**). However, no differences in the abundance of CD117+, Sca1+, Sca1-, GMP and MEP cells were detected (**Figure S5A-C**). Given these results we investigated other mechanisms by which monocyte subsets may be altered in response to WATA-CM and BATA-CM.

We first explored whether the proportion of monocyte subsets was altered through conversion of one subset to another. Ly6C^{high} monocytes have been shown to give rise to Ly6C^{low} monocytes in mouse blood and BM, and this is paralleled by the human homologous CD14 and CD16 monocyte subsets (Kratofil et al., 2017; Lessard et al., 2017; Patel et al., 2017). However, while there has been speculation about the conversion of Ly6C^{low} to Ly6C^{high} monocytes, there is little experimental evidence for this phenomenon. To explore this possibility, we first separately isolated Ly6C^{low} and Ly6C^{high} monocytes through FACS. Each FACS-sorted homogenous monocyte population was treated with WATA-CM, BATA-CM or left untreated for 18 hours. Following treatments, we evaluated the presence of the alternative monocyte subtype relative to that in the respective starting homogenous populations. Surprisingly, no conversion from

Ly6C^{high} to Ly6C^{low} monocytes was observed in any condition (**Figure 7A-F**), but, remarkably, there was conversion of the FACS sorted Ly6C^{low} into Ly6C^{high} monocytes following exposure to WATA-CM (**Figure 7B,E**). While the FACS-sorted starting cells were all Ly6C^{low} monocytes, following the 18-hour WATA-CM treatment the monocyte population consisted of 49.7% Ly6C^{high} monocytes (only Ly6C^{low} monocytes 50.3%) (**Figure 7E**). This was in stark contrast to all other isolated cell and CM treatment combinations tested, which gave rise to a maximum of 16.3% of the opposite monocyte subset compared to the starting population. These results revealed the interesting phenomenon of Ly6C^{low} into Ly6C^{high} monocyte conversion induced by white adipocytes.

Monocyte precursor cells in the BM and their derivative macrophages in the tissues maintain their cell numbers by cell proliferation (Cassado et al., 2015). Thus, we asked whether proliferation might contribute to the higher proportion of terminally differentiated Ly6C^{high} or Ly6C^{low} monocyte subsets observed following treatment with WATA-CM (in addition to the conversion of Ly6C^{low} into Ly6C^{high}) or BATA-CM, respectively. To this end, we treated BM-generated monocytes with WATA-CM, BATA-CM or left them untreated for 18 hours, with or without the presence of bromodeoxyuridine (BrdU). Using flow cytometry analysis, DNA-incorporated BrdU was detected in the Ly6C^{low} or Ly6C^{high} monocyte gates of the CD11b versus Ly6C plots, with BrdU as the third colorimetric axis. BrdU incorporation is illustrated by a scale of yellow to red hues compared to the corresponding cells not exposed to BrdU. Proliferating cells were quantified as percentage of CD11b⁺ Ly6C⁺ cells that were highly positive for BrdU incorporation. Interestingly, BATA-CM increased DNA-incorporated BrdU in the sorted Ly6C^{low} monocyte subset, present as yellow to red hues in the CD11b⁺ Ly6C^{low} cell gate (**Figure 7G**). This treatment with BATA-CM increased the number of proliferating cells by 25-fold (**Figure 7H**). In contrast, BrdU incorporation in the sorted Ly6C^{high} monocytes did not change with any treatment conditions. These findings revealed that brown adipocytes promote an abundance of Ly6C^{low} monocytes by inducing their proliferation. Moreover, this replicative behavior is consistent with recent findings that proliferative cells have higher glucose consumption, lactate excretion and oxidative phosphorylation relative to quiescent cells (Yao et al., 2019).

Discussion

In this study, we demonstrate a temporal correlation between HFD-induced whitening of BMA and a BM myeloid lineage bias towards Ly6C^{high} monocytes *in vivo*. The latter present a more glycolytic metabolism underpinned by a reduction in mitochondrial membrane potential, a rise in mitochondrial fission genes and a reduction in mitochondrial fusion genes. Causatively, we show that CM from BMA of HFD-fed mice promotes the generation of Ly6C^{high} monocytes *ex vivo*. Phenocopying the effect of BMA derived from HFD-fed mice, *bona fide* WATA favoured a Ly6C^{high} monocyte preponderance, while BATA promoted a dominance of Ly6C^{low} monocytes. Interestingly, these differential influences by the respective adipocytes are induced by distinct cellular pathways. Mechanistically, white adipocytes induced conversion of Ly6C^{low} to Ly6C^{high} monocytes, while brown adipocytes favoured Ly6C^{low} predominance by inducing their proliferation. These changes in monocytes were underpinned by alterations in the monocytes metabolism, as WATA blunted the monocyte's mitochondrial membrane potential and OCR. Overall, our findings reveal a regulation of monocyte subsets directly influenced by adipocytes, whether from the BM or from AT. Along with the observation of dynamic whitening of BMA during HFD, our results contribute to the emerging evidence that BMA are not mere space fillers, rather they are important contributors to cellular metabolism and hematopoietic lineage decisions (Boroumand and Klip, 2020). That these changes occur early on in the course of diet-induced obesity suggests that the BM is an early responder to a change in feeding status and that adipocytes play a significant role in directing monocyte subset skewing. Moreover, they reveal novel mechanisms regulating the genesis of such skewing.

BM as an early sensor and an active contributor to HFD-induced AT macrophage accumulation

It is now widely recognized that many factors contribute to the accumulation of macrophages in AT that includes the contribution of monocytes. There is mounting evidence for both HFD-induced EWAT monocyte chemo-attraction and for enhanced BM myelopoiesis, that work together to increase the monocyte infiltration and pro-inflammatory AT macrophage accumulation (Singer et al., 2014; Trottier et al., 2012 and Fig. 1D-H). Singer *et al.* demonstrated that transplantation of BM from 18-week HFD-fed

mice into control recipients increased the number of invasive circulating monocytes and pro-inflammatory AT macrophages (Singer et al., 2014). These findings support the notion that HFD influences hematopoietic myeloid skewing in the BM upstream of monocyte tissue invasion, thereby contributing to downstream systemic and peripheral tissue inflammation. Furthermore, HFD-driven chemoattracting signals from the homeostatically-disrupted EWAT initiate tissue influx and depletion of invasive Ly6C^{high} monocytes from the circulation, which in turn may acts as a signal for their increased production in the BM (Kamei et al., 2006; Nagareddy et al., 2014; Nov et al., 2013; Renovato-Martins et al., 2017).

In the present study, by analyzing chronological changes in EWAT and BM myeloid composition, we observed that total BM monocytes increase by 3 weeks of HFD, whereas the proportion of Ly6C^{high} monocyte predominance was evident only at 8 weeks coinciding with a gain in AT macrophages that were also pro-inflammatory skewed. We did not observe statistical significance in HFD-dependent changes in myeloid precursors, likely due to their scarcity since HSC make up only 0.01% of the entire BM nucleated cells (Challen et al., 2009). The rise in total monocytes observed at 3 weeks of HFD and prior to elevation of AT macrophages identifies the BM as an earlier-responding *locus* during HFD. Interestingly, the increase was only apparent in Ly6C^{low} patrolling monocytes and not the Ly6C^{high} tissue invasive monocyte subset that is known for tissue infiltration in chronic obesity. This might be explained by an initial non-specific upregulation of total myelopoiesis and an increased Ly6C^{high} monocyte BM extravasation. Therefore, it appears that only Ly6C^{low} monocytes are elevated in the BM at 3 weeks of HFD. By 8 weeks of HFD, we observed a shift in the monocyte subset proportion, favouring Ly6C^{high} monocytes. Thus, by 8 weeks, the BM has adopted the ‘typical’ HFD-induced hematopoietic phenotype, in so far as it already shows the increased Ly6C^{high} monocytes previously reported at the habitually studied 16-18 weeks of HFD. Importantly, we detected a gain in pro-inflammatory AT macrophages by 8 weeks of HFD that is concurrent with the predominance of invasive Ly6C^{high} monocytes in the BM. In contrast, the embryonic-derived tissue resident AT macrophage markers, *Timd4* and *Marco* (Beattie et al., 2016; Gibbings et al., 2015), were not elevated until 18 weeks of HFD. This brings to light the importance of the contribution of the BM-derived myeloid cell pool to early EWAT pathology and AT macrophage accumulation.

Changes in BMA influence monocyte subset skewing

BMA are a distinctive member of the BM cellular suite that is sensitive to HFD effects (Pham et al., 2020). In lean mice, BMA present characteristics that are similar to those of beige or brown adipocytes in peripheral AT (Cuminetti and Arranz, 2019). With HFD, BMA undergo hyperplasia and hypertrophy triggered by PPAR γ activation (Rosen and MacDougald, 2006). Furthermore, with obesity, adipocyte precursor mesenchymal stem cells tilt their lineage commitment towards adipogenesis (Silva et al., 2016). While BMA are now known to be a distinct adipocyte population, they express several characteristics of white and brown adipocytes (Boroumand and Klip, 2020; Krings et al., 2012; Sulston and Cawthorn, 2016; Tratwal et al., 2020). Brown adipocyte markers of BMA are reduced in obese mice and a parallel adoption of more white adipocyte markers has been observed (Adler et al., 2014b; Doucette et al., 2015; Krings et al., 2012). The amalgamation of upregulated white adipocyte proteins and elevated fat storage capacity is known as BM adipocyte whitening. Obesity-induced gains in whole body adiposity was causally linked to elevated circulating CD11b⁺ monocytes (Takahashi et al., 2003). However, the influence of BMA from either lean or obese mice on the expansion of CD11b⁺ monocytes had not been previously investigated.

The limited spatial capacity of the BM interior as well as the proximity of the cellular lineages in the various BM niches lead us to anticipate that the obesity-linked BMA whitening would influence the monocytic lineage. Here, we found a temporal, positive link between BMA hypertrophy and monocytosis at 3 weeks of HFD. By 8 weeks of HFD, BMA whitening also positively correlated with the rise in tissue invasive Ly6C^{high} monocytes in the BM. Interestingly, these findings were not associated with changes in pro-inflammatory signals in either monocyte subset. As a proof of concept for a direct and distinctive influence of adipocytes on monocyte number and subsets, we show that exposure to soluble factors from *bona fide* white adipocytes favours Ly6C^{high} monocyte preponderance while exposure to soluble factors from brown adipocytes favours Ly6C^{low} monocytes. Mechanistically, we discovered that white adipocytes induced conversion of Ly6C^{low} to Ly6C^{high} monocytes, a process that is not well understood at present. Brown adipocytes however induce their influence on Ly6C^{low} monocytes by increasing their proliferation. Interestingly, secretome studies have reported that BAT-derived VEGFA and VEGFB can stimulate

proliferation in adipocyte progenitors and endothelial cells (Bagchi et al., 2013; Leung et al., 1989). Adipocyte-specific VEGFA transgenic mice were protected from the effects of HFD and presented altered macrophage polarization (Elias et al., 2012). Therefore, it is conceivable that a reduction in brown adipocyte characteristics of BMA also leads to a drop in the factors that would normally favour proliferation of Ly6C^{low} monocytes. While the identification of the soluble unknown factor(s) responsible for the adipocyte-specific myeloid influence is outside the focus of the present study, we have laid the foundation for a future, comprehensive investigation of the molecular mediators involved.

How do adipocytes skew myeloid lineage subsets?

While the designation of macrophages as ‘M1 and M2’ (of pro- or anti-inflammatory characteristics, respectively) represents the extremes of a wide spectrum of phenotypes, the ‘M1’ pro-inflammatory orientation has been linked to glycolytic activation while the ‘M2’ anti-inflammatory orientation has been linked to oxidative phosphorylation (Castoldi et al., 2016; Kelly and O’Neill, 2015). A similar glycolytic metabolic switch with obesity has been reported for CD8 pro-inflammatory T cells (Buck et al., 2017). Surprisingly, while pro-inflammatory pathways in monocytes are activated by hyperglycemic conditions (Shanmugam et al., 2003), the metabolic preference of the different monocyte subsets had not been explored. This is a compelling question, given that Ly6C^{high} monocytes penetrate tissues and differentiate to pro-inflammatory ‘M1’ AT macrophages while Ly6C^{low} monocytes enter tissues to promote healing and repair (Geissmann et al., 2003; Lin et al., 2009). Therefore, we hypothesized here that, in the context of metabolic inflammation, there is a change in the metabolic phenotype of monocyte subsets.

Our findings show that Ly6C^{high} and Ly6C^{low} monocytes had different metabolic preferences at baseline, with Ly6C^{low} having higher mitochondrial membrane potential indicative of more active oxidative phosphorylation. Interestingly, HFD consumption lowered the mitochondrial membrane potential of both Ly6C^{low} and Ly6C^{high} monocytes, implicating a reduction in their mitochondrial oxidative capacity. This change aligned with a HFD-induced reduction in oxygen consumption by Ly6C^{low} monocytes. Importantly,

this difference was observed as early as 3 weeks and exacerbated by 8 and 18 weeks of HFD. The elevated lactate concentration measured in the BM at all HFD durations may be reflective of metabolic changes in cells of the BM cavity. Since we did not observe changes in the lactate levels in WATA-CM or BATA-CM, the higher lactate in BM of HFD-fed mice may not originate from changes in BMA.

Along with the change in monocyte subset proportions, we observed an increase in genes responsible for mitochondrial fragmentation and a decrease in those involved in mitochondrial elongation in monocytes from HFD-fed mice and treated with WATA-CM and BMA-CM. The physiological consequence of these changes was revealed by the observation of a striking mitochondrial fragmentation in monocytes treated with WATA-CM in comparison to control or BATA-CM treated monocytes. HFD is known to induce mitochondrial fission in a number of cells that correlates with insulin resistance. A recent report linked the two phenomena through *Drp1* action, as *Drp1* inhibition reversed HFD-induced insulin resistance (Filippi et al., 2017). Furthermore, obese and diabetic humans have reduced *Mfn2* expression in skeletal muscle. *Mfn2* positively correlates with insulin sensitivity (Bach et al., 2005), and *Mfn2* gene and protein are significantly downregulated in liver cells of HFD-fed rats (Gan et al., 2013). In our analysis, the expression of *Mfn2* negatively correlated with the abundance of Ly6C^{high} monocytes, while, the expression of *Drp1* positively correlated with the BM abundance of Ly6C^{high} monocytes. Overall, our findings reveal novel and distinct effects of adipocyte types on monocyte subset development that may occur through changes in mitochondrial fitness.

Given that the proportion of Ly6C^{low} monocytes dropped in favour of a predominance of Ly6C^{high} monocytes by 8 but not 3 weeks of HFD, it is possible that this subset change obeys a prior shift in their metabolic orientation. Supporting these observations, exposure of monocytes to white adipocyte CM also reduced their mitochondrial membrane potential. Furthermore, BMA-CM from 8-week HFD-fed mice also lowered monocyte mitochondrial membrane potential compared to control BMA-CM and favoured Ly6C^{high} predominance. Mechanistically, these data suggest that white adipocytes induce a monocyte metabolic shift that may guide Ly6C^{high} monocyte prevalence.

In conclusion, we demonstrate an interconnectivity of cellular networks within the BM, a vulnerability of the BM microenvironment to HFD, and a consequential myeloid lineage determination likely due to BMA whitening. Given the vastly different metabolic and soluble factors between *bona fide* white and brown adipocytes, it is easy to imagine that phenotypic changes in BMA can vastly alter the BM microenvironment and the factors that surround hematopoietic cells. BMA whitening during HFD feeding contributes to myelopoiesis and predominance of invasive Ly6C^{high} monocytes mediated by changes in monocyte metabolism.

Materials and Methods

Animal Procedures

Mouse protocols were approved by the animal care committee (Protocol #20011850 to S.E.G. and D.J.P., University of Toronto and #1000047074 to A.K., The Hospital for Sick Children). Three groups of 9-week-old male C57BL/6 mice were fed either low fat diet (10% kcal from fat; D12450Ji, Research Diets) or HFD (60% kcal from fat; D12492i, Research Diets) for 3, 8, or 18 weeks. Mice were singly caged to avoid stress and weight inequalities induced by aggression of the dominant male to the subordinate male (Kappel et al., 2017). The cages were maintained at 22°C on a 12h light/dark cycle, the diets were replaced once per week and various cage enrichments were provided including switching the positioning of the cages throughout the study.

Body weight was measured twice per week. Upon completion of the respective feeding durations, mice were fasted for 4 hours, blood was drawn via tail vein and fasting blood glucose was measured with a glucometer. Mice were euthanized by cervical dislocation. Tissues were dissected and flash frozen in liquid nitrogen for RNA isolation. EWAT, blood and BM were immediately processed for flow cytometry analysis.

EWAT-Derived Stromal Vascular Fraction (SVF) Isolation

EWAT-derived SVF was collected as previously described (Orr et al., 2013). Excised EWAT was rinsed in PBS, minced and digested with 0.47wv/mL Liberase solution (Millipore Sigma, Cat No 5401054001) shaking at 200 rpm for 20 mins at room temperature. SVF was isolated using a 70 µm cell strainer, the enzymatic activity of Liberase was neutralized with DMEM containing HI-FBS for 5 minutes and centrifuged at 500 ×g for 10 minutes. The supernatant containing adipocytes was removed, the pellet was re-suspended in red blood cell lysis buffer for 5 minutes to remove erythrocytes and washed with PBS three times.

BM Histology

Extracted tibia and femur bones were fixed in 10% formalin for 48 hours, decalcified in 14% EDTA solution for 14 days, paraffin wax-embedded, and longitudinal sections were stained with H&E and Oil Red O (Millipore Sigma; Cat No O0625) to visualize BM adiposity.

BM-Derived Monocyte Suspension Cell Culture and Differentiation

Murine primary BM cells were plated at 500,000 cells/mL on Thermo Scientific™ Nunc™ Non-Treated 6-well plates (Cat No 1256680) with complete RMPI (company) and supplemented with recombinant mouse M-CSF (Peprotech Inc. Cat No 315-02) at 20ng/mL for 5 days (Francke et al., 2011). Following 5 days of BM cell incubation on the ultra-low attachment surface plates, the suspended cells have the signature of mature monocytes and can be used for experimentation. These BM-derived differentiated monocytes were then collected, washed with RMPI, centrifuged and re-suspended with various adipocyte CM for 18 hours.

Primary Adipocyte Isolation and Culture

Epididymal white and interscapular brown AT were dissected in sterile conditions, the tissues underwent mechanical mincing and enzymatic digestion by collagenase type I (Worthington Biochemical Corp, Cat No LS004197) at 4mg/mL shaking for 1 hour. The digested adipocytes were then filtered through a 200 µm cell strainer and centrifuged, washed twice and plated in adipocyte media: high glucose DMEM, 1% BSA, 0.5% FBS, and 1% Penicillin and Streptomycin for 24 hours. BM adipocytes were isolated from the floating fraction following BM isolation via centrifugation. The cells were counted and cultured in adipocyte media overnight. Tissues from three mice were collected and the isolated cells were pooled in each CM generating experiment.

Flow Cytometry

Following red blood cell lysis, SVF, blood and the BM were washed with FACS buffer, stained with Aqua Live/Dead Antibody (ThermoFisher, L34957) for 15 minutes in the dark, washed with FACS

buffer again, blocked with anti-mouse CD16/32 antibody (eBioscience, 14-0161-86) for 15 minutes in the dark following a 30 minutes' incubation with corresponding antibody for each tissue (**Table 1**). The samples were then fixed, read on LSR Fortessa cytometer (BD Biosciences) and data gated based on appropriate fluorescence minus one (FMO) controls and analyzed on the FlowJo software version 10 (Becton, Dickinson & Company).

Quantitative PCR (qPCR)

RNA was extracted from cells or tissues using TRIzol™ (Life Technologies, Cat No 15596018), then cDNA synthesized using the SuperScript VILO cDNA kit (Life Technologies, Cat No 11754250) according to manufacturer's instructions. qPCR reactions were performed with predesigned TaqMan probes (Life Technologies) on a StepOne Plus Real-Time PCR System (Life Technologies). Gene expression was normalized to that of housekeeping genes, Abt1, Hprt, and Eef2 by the $\Delta\Delta C_T$ method.

BrdU incorporation

BM derived monocytes were labelled with 10 μ M BrdU (abcam, ab6326) during the 18-hour treatment at 37C. The cells were then washed with FACS buffer, surface markers were stained as described above and incubated in Foxp3 Fixation/Permeabilization Buffer (eBioscience, 00-5523-00) overnight. They were then treated with 30 μ g of DNase I.9 (Sigma, 260913). for 1 hour, stained with anti-BrdU antibody for 1 hour and then washed with FACS buffer in preparation to run on LSR Fortessa Cytometer.

Fluorescent Activated Cell Sorting of Monocytes

BM from two femurs and tibias of 10-week-old chow-fed mice was used in the isolation of monocyte subsets via fluorescent activated cell sorting using the FACS Aria III instrument. Gr1⁻ CD115⁺ CD11b⁺ Ly6C⁺ surface markers were used in identifying total monocytes and the two subsets were subsequently distinguished by the relative surface abundance of Ly6C protein. Isolated Ly6C^{low} and Ly6C^{high} monocytes were then validated using antibodies to CX3CR1 and CCR2 (**Table 1**), respectively.

Cell Metabolism Analysis

Oxygen consumption (OCR) and extracellular acidification rates were measured using a Seahorse XFe96 analyzer (Seahorse Bioscience). Seahorse 96-well cell culture plates were pretreated with 1 $\mu\text{g}/\text{cm}^2$ Corning™ Cell-Tak Cell and Tissue Adhesive (Fisher Scientific, Cat No CB40240) and FACS sorted Ly6C^{low} or Ly6C^{high} monocytes were seeded at 100,000 cells/well, allowed to equilibrate for 1h in XF base medium supplemented with 11 mM glucose, 1 mM sodium pyruvate and 2mM Glutamine. Following baseline measurements, FCCP was injected at 1mM and thereafter an injection of Antimycin A/ Rotenone. Cells were counted following the seahorse assay to test for changes in cellular detachment between the conditions.

Immunofluorescence Staining for Super-Resolution Microscopy

Monocytes were spun down onto coverslips and fixed with 4% paraformaldehyde for 15 minutes. Thereafter, the cells were rinsed with PBS and permeabilized with 0.1% Triton X-100 for 10 minutes. The cells were then blocked with 2% BSA in PBS at 4°C overnight. The cells were then incubated with the primary antibody, Tom20 (FL-145, Santa Cruz, sc-11415), in a 1:100 dilution in blocking buffer at 4°C overnight. Thereafter, the cells were incubated with Alexa Fluor 488 anti-rabbit secondary antibody (1:100) for 2 hours at room temperature. Cells were incubated with Alexa Fluor 568 Phalloidin (1:100) for 3 hours at room temperature. Coverslips were then mounted by ProLong Diamond (Thermo Fisher Scientific) and allowed to dry for two days. The images were acquired via Zeiss LSM880 Airyscan super resolution microscopy and the images were processed via Fiji software.

ELISA

The BM content was fractioned into hematopoietic and supernatant portions by centrifugation at 1,600 rpm. The supernatant fraction was re-suspended in 500ul of 1X PBS (supplemented with Protease Cocktail Inhibitor, Sigma). Total protein content was estimated by Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). Subsequently, mouse TNF, IL-1b, IL-6, CXCL1 and CCL2 ELISAs were

carried out according to the manufacturers' instructions. All Mouse DuoSet ELISA kits were purchased from R&D systems. The individual protein contents were finally normalised to total protein content.

Statistical Analysis

Data are expressed as means $\pm$ SEM. An unpaired Student's T test was used to detect differences in data sets with one variable. A one-way ANOVA with Tukey post-test was used to detect differences in data sets of two or more variables. Statistical significance was set at $P < 0.05$. Data analysis and statistics tests were done using GraphPad Prism version 6 (GraphPad Software), Flow Jo Software version 10.4 and figures were prepared using Adobe Illustrator CS6 version 16.0.

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Author Contribution

P.B., S.E.G. and A.K. conceived the study design. P.B. performed experiments and analyzed the data. M.W., J.S., I.T., N.S., L.Z., Z.L., A.L., T.M. and Y.Z. participated in experimental work and data collection. A.K., D.J.P., and S.E.G. provided access to resources and facilities. P.J.B. contributed with advice and trouble-shooting. P.B. and A.K. wrote the manuscript, and all authors read and approved it.

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Figure 1. Mice fed HFD increased body weight, fasting blood glucose, visceral and bone marrow adipose tissue. [A] Body weight was measured twice a week throughout the course of feeding. [B] Mice were fasted for 4 hours after which fasting blood glucose was measured. [C] Epididymis white adipose tissue was weighed the day of sacrifice. [D] Area covered by bone marrow adipocytes were measured

808 following hematoxylin and eosin (H&E) to visualize adipocyte ghosts using quantified using ImageJ. [E]
 809 Bone marrow adipocyte numbers were blindly counted following H&E staining. [F] Femurs of control
 810 and HFD-fed mice were fixed in formalin for 2 days followed by 14 days EDTA decalcification and
 811 subsequently stained with ORO and [G] H&E. Scale bar is 25 μ m. Results are expressed as mean $\pm$ SEM,
 812 n=4-14 mice per group. One-way ANOVA was used for statistical analysis at each dietary group and
 813 across feeding periods. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and ns = not significant.

Figure 2

Figure 2. Bone marrow monocytes increased prior to adipose tissue macrophages. Quantification of stromal vascular fraction (SVF) of [A] adipose tissue monocytes [B] total macrophages and [C] pro-inflammatory macrophages via flow cytometry analysis identified first by size and granularity through forward and side scatter and then using the following markers: CD11b CD115 F4/80 Ly6C GR1 CD11c. [D-F] Whole adipose tissue gene expression of the myeloid marker *Itgam*, macrophage marker *Adgre* and

820 pro-inflammatory myeloid marker *Itgax* by RTqPCR. [G-H] Gene expression analysis of embryonic
 821 derived tissue resident macrophage markers *Timd4* and *Marco* by RTqPCR. [I] Flow cytometry analysis of
 822 total and [J] monocyte subsets in the bone marrow with HFD-feeding. Results are expressed as mean $\pm$
 823 SEM, n=5-10 mice per group. One-way ANOVA was used for statistical analysis at each dietary group and
 824 across feeding periods. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and ns = not significant.

Figure 3

Figure 3. HFD blunted the mitochondrial oxidation of monocyte subsets. Flow cytometry detection of [A-C] MitoTracker Red CMXRos in bone marrow monocyte subsets of control and HFD-fed mice. [D-F]

828 OCR of FACS-sorted Ly6C^{low} and Ly6C^{high} monocytes from control and HFD-fed mice measured by the
 829 Seahorse Assay. [G-I] Area under the curve of OCR of FACS-sorted Ly6C^{low} and Ly6C^{high} monocytes from
 830 control and HFD-fed mice measured by the Seahorse Assay. [J] Area under the curve of ECAR
 831 measurement from FACS-sorted Ly6C^{low} and Ly6C^{high} monocytes of control and HFD-fed mice measured
 832 by the Seahorse Assay and pooled from all feeding durations. [K] BM lactate measurement of the
 833 supernatant fraction in HFD and control samples. Results are expressed as mean $\pm$ SEM, n=3 mice per
 834 group where applicable. One-way ANOVA was used for statistical analysis. *P < 0.05 and **P < 0.01.
 835 Graph in panels C was created using FlowJo software (Becton, Dickinson Company).

Figure 4

Figure 4. HFD downregulated gene expression of mitochondrial fusion genes and upregulated mitochondrial fission genes. Gene expression of mitochondrial fusion genes *Mfn1*, *Mfn2*, *Opa1*, *Tomm20*, and *Tomm40* in monocytes isolated from control and HFD-fed mice for [A] 3, [B] 8, [C] 18 weeks. Gene expression of mitochondrial fission genes *Drp1*, *CypD* and *Fis1* in monocytes isolated from control and HFD-fed mice for [D] 3, [E] 8, [F] 18 weeks. [G] Correlative analysis of monocyte subset BM cellular

842 abundance and BM-derived monocyte *Mfn2* gene expression. [H] Correlative analysis of monocyte subset
 843 BM cellular abundance and BM-derived monocyte *Drp1* gene expression. Results are expressed as mean $\pm$
 844 SEM, n=3-9 mice per group. One-way ANOVA was used for statistical analysis. *P < 0.05.

Figure 5

Figure 5. Bone marrow adipocyte whitening detected at 8 and 18 weeks of HFD favours increased

bone marrow Ly6C^{high} monocytes and induced changes in mitochondrial dynamic gene expression.

Volcano plots showing the gene expression of white and brown adipocyte markers in red and blue respectively [A] at 3, [B] 8 and [C] 18 weeks of HFD. Correlative graphs of the [D] white adipocyte marker ASC1 and [E] brown adipocyte marker UCP1 with Ly6C^{low} and Ly6C^{high} monocyte subsets. Flow cytometry quantification of naïve Ly6C^{low} and Ly6C^{high} monocytes upon 18 hours of treatment with BMA-CM from control and HFD-fed mice for [F] 3, [G] 8 and [H] 18 weeks. [I] Gene expression of mitochondrial fusion genes *Mfn1*, *Mfn2*, *Opa1*, *Tomm20*, and *Tomm40* in naïve monocytes treated with BMA-CM from control and HFD-fed mice. [J] Gene expression of mitochondrial fission genes *Drp1*, *CypD* and *Fis1* in naïve monocytes treated with BMA-CM from control and HFD-fed mice. Results are expressed as mean $\pm$ SEM, n=3-9 mice per group. One-way ANOVA was used for statistical analysis at each dietary group and across feeding periods. *P < 0.05, ****P < 0.0001 and ns = not significant.

Figure 6

Figure 6. *Bona fide* white adipocytes favoured Ly6C^{high} monocytes preponderance, blunted

monocyte mitochondrial membrane potential, and induced mitochondrial fragmentation *in vitro*.

Naïve monocytes were treated with CM from isolated *bone fide* white and brown adipocytes from EWAT

and inter-scapular BAT for 18 hours. [A] Flow cytometry dot plots detecting the effects of 18 hours of

treatment with media alone, WATA-CM and BATA-CM on monocyte subset abundance with the [B] quantification of the gates drawn for Ly6C^{low} and Ly6C^{high} monocytes. [C] MitoTracker Red CMXRos detected by flow cytometry in Ly6C^{low} monocytes treated with media alone, WATA-CM and BATA-CM for 18 hours. [D] Gene expression of mitochondrial fusion genes *Mfn1*, *Mfn2*, *Opa1*, *Tomm20*, and *Tomm40* in naïve monocytes treated with Media, WATA-CM and BATA-CM. [E] Gene expression of mitochondrial fission genes *Drp1*, *CypD* and *Fis1* in naïve monocytes treated with Media, WATA-CM and BATA-CM. [F] Super-resolution microscopy analysis of mitochondrial morphology via TOM20 staining in monocytes treated with Media, WATA-CM and BATA-CM. Scale bar = 5 μ m. Images analyzed in Fiji (version 2.1.0/1.53c). WATA-CM = white adipose tissue adipocyte-conditioned media; BATA-CM = brown adipocyte tissue adipocyte-conditioned media. EWAT = epididymal white adipose tissue; BAT = brown adipose tissue. Results are expressed as mean $\pm$ SEM, n=3-9 mice per group. One-way ANOVA was used for statistical analysis. *P < 0.05, **P < 0.01 and ***P < 0.001, ****P < 0.0001 and ns = not significant. Graphs in panels A-C were created using FlowJo software (Becton, Dickinson Company).

Figure 7

Figure 7. White and brown adipocytes favour Ly6C^{high} and Ly6C^{low} monocytes through different

mechanisms. FACS-sorted Ly6C^{low} and Ly6C^{high} monocytes from control mice were independently treated with [A] media alone, [B] WATA-CM and [C] BATA-CM for 18 hours after which the presence of the alternate monocyte subset was assessed via flow cytometry. [D-F] Quantification of Ly6C^{low} and Ly6C^{high} gates in panels A-C. [G] *In vitro* generated monocytes were treated with media alone, WATA-CM or BATA-CM in the presence of the pyrimidine analogue BrdU to detect cellular proliferation. [H] Quantification of BrdU abundance in Ly6C^{low} and Ly6C^{high} with treatments in panel C. Results are expressed as mean ± SEM, n=3-6 mice per group. One way ANOVA test was used for statistical analysis. ****P < 0.0005. Graphs in panels A-C and G were created using FlowJo software (Becton, Dickinson Company).

Figure S1

A 3 weeks

B 8 weeks

C 18 weeks

889

890 **Figure S1. Monocyte precursor and stem cells were not significantly altered with HFD *in vivo*.** Flow

891 cytometry analysis of HSC and monocyte precursor cells in mice fed a Control or HFD for [A] 3, [B] 8,

892 and [C] 18 weeks. Flow cytometry antibodies used are listed in Table 1. Student's T test was used and no

893 statistical significance was observed.

Figure S2

Figure S2: BM pro-inflammatory factors did not change with HFD. No changes in pro-inflammatory cytokines were detected in BM supernatant fraction using the ELISA assay in mice fed control or HFD for [A] 3, [B] 8, or [C] 18 weeks. FACS-sorted monocyte subsets were analyzed for changes in pro-inflammatory gene expression; shown in [D] TNF α gene expression increased with 18 weeks of HFD in Ly6Chigh monocytes but no other changes were detected. Results are expressed as mean $\pm$ SEM, n=3-6 mice per group. One way ANOVA test was used for statistical analysis. *P < 0.05 and **P < 0.01.

Figure S3

Figure S3. BMA precursor cells with HFD. RT qPCR of isolated pelleted fraction were assessed for pre-adipocyte markers of mice fed a Control or HFD for [A] 3, [B] 8, and [C] 18 weeks. [D] classical white and brown adipocyte markers were confirmed in isolated WATA and BATA.

Figure S4

A

B

C

D

Figure S4: Flow cytometry gating strategy for identification of Ly6C^{low} and Ly6C^{high} monocytes and their precursor cells. [A] Using side and forward scatter to identify singlet cells, CD115+Gr1- cells were

identified that were further analyzed using CD11b and Ly6C. [B] Ly6C^{low} and Ly6C^{high} monocyte gates were drawn using fluorescence minus one (FMO) controls for CD11b and Ly6C. [C] The subsets were further confirmed via CX3CR1 and CCR2, respectively. [D] CD45⁺ cells were analyzed using a lineage cocktail (table 1) and CD117⁺, further broken down into Sca⁺ and – cells. Sca⁺ cells were further classified using CD48 and CD150 while Sca⁻ cells were further characterized via CD16/32 and CD34. Graph in panels C was created using FlowJo software (Becton, Dickinson Company).

Figure S5

Figure S5: HSC and their progenitors using the panel of markers listed in table 1 following the white and brown CM treatment for 18 hours. [A-C] Flow cytometry analysis was used to test for the presence and changes in HSC and monocyte precursor cells in cultured monocytes treated with media, WATA-CM, and BATA-CM. Flow cytometry antibodies used are listed in Table 1. One way ANOVA test was used for statistical analysis. *P < 0.05.

Table 1. List of Antibodies used for flow cytometry analysis.

Markers	Fluorophore	Manufacturer	Clone	Catalog Number
CD11b	APC-eFluor 780	eBioscience	M1/70	47-0112-82
Ly6C	PE-Cy7	Biolegend	HK1.4	128018
Gr-1	Alexa Fluor 700	eBioscience	RB6-8C5	56-5931-82
CX3CR1	Brilliant Violet 421	Biolegend	SA011F11	149023
CCR2	Alexa594	R&D Systems	475301	FAB5538T
CD115	APC	eBioscience	AFS98	17-1152-82
F4/80	PerCP-Cy5.5	BioLegend	BM8	123128
CD11c	FITC	eBioscience	N418	11-0114-81
CD34	FITC	BD Biosciences	RA1434	553733
CD16/32	APC-R700	BD Biosciences	2.4G2	565502
CD45.2	BV650	BD Biosciences	30-F11	563410
CD150	PEPCP710	invitrogen	mShad150	46-1502-82
CD117	APC/Fire 750	BioLegend	2B8	105838
SCA1	PE-Cy7	invitrogen	D7	25-5981-82
CD48	BV605	BioLegend	MM48-1	103441
CD3e*	PE	BD Biosciences	17A2	555725
B220*	PE	BD Biosciences	RA3-6B2	553089
Ly6G*	PE	BD Biosciences	1A8	551461
CD11b*	PE	BD Biosciences	M1/70	553311
CD11c*	PE	BioLegend	N418	117308
Ter119*	PE	BD Biosciences	TER-119	553623
CD127*	PE	eBioscience	A7R34	12-1271-82
NK1.1*	PE	BD Biosciences	PK138	553165

*LINEAGE is a cocktail of antibodies containing CD3e, B220, Ly6G, CD11b, CD11c, Ter119, CD127, NK1.1 with the same fluorophore to exclude all terminally differentiated cells.