High fat diet triggers a prompt and transient increase in adipose tissue G-CSF and circulating myeloid cells in mice.

ABSTRACT

Scope: The short-term effects of feeding high fat diet (HFD) to mice was investigated with focus on the effect on myelopoesis, circulating neutrophils and the induction of G-CSF.

Methods and Results: Mice were fed HFD (45%) during a period of 5 weeks with samples taken after 3 days and 1, 3, 4 and 5 weeks. Three days after commencement of HFD, the number of circulatory neutrophils and monocytes increased but returned to baseline-level at day 8. This transient increase coincided with an increased blood concentration of G-CSF and a transient increase in bone marrow and spleen neutrophils. In supernatant from cultivated visceral adipose tissue isolated from HFD fed mice on day 3 and 8, G-CSF was increased. The expression of Toll-like receptor 4 in adipose tissue was down-regulated from week 4. *In vitro*, LPS was a poor stimulator of G-CSF, while G-CSF or LPS together with G-CSF or GM-CSF induced increased G-CSF production. G-CSF suppressed production of LPS-induced TNFα and increased IL-10 production in dendritic cells suggesting that G-CSF down-regulates LPS-induced inflammation.

Conclusion: HFD induces a transient increase in adipose tissue G-GSF and circulating myeloid cells in mice. We suggest G-CSF induces increased myelopoiesis and simultaneously down-regulates LPS-induced inflammation.

Key words: Adipose tissue, G-CSF production, High fat diet, monocytes, neutrophils,

1. INTRODUCTION

Daily or regular intake of high fat meals is common in large parts of the world and may lead to overweight. Alongside the weight gain, a state of systemic low-grade inflammation (LGSI) often emerges, which in the long run may lead to the development of metabolic syndrome and further on, to chronic diseases e.g. type 2 diabetes (T2D) and cardiovascular diseases.[1] However, the precise relationship between high fat diet (HFD) consumption, inflammation and obesity, is still not fully understood. Also consumption of a single high fat meal has been reported to induce an inflammatory response and has been suggested to jumpstart the low-grade inflammation.[2] A systematic review on the postprandial inflammatory response to a high fat meal in healthy adults did only reveal an effect on blood IL-6 but not on other pro-inflammatory markers and the 47 studies included in the review revealed large variations in the post-prandial inflammatory response.[2] The reason for this variation remains obscure. Very little has been described regarding the effects of the early course of a HFD before the onset of diet-induced weight gain and obesity as regard the inflammatory events taking place in bone marrow, adipose tissue and blood, with potency to jumpstart the development of LGSI and comorbidities.

Microbiota has been suggested to play a role in HFD-induced inflammation via its production of endotoxin.[3, 4] Increased dietary lipid increases the lymphatic flow and leads with it an increased amount of endotoxin from the gut.[5, 6] The full consequences of increased endotoxin influx are however not fully understood. The effect of an LPS injection on granulopoiesis and increased G-CSF levels is well established[7, 8], but the consequences of a continuous LPS influx from the gut as expected from a permanent or regular HFD have only been sparsely reported. Cani et al. showed that a HFD fed to mice for four weeks lead to an increase in the circulatory endotoxin level and provided evidence that LPS was responsible for the onset of metabolic disease leading to T2D in mice.[9] Apart from LPS, also free fatty acids (FFA), in particular saturated FFA stimulate inflammation through the binding to TLR4.[10] Hence, as both FFA and LPS influx is presumed to be increased in HFD fed mice both may contribute in the stimulation of an inflammatory response [11, 12] indicating that solely measuring the LPS concentration in e.g. blood, may not directly correlate with an inflammatory response.

Mice fed a diet rich in fat (45-60%) serve as a frequently used model for studying the physiological effects of an excess energy intake and obesity. Obesity and firmly established LGSI typically emerge after HFD feeding for eight weeks or more whereas comorbidities such as T2D establish even later.[13] The inflammatory response is complex and studies of the relationship between obesity and physiological changes have revealed that a connection between visceral adipose tissue (VAT), hematopoiesis (bone marrow) and blood exists.[14, 15] Whether shorter periods of feeding HFD and inflammation exhibit similar relationships is unclear.

Talukdar et al. (2012) showed that as early as three days after the onset of feeding a HFD, neutrophil infiltration in VAT, which remained high during 90 days of feeding was seen.[16] Others have reported a transient neutrophil infiltration in the VAT ceasing one week after the onset of feeding a HFD.[17] Hence, neutrophil influx in VAT does take place, however the length of this event and how it is effectuated and perhaps abrogated remains obscure. Due to the short lifespan of neutrophils, a constant infiltration into adipose tissue requires a constant supply of neutrophils from the bone marrow. An increase in circulatory neutrophils may thus require a steady mobilization signal and an increased production in the bone marrow.

Here, we investigated the effect of short-term feeding a HFD to young mice on bone marrow and blood cell composition and further assessed the circulatory and VAT level of G-CSF and GM-CSF as well as the expression of genes involved in LPS-induced inflammation in VAT. We found that a transient neutrophil infiltration in VAT coincided with a transiently increased VAT production of G-CSF. We suggest that G-CSF holding dual activity stimulates neutrophil recruitment while simultaneously suppresses inflammatory responses of other myeloid cells. This may explain the transient nature of myelopoiesis and advocate against LPS as a key initiator of HFD-induced LGSI.

2. MATERIALS AND METHODS

Animals

Male C57BL/6 mice (Taconic, Lille Skensved, Denmark) 6 weeks of age, were housed with 4 mice per cage and fed *ad libitum* either a high-fat diet (HFD) (Altromin C-1000 rodent diet modified as HFD with 45% energy from fat, Brogaarden, Denmark) or a standard rodent diet with 5% kcal from fat (Altromin C-1000, Brogaarden). At study day 1, all mice were weighed and allocated to HFD (40 mice) or the standard diet (20 mice). The mice were euthanized by cervical dislocation and tissue samples collected under sterile conditions. The experiment was carried out in accordance with the Council of Europe Convention European Treaty Series 123 on the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and the Danish Animal Experimentation Act (LBK 1306 from November 23, 2007) approved by the Animal Experimentation Inspectorate, Ministry of Justice, Denmark (Licence number: 2012-15-2934-00256 C1-6).

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Sample Collection

At day 3, 8, 23, 30 and 37, eight mice fed HFD and four fed standard diet were weighed and anesthetized. Whole blood was collected from the retro-orbital vein with heparin-coated capillaries into EDTA-coated eppendorf tubes. Thirthy µL blood was transferred to cryotubes containing 180 µL lysisbuffer (50% Lysis/Binding Solution Concentrate, 50% isopropanol, Thermo Fisher, Waltham, MA, USA) and stored at -80°C until RNA extraction. The remaining blood was used for complete blood count (CBC) and leukocyte differentiation and isolated plasma was transferred and frozen at -80°C until ELISA was performed. Immediately after euthanasia, spleen, epididymal adipose tissue (VAT) and femur were collected. The spleen was cut in two and one part was placed in RNAlater (Ambion by Life Technologies, Thermo Fisher) for qPCR and the other part was placed in cold sterile PBS for cell isolation and flow cytometry. Bone marrow (BM) cells were isolated from the femur by flushing them out from cleaned bones with cold PBS followed by flow cytometry analysis.[18] The VAT was placed in cold PBS and weighed. Hereafter, a small part of the VAT was placed in RNAlater for qPCR and the rest used for culturing.

Cell isolation and flow cytometric analyses

Blood neutrophilic granulocytes and monocytes were assessed by blood leukocyte counting by an automatic cell counter (Advia 2120i Hematology System, Siemens, Germany). To analyze single cell suspensions of spleen and BM cells by flow cytometry, cells were counted, washed and suspended in FACS wash buffer (PBS, 1% fetal bovine serum). For the identification of neutrophils and mature monocytes, a cocktail of anti-mouse Ly6G (1A8), Ly6C (AL-21), CD11b (M1/70) and CD115 (T38-320) antibodies were used (BD pharmingen, BD Biosciences, San Jose, CA, USA).[18] For the identification of mature myeloid cells, Fc-receptors were first blocked with Fc-block (BD pharmingen, BD Biosciences) for 10 min at 4°C, washed in FACS wash buffer and incubated with the antibody cocktail at 4°C for 30 min. After washing with FACS wash buffer, cells were analyzed using a BD FACS CANTO II (BD Biosciences). Data were analyzed with FlowJo™ software (BD Biosciences) and the number of neutrophils and monocytes was calculated from total number of cells for each sample.

RNA isolation and qPCR

Adipose tissue was homogenized in lysis buffer (MagMAX-96 RNA Isolation Kit; Ambion, Thermo Fisher) by using glass beads and the The FastPrep®-24 Instrument (MP Biomedicals, Thermo Fisher). Total RNA from homogenized adipose tissue, BM cells and blood cells was extracted by MagMAX Express (Applied Biosystems, Foster City, CA, USA) using the MagMAX-96 RNA Isolation Kit (Ambion, Thermo Fisher) for tissues and the MagMAX-96 Blood RNA Isolation Kit (Ambion, Thermo Fisher) for blood cells, as previously described[19] and according to manufacturer's instructions. cDNA was produced from ~200 ng total RNA using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems) according to the manufacturer's instructions. Gene expression of selected genes listed in Table 1 was analyzed on the StepOnePlus instrument (Applied Biosystems) using universal fast thermal cycling parameters (Applied Biosystems) and TaqMan Fast universal PCR Mastermix (Applied Biosystems). Relative quantification of the gene expression was calculated by the comparative cycle threshold (C_T) method. The expression of target genes was normalized to the gene expression of *Actb* (beta-actin) as the reference gene: [$\Delta C_T = C_{T(target)} -$

$C_{T(reference)}$]. Relative quantification of gene expression was calculated as $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = [\Delta C_{T(sample)} - \Delta C_{T(calibrator)}]$. Mean ΔC_T of samples from control mice was used as the calibrator.

Culturing of adipose tissue

The epididymal fat pads (VAT) were washed in PBS and two 50 mg pieces were placed in two different wells of a 6-well-plate and cut into 5 smaller pieces and cultured for 24 hours in 2 ml M199 media with glutamine, 25 mM HEPES and 1% pen/strep.[20] After culturing the adipose tissue supernatant from each mouse was frozen at -80°C until cytokine quantification.

Cytokine quantification

Plasma G-CSF and G-CSF, GM-CSF, CXCL-2, TNFα, IL-10 and IL-1β in the supernatant of cultured adipose tissue and of stimulated cells were quantified by DuoSet ELISA kits from R&D Systems (!Minneapolis, MN, USA) according to manufacturer's instructions.

***In vitro* treatment of murine bone marrow cells**

Bone marrow cells were isolated as described elsewhere.[21] Briefly, bone marrow from C57BL/6 mice was flushed from the femur and tibia and washed twice in sterile PBS. Cells (2.9×10^6) were seeded in 12-well-plates in 1 mL RPMI 1640 (Sigma-Aldrich, St. Louis, MO), containing 10% (v/v) heat-inactivated fetal calve serum, penicillin (100 U/ml), streptomycin (100 µg/ml), glutamine (4 mM) and 50 µM 2-mercaptoethanol. Cells were then treated with LPS (Sigma-Aldrich), mouse G-CSF (R&D Systems) and/or GM-CSF (from a GM-CSF-transfected myeloma cell line[21] in a final concentration of 0.1 µg/ml LPS, 10 ng/ml G-CSF and GM-CSF. The cells were incubated for 1 or 4 days at 37°C in a 5% CO_2 humidified atmosphere. BM cells were harvested and examined for neutrophils and mature monocytes using flow cytometry and the concentration of cytokines in supernatant was determined by ELISA. Dendritic cells were prepared as described.[21]

Statistical analysis

Statistical analysis was performed in GraphPad Prism version 5.03 (GraphPad Software). Significance was evaluated by 2-way ANOVAs, 1-way ANOVAs or t-tests to determine differences between treatment groups

and time points. Relative quantification (RQ) is plotted in gene expression experiments and statistical analysis was performed on ΔC_T values. In experiments where control groups showed constant expression level all data from control mice were pooled.

3. RESULTS

High fat diet-increased body and epididymal fat pad weight is preceded by a transient increase in blood myeloid cells in mice

Male mice aged 6 weeks at the start of the experiment, were fed either a 45% HFD or a control diet with 4% fat. The mice were weighed regularly during the 37 days feeding period. Both groups gained weight during the first three weeks, indicating that the mice were not fully outgrown at the beginning of the experiment.[22] At Day 30 and 37, the HFD mice had increased their body weight and the weight of the epididymal fatpads significantly compared to the control mice (figure 1a-b). Percentages of blood monocytes and neutrophils from the mice as determined by CBC revealed a constant percentage of monocytes and granulocytes in the control group (0.6% and 10.5%, respectively), (figure 1c-d). In the HFD group, at Day 3, the level of monocytes increased more than twofold, but decreased to the base-level at Day 8 (figure 1c). Also the level of granulocytes showed an indication of an increase at Day 3, this was however not significant (p=0.16). When analyzing the concentration of the growth factor G-CSF in the plasma, the control groups showed a fairly constant level around 75 pg/ml. In the HFD group at Day 3, the G-CSF concentration was increased but dropped thereafter to that of the control mice (figure 1e).

Myelopoiesis is transiently increased early in the high fat fed feeding period

We found no differences between the HFD and the control group in the proportions of myeloid progenitor cells (supplementary figure 1). The fully differentiated myeloid cells (gating strategy in figure 2a), however, exhibited increased level of neutrophils ($Ly6G^+Ly6C^+CD11b^+$) at Day 8 ($1.9 \cdot 10^7$ vs $1.4 \cdot 10^7$ per BM, p=0.012) but returned to that of the control mice at Day 23 (figure 2b). At day 37, a significant increase in neutrophils was seen in the HFD group compared to control ($1.6 \cdot 10^7$ vs. $1.3 \cdot 10^7$, p=0.03). Also the monocyte population ($Ly6G^-Ly6C^+$ $CD11b^+CD115^+$) in the HFD group showed a trend towards increased numbers at Day 8 (p=0.18) and Day 23 (p=0.09) but not at later time points. Of note, from day 30 the proportion of monocytes increased markedly in both groups, presumably due to the outgrowth of the mice at this age. A number of genes including *Elane, Arg1, Haptoglobin (Hp) and Itgam (CD11b)*, are expressed in neutrophils

at specific differentiation stages (figure 2d).[23] The expression of these genes in the BM cells was analyzed. In line with the increased neutrophil population detected in BM at day 8, a significant increase in the expression of *Hp* and *CD11b* indicative of neutrophils in the late differentiation state or mature neutrophils was detected at Day 8 (figure 2e). No differences between the two groups were detected in the expression of *Arg1* or *Elane*. Thus, HFD causes a prompt but transient increase in mature myeloid cells in the BM.

Spleen myeloid cells are transiently increased early in the HFD feeding period

When analyzing the myeloid population in the spleen during the course of HFD, we saw a non-significant increase in neutrophils ($Ly6G^+Ly6C^+CD11b^+$) at Day 8 (p=0.081) compared to control group (figure 3b). Mature monocytes ($Ly6G^-Ly6C^+$ $CD11b^+CD115^+$) showed a trend towards increased numbers in the HFD group at Day 3 and Day 23 (p=0.11 and p=0.12, respectively) (figure 3c). These data support the finding from the blood leukocyte counting (figure 1c and d) showing that an early increase in blood neutrophils is induced upon HFD feeding.

Expression analysis of blood was performed on *Hp* to identify immature neutrophils, on *Il1b* and *Stfa2l1* to identify mature neutrophils, and *Cd14* to identify monocytes. The expression of *Hp* showed a tendency to be increased on Day 3 (p=0.15) in HFD fed mice while on Day 8 (p=0.061) and Day 23 (p=0.019) the expression dropped to become lower than the chow fed mice and then increased again on day 37 to the level of the control group (figure 3d). In the HFD group, the expression of *Il1b* and *Stfa2l1* was significantly increased on Day 8 compared to the control group, while from Day 23 the expression of these genes was not different from the control group. The expression of *Cd14* showed a significantly higher level at Day 23 compared to control group, while the expression did not significantly differ from the control group at any other day. Hence, during the first week of HFD feeding, the number of mature neutrophils in circulation transiently increases but drops subsequently to the level of the chow fed control group. These data indicate an early but transient recruitment of neutrophilic granulocytes from the bone marrow to the blood upon the onset of HFD feeding.

Cultured adipose tissue from mice at the onset of high fat feeding secretes elevated G-CSF

To investigate if adipose tissue could contribute to the enhanced G-CSF level measured in the blood at Day 3, VAT isolated from mice fed HFD or control diet were cultured for 24 hours and the concentration of released G-CSF and GM-CSF was measured in the supernatant (figure 4a-b). Adipose tissue from mice on control diet showed a constant production of G-CSF and GM-CSF and data from different time points was therefore pooled. The G-CSF concentration from cultured HFD VAT showed on Day 3 a trend to be increased ($p=0.08$) and at Day 8 a significantly increased G-CSF production ($p=0.01$) (figure 4a). Adipose tissue from 23, 30 and 37 days did not show increased G-CSF production. The HFD did not result in a changed GM-CSF production, which showed a stable level around 0.5 ng per gram cultured adipose tissue during the entire experiment (figure 4b). Also the concentration of IL-1□ was analyzed but the concentration in all samples was at or below the detection limits (data not shown).

Pro-inflammatory as well as anti-inflammatory events occur in adipose tissue of high-fat fed mice

To investigate which factors were induced in adipose tissue during the five weeks of HFD feeding, the expression of different genes was analyzed in the VAT of HFD fed and control mice (figure 5). For the control group, all the genes tested showed a stable expression during the entire experiment, accordingly expression data from the various days were pooled. In the HFD group, the expression of *Ccl2* encoding the monocyte-recruiting chemokine CCL2[24] was slightly increased at Day 8 ($p=0.065$) compared to control and was increased at Day 23, Day 30 and 37 (figure 4). The expression of *Cxcl2* encoding the neutrophil-recruiting chemokine CXCL2 first showed a decreased expression on Day 3 ($p=0.08$) and then returned to the control level from Day 8 though with an increased expression at Day 30. Hence, the chemokines exhibited distinct expression profiles indicating a gradual increase of the monocyte-recruiting CCL2 and an initial reduction of the neutrophil-recruiting CXCL2. The expression of *Hp* was transiently up-regulated at Day 3 and Day 8, while the expression of *Stfa2l1* transiently showed a tendency to be increased on Day 8

(not significant). The expression of *F4/80* signifying the presence of macrophages[25] did not at any time point deviate from the expression of the control group, while *S1008a* expressed constitutively by monocytes and neutrophils [26] showed an up-regulation at Day 30 and 37. While the expression of *Cd8a* did not at any time point differ from the control group, the expression of *FoxP3* increased at the end of the study (Day 37). Also the expression of TLR4 was analyzed and showed a significantly decreased expression at Day 30 and 37 compared to the control group.

G-CSF stimulates the production of G-CSF in BM cells but inhibits an inflammatory response in monocyte-derived cells

To investigate how G-CSF, alone or in concert with LPS affected the development of myeloid cells, LPS was added to freshly isolated BM cells, with or without concomitantly added G-CSF or GM-CSF, and incubated for 1 or 4 days, where after the cellular composition was assessed by flow cytometry (figure 6a-b). After 1 day of stimulation with G-CSF or GM-CSF, the number of neutrophils doubled compared to cells cultured in media only, and after 4 days neutrophils were almost tripled compared to media-grown cells at day 1 (Figure 6a). The number of monocytes also increased in the presence of G-CSF or GM-CSF, after 1 day to 1.5-2 fold and after 4 days to 4-6 fold the number in media. In the presence of LPS, the number of neutrophils at day 1 and 4 gradually dropped to around 60 and 20% of the media Day 1 control. Likewise, when LPS was added together with either G-CSF or GM-CSF, the levels of neutrophils reached about 75% of the level obtained without LPS at day 1 and, at day 4, the number of granulocytes dropped to below the Day 1 media control level. In contrast, LPS stimulated the development of monocytes leading to more than the doubled number of monocytes at Day 4 compared to media controls at day 1 and 4 (figure 6b). LPS added together with G-CSF or GM-CSF did not lead to further increase in the number of monocytes as compared to stimulation with G-CSF or GM-CSF alone.

Neutrophils have a short lifespan, thus it is not possible from this experiment to establish whether LPS stimulates a faster development and thereby deplete the number of cells or halts the differentiation of these cells. To establish whether LPS stimulation affected the production of G-CSF or GM-CSF in the isolated

bone marrow cells, which could represent an indirect way to stimulate myelopoiesis, we tested the concentration of G-CSF and GM-CSF in the supernatant of the stimulated bone marrow cells. LPS alone did not induce production of G-CSF, but addition of G-CSF led a strong production of G-CSF, and G-CSF together with LPS further increased the concentration (figure 6c). Compared to Day 1, the G-CSF level at Day 4 measured after addition of G-CSF alone or in combination with LPS decreased indicating that the G-CSF is spend during the development of neutrophils. GM-CSF did not induce significant G-CSF production, but together GM-CSF and LPS induced a modest level of G-CSF (figure 6c). Neither LPS nor G-CSF or GM-CSF induced production of GM-CSF in the cells (figure 6c). We also tested the production of CXCL-2 known to be induced by LPS (figure 6c). LPS induced a clear increase in CXCL-2, while G-CSF or GM-CSF did not stimulate CXCL2 production. Interestingly, in the presence of GM-CSF, the LPS-induced CXCL2 production doubled. Prestimulating BM-derived dendritic cells with G-CSF prior to LPS stimulation resulted in reduced TNF induction while the production of IL-10 was increased (figure 6d).

Together, these results demonstrate that the presence of LPS influences the G-CSF and GM-CSF-induced development of myeloid cells. Moreover the production of G-CSF depends on the presence of G-CSF and is enhanced by LPS and affects the inflammatory response through its anti-inflammatory effect on monocyte-derived cells.

4. Discussion

It is well-established that a constant or regular intake of an energy surplus in the form of fat may lead to overweight or obesity and in turn systemic low-grade inflammation, which includes increased numbers of circulating neutrophils and monocytes.[1] However, how such diets influence the hematopoiesis in young and normal weight individuals has only been sparsely studied. In young mice, a moderate high-fat (45%) diet induced increased weiht after 4-5 weeks of HFD feeding. This was preceded by a prompt and transient increase in circulatory G-CSF, neutrophils and monocytes, in G-CSF production by VAT and in increased production of neutrophils and monocytes in bone marrow. Of note, at the time where weight gain became significant, no signs of elevated circulatory neutrophils were evident in the mice.

A transient increase in circulating neutrophils has been reported previously in mice fed a high-fat (60%) diet.[15, 27] Our data shows that also a considerably lower fat content in the diet (45%) may lead to this transient increase in neutrophils and further makes it probable that an elevated transient level of circulating G-CSF preceding the increase in neutrophils is the cause. G-CSF is the prime signaling molecule for mobilizing neutrophilic granulocytes from the bone marrow into circulation.[28] The early and transient up-regulation of G-CSF in blood coincided with the transient increase in granulocytes measured in blood and in spleen during the first week of HFD feeding and may explain the increased level of neutrophils in circulation. Like others, [27] we did not find any changes in the proportions of myeloid progenitor cells upon HFD feeding. This is in contrast to Nagareddy et al. (2014) and Singer et al. (2014) who found increased numbers of myeloid progenitors concomitantly with increased number of neutrophils and monocyte in bone marrow of obese mice.[14, 15] Of note, these studies investigated either obese mice or ob/ob mice, where the increased number of circulating neutrophils and monocytes seem to be caused by metabolic conditions rather than a change in diet able to increase the endotoxin absorption.

The cause of the increased blood G-CSF concentration was not established. However, one possibility is that the high content of dietary fat in the gut leads to an increased lymphatic flow, in turn leading to an increased influx of LPS from the intestinal lumen through the lymphatic system. An increased lipid content in the diet

is reported to increase the lymphatic flow[29] and may also increase the absorption of LPS.[1, 30] LPS may stimulate an increased production of G-CSF in various tissue[31] in turn leading to recruitment of granulocytes into circulation. We demonstrated here that VAT from mice fed HFD for 3-8 days had an increased production of G-CSF while at later time points, the production was reduced to the same level as VAT from mice on a standard diet. Other tissues than VAT may also induce increased production of G-CSF and thus contribute to a transiently increased G-CSF level in the blood. The most potent G-CSF-producing cells comprise monocytes and macrophages, but G-CSF is also produced by fibroblasts and endothelial cells [32], all being ubiquitously present in the various tissues of the body.

In the adipose tissue, we found a transient increase in the expression of *Hp* and *Stfa2l1* during the first week of HFD feeding indicating an increased number of neutrophils in the last differentiation stages.[23] This corresponds to the findings in earlier studies showing an early transient influx of neutrophils upon feeding a HFD.[17, 27] After this initial stage and after the G-CSF peak, we found a down-regulation of *Tlr4* expression in the HFD fed group at the time where mice on HFD had increased their weight compared to control mice, which might indicate a state of LPS tolerance, however such direct relation is purely speculative.

Apart from playing a key role in the mobilization of neutrophils from the bone marrow into circulation, G-CSF is important in the promotion of the granulocytic lineage, mainly neutrophils.[33] We found an increase in both neutrophils and monocytes in BM from HFD fed mice during the first week(s), and speculated that the increased G-CSF could stimulate the promotion of these cells. To investigate this, we stimulated freshly isolated BM cells with G-CSF and GM-CSF, alone or together with LPS, and found that in contrast to G-CSF and GM-CSF, LPS was a poor stimulator of neutrophils and monocytes; rather it seemed to halt the generation of neutrophils and had only modest effect on the generation of monocytes. Of note, the addition of G-CSF induced significant production of G-CSF in the bone marrow cells. LPS alone did not induce G-CSF production but slightly enhanced the production induced by G-CSF. Isolated BM cells are primarily comprised by hematopoietic cells but the presence of some endothelial cells cannot be excluded. The endothelial cells were previously identified as the only cells in BM producing G-CSF in response to LPS.[7] This is in agreement with our data showing only modest effect of LPS on G-CSF production. However, the

epithelial cell-produced G-CSF might induce G-CSF production in the myeloid cells thus leading to an enhanced effect and probably increased generation of neutrophils and monocytes as indicated from our results. In contrast to G-CSF, GM-CSF did not stimulate G-CSF production but together with LPS, the induction of both G-CSF and CXCL2 was enhanced. This illustrates the difference between the two growth factors; while GM-CSF is purely pro-inflammatory, G-CSF holds pro-inflammatory (through its neutrophil mobilizing and promoting property) as well as immune-regulating properties.[34]

The increased production of G-CSF readily ceased within the first week of HFD feeding indicating the action of immune-regulating properties. We did not demonstrate direct causality between the G-CSF and immune regulation *in vivo*, but we could show that G-CSF inhibited TNFα production while increasing IL-10 production *in vitro*. Especially monocytes and their derivatives expressing the G-CSF receptor are influenced by G-CSF in an anti-inflammatory way leading to attenuated production of pro-inflammatory cytokines upon LPS stimulation.[35] Ligation of the G-CSF receptor induces internalization and degradation of the receptor resulting in down-regulation of the responsiveness to G-CSF. Mice challenged with LPS after preteatment with G-CSF, were protected against the dose of LPS that killed non-pretreated mice through a mechanism involving inhibition of TNFα.[36] Blood taken from healthy humans injected with G-CSF responded with less cytokine production when stimulated *in vitro* with LPS[37] and volunteers pretreated with G-CSF before injection of minute doses of LPS exhibited lower levels of the pro-inflammatory cytokines IL-6 and TNF□.[38] Together, this points towards a strong anti-inflammatory activity of G-CSF, however, for how long the elevated serum G-CSF found in mice fed a HFD may prevent inflammation remains to be investigated as does the possibility of other mechanisms taking over in preventing HFD-induced inflammatory responses. To this end, IL-10 produced by monocytes and macrophages holds even stronger anti-inflammatory activity and influences monocytes as well as adipocytes[39, 40] and IL-10 from monocytes was recently demonstrated to induce epigenetic chances in adipocytes.[40] Whether IL-10 is involved in mechanisms causing establishment of long-term endotoxin tolerance is however purely speculative and the presented data calls for new studies that investigate a possible long term anti-inflammatory effect of G-CSF.

In summary, the presented data confirm previous reports showing that HFD fed to mice induce a transient increase in circulatory neutrophils and monocytes. This is paralleled with a transient peak in G-CSF. The VAT contributes to the transient production of G-CSF, which is followed by down-regulation of TLR4 expression. As G-CSF inhibits production of TNFα and up-regulates IL-10 production we suggest that the transient G-CSF peak stimulates endotoxin tolerance. This may confer an anti-inflammatory state to the body upon high-fat meals that may influence post-prandial responses and might contribute to the high variations seen in the inflammatory response to a single meal. Further studies are warranted to pursue the possible importance of G-CSF in post-prandial inflammatory response.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Legends to figures

Figure 1. *HFD induces a prompt transient rise in myeloid cells in the blood which is absent at the first signs of weight gain.* **A:** Body weight and **B:** VAT weight in mice fed high fat diet (HFD) or chow for 37 days (HFD n=8, control n=4 for each time point). Two-way ANOVA performed. Percentages of **C:** neutrophils and **D:** monocytes in blood as determined by CBC. All Control groups pooled n=20. One-way ANOVAs performed. **E:** Concentration of G-CSF in blood determined by ELISA. One-way ANOVA performed. All data is shown as mean +/-SEM, ***<0.001, **<0.01, *p<0.05.

Figure 2. *High fat feeding reveals an early and transient up-regulation of neutrophils in bone marrow.* **A:** Gating strategy for identification of neutrophils and monocytes in isolated bone marrow cells. **B:** The number of neutrophils ($Ly6C^+Ly6G^+CD11b^+$) and **C:** monocytes ($Ly6C^+Ly6G^-CD11b^+CD115^+$) in bone marrow from HFD fed mice and control mice, HFD n=8, C n=4, individual unpaired t-tests performed. **D:** Schematic overview over the expression of selected genes in various differentiation stages of neutrophil granulocytes. **E:** Expression of genes at different time points in the HFD fed group (n=8) compared to control mice (n=4) by unpaired t-tests performed on dCt values. Mean+/-SEM, **p<0.01, *p<0.05.

Figure 3. *High fat feeding induces an early transient up-regulation of neutrophils and monocytes in spleen.* **A:** Gating strategy for identification of neutrophils and monocytes in spleen. The proportion of **B:** neutrophils ($Ly6C^+Ly6G^+CD11b^+$) and **C:** monocytes ($Ly6C^+Ly6G^-CD11b^+CD115^+$) in spleen from HFD fed mice and control mice. **D:** Expression of genes at different time points in the HFD fed group compared to control mice by unpaired t-tests on dCt values. Mean+/-SEM, **p<0.01, * p<0.05.

Figure 4. *HFD induces a prompt but transient increase in the production of G-CSF from adipose tissue.* The concentration of **A:** G-CSF and **B:** GM-CSF in supernatant from cultured isolated VAT as measured by ELISA. Concentration in the HFD fed group compared to the average of all control mice by One-way ANOVAs. Mean+/-SEM, * p<0.05.

Figure 5. *HFD increases simultaneous expression of pro-inflammatory and anti-inflammatory genes in adipose tissue.* Expression of genes in VAT at different time points in the HFD fed group compared to the average of all control mice by One-way ANOVA on dCt values. Mean+/-SEM, ***<0.001, **p<0.01, * p<0.05.

Figure 6. *LPS enhances the G–CSF and GM-CSF-induced G-CSF, which holds anti-inflammatory properties.* The number of **A:** neutrophils ($Ly6G^+Ly6C^+CD11b^+$) and **B:** monocytes ($Ly6C^+Ly6G^-CD11b^+CD115^+$) after stimulation with LPS alone or together with G-CSF or GM-CSF of bone marrow for 1 or 4 days relative to the number measured in unstimulated bone marrow cells on Day 1. **C:** The concentration of G-CSF**,** GM-CSF and CXCL-2 in the supernatant of bone marrow cells after stimulation with LPS alone or together with G-CSF or GM-CSF for 1 or 4 days. **D:** The production of TNFα and IL-10 in LPS stimulated dendritic cells +/-preincubation with G-CSF.

Authors contributions: SMB and HF planned and supervised the study, HMSE, LM, CHL performed the experimental work, HMSE and HF wrote the manuscript, all authors critically reviewed and accepted the manuscript.

Table 1: Taqman assays used for gene expression analysis

Gene	Gene name	Assay ID
Actb	Actin, beta	Mn00607939_s1
Elane	Neutrophil Elastase	Mm01168929_g1
Arg1	Arginase 1	Mm00475988
Ccl2	Chemokine (C-C motif) ligand 2	Mm00441242_m1
Cxcl2	Chemokine (C-X-C motif) ligand 2	Mm00436450_m1
Hp	Haptoglobin	Mm00516884_m1
Stfa2l1	Stefin A2 like 1	Mm04212095_mH
Foxp3	Forkhead box P3	Mm00475162_m1
Tlr4	Toll-like receptor 4	Mm00445273_m1
S100A8	S100 calcium binding protein A8 (calgranulin A)	Mm00496696_g1
Itgam/CD11b	Integrin alpha M	Mm00434455
CD14	Cluster of Differentiation 14	Mm00438094
CD8a	Cluster of Differentiation 8 a	Mm01182107-g1
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1	Mm00802529_ml

Figure 1

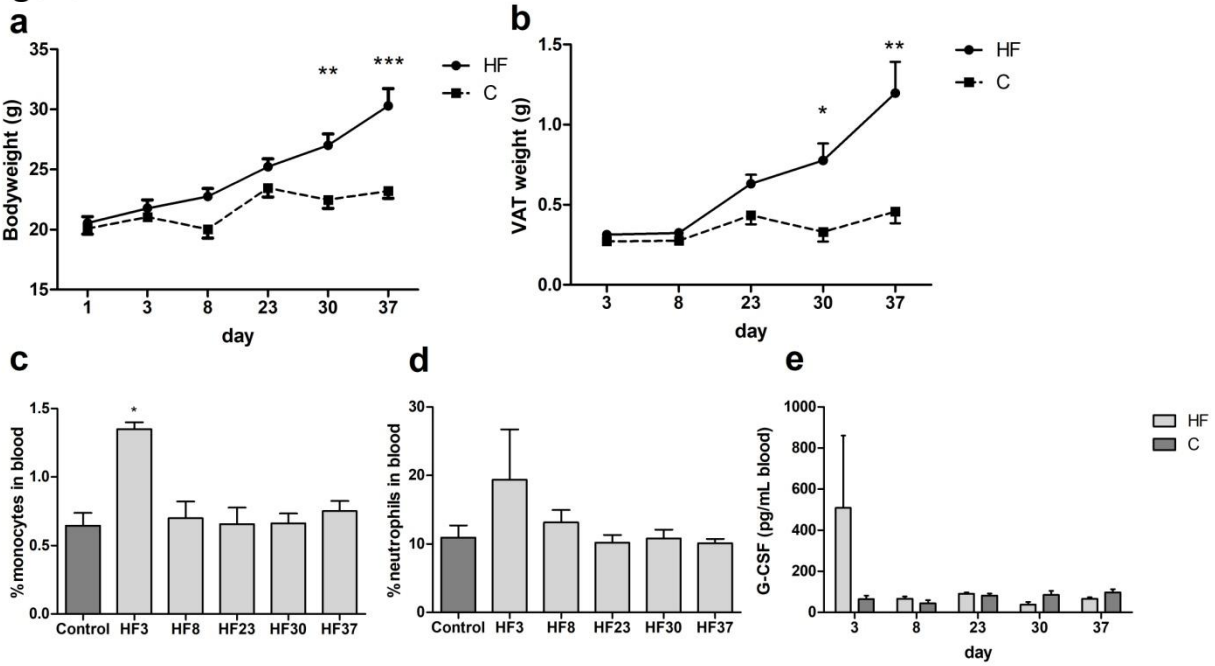
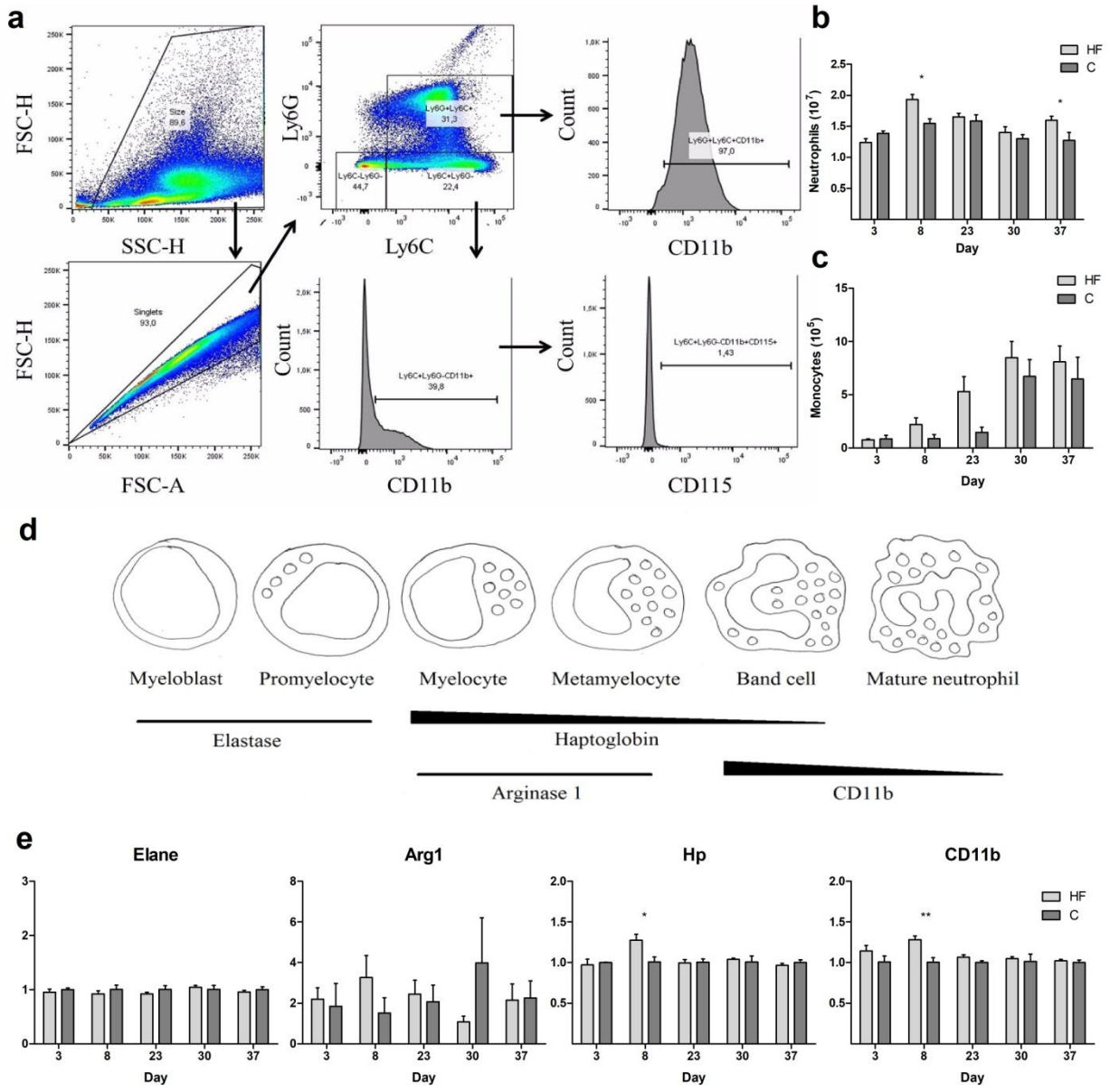


Figure 2


a
FSC-H
SSC-H
Size
89,6
Ly6G
Ly6C
Ly6G+Ly6C+
31,3
Ly6C-Ly6G-
44,7
Ly6C+Ly6G-
22,4
Count
CD11b
Ly6G+Ly6C+CD11b+
97,0
FSC-H
FSC-A
Singlets
93,0
Count
CD11b
Ly6C+Ly6G-CD11b+
39,8
Count
CD115
Ly6C+Ly6G-CD11b+CD115+
1,43
b
Neutrophils (10^7)
HF
C
Day
c
Monocytes (10^5)
HF
C
Day
d
Myeloblast
Promyelocyte
Myelocyte
Metamyelocyte
Band cell
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Elane
Arg1
Hp
CD11b
HF
C
Day

Figure 3

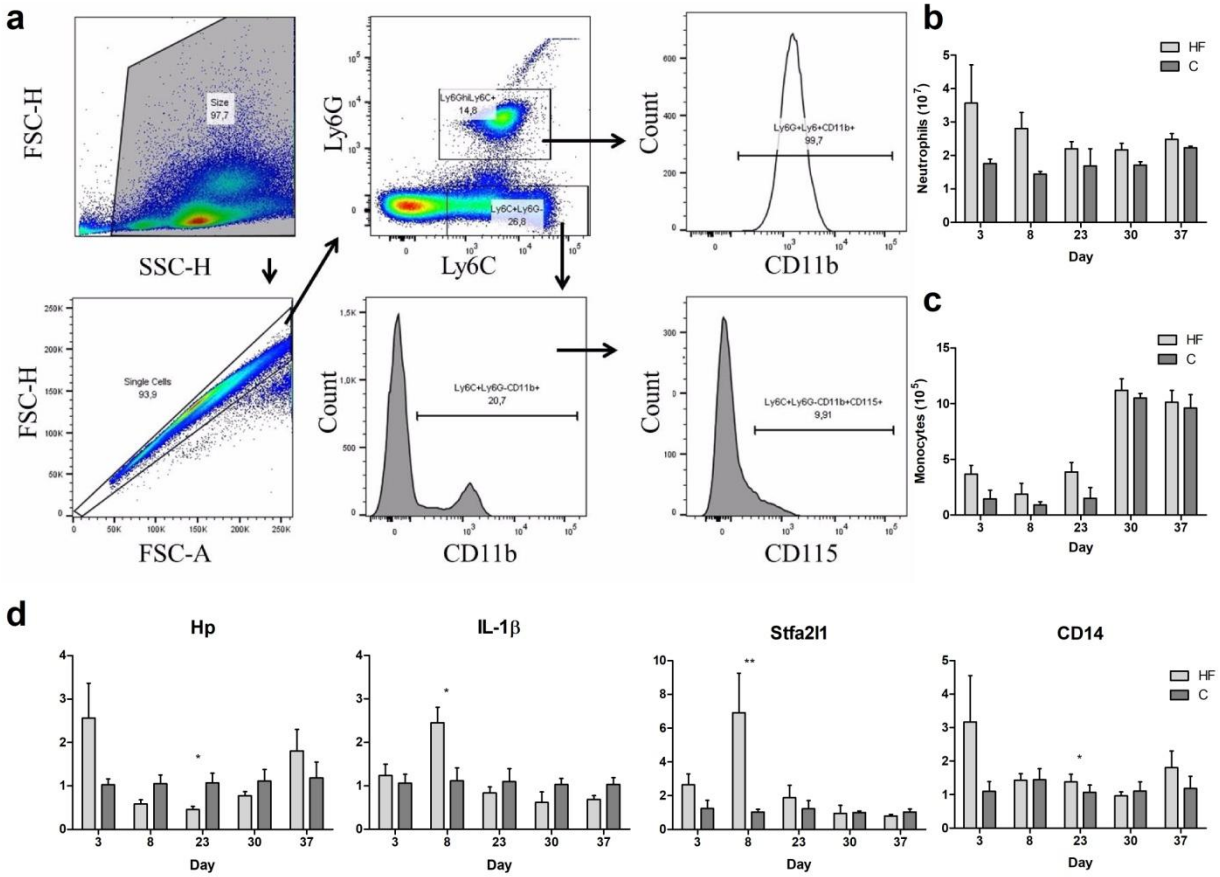


Figure 4

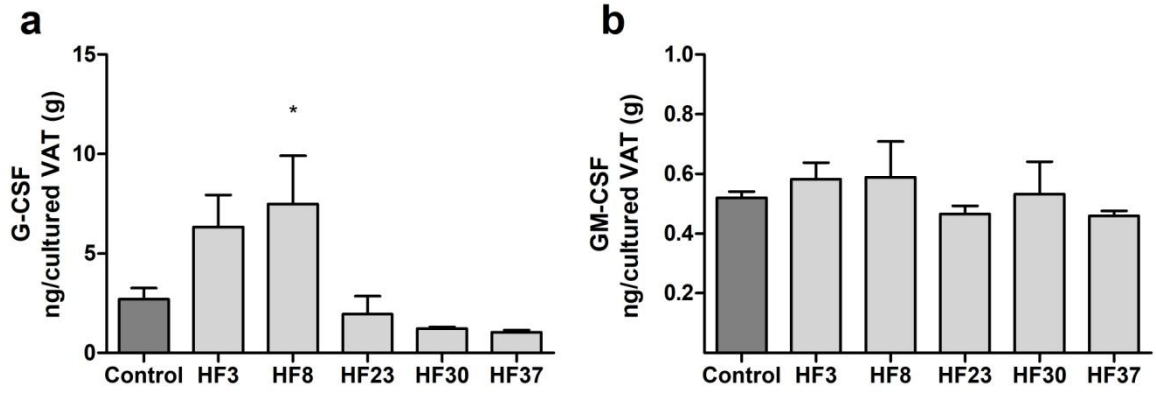


Figure 5

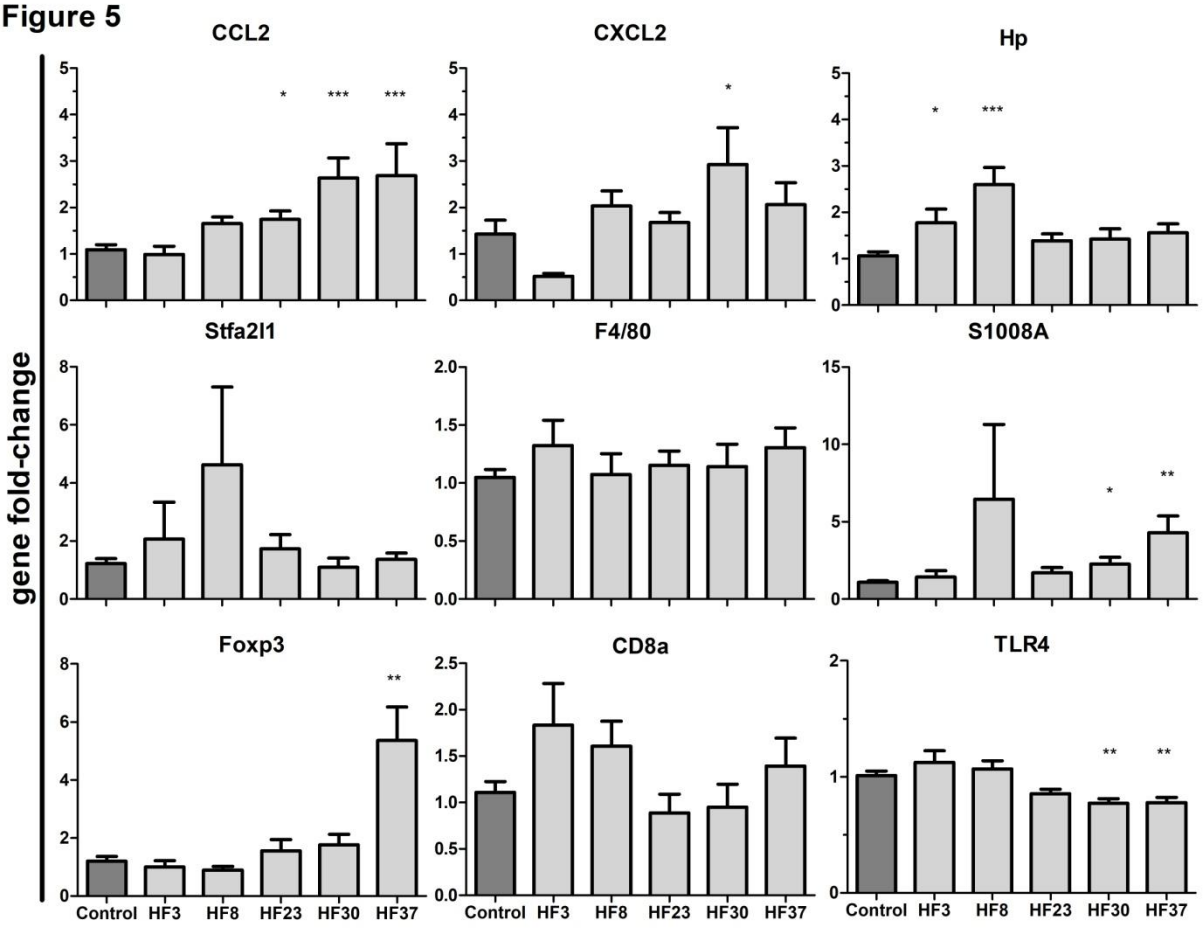
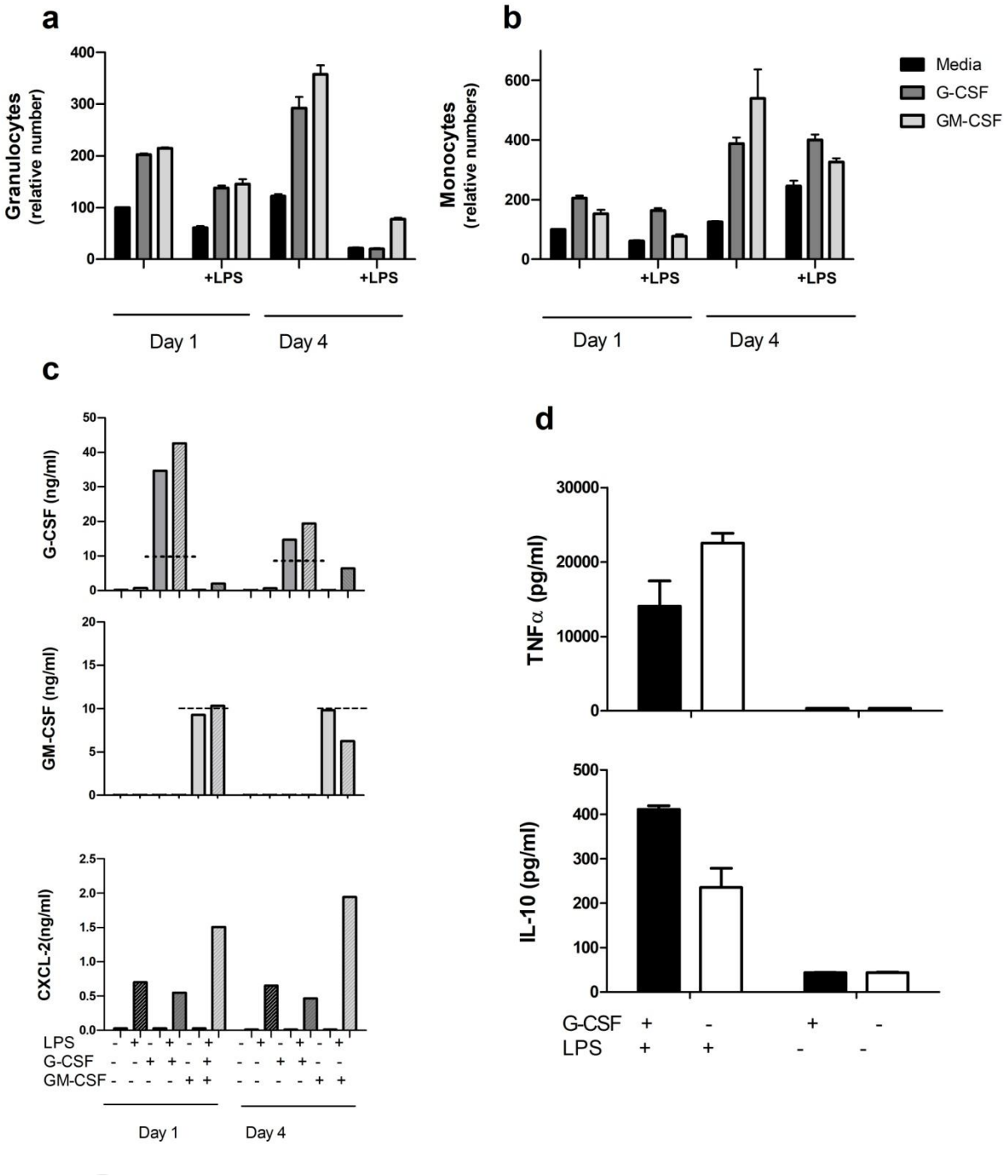
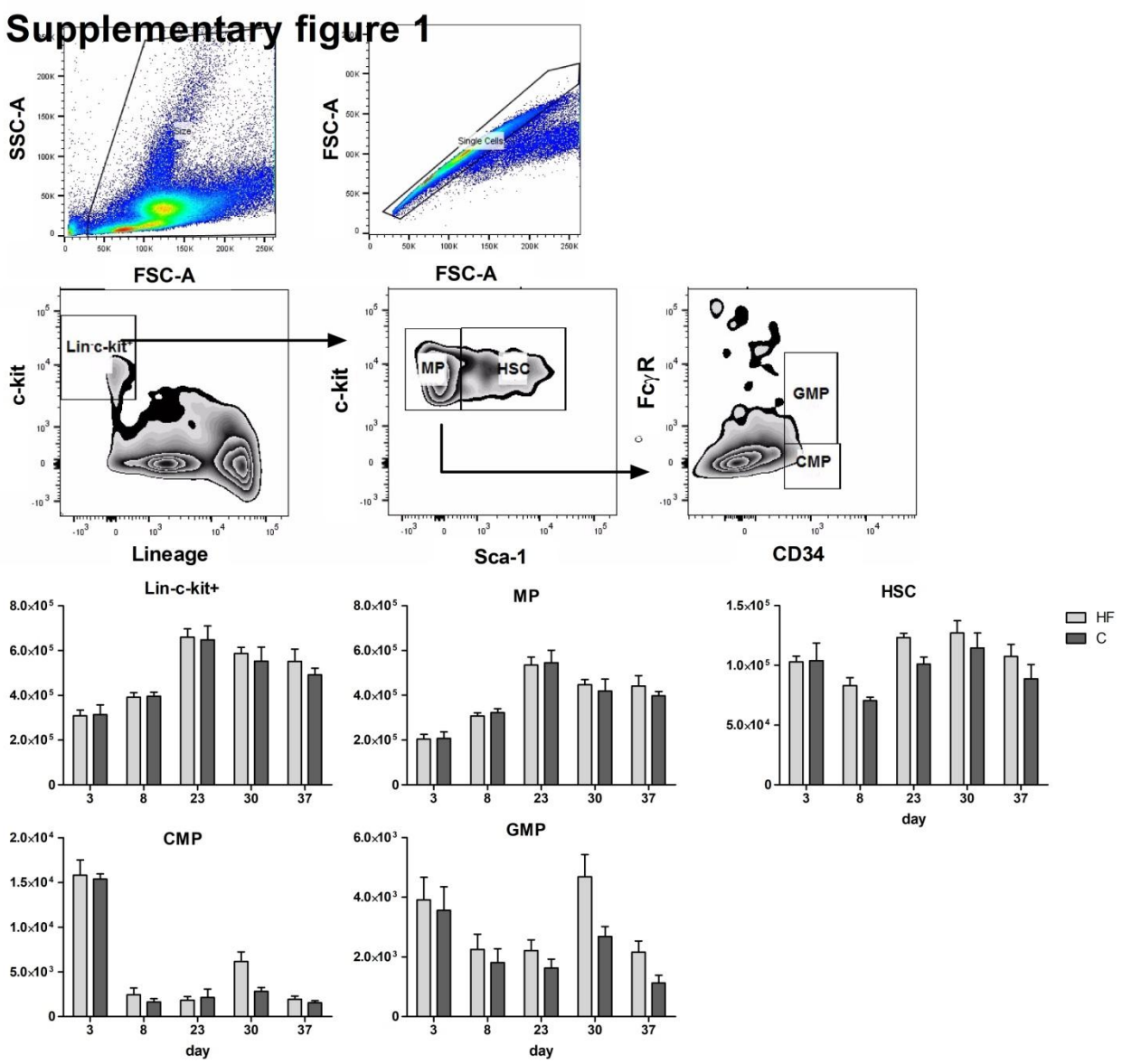


Figure 6


a
Granulocytes (relative number)
400
300
200
100
0
+LPS
+LPS
Day 1
Day 4
b
Monocytes (relative numbers)
600
400
200
0
+LPS
+LPS
Day 1
Day 4
Media
G-CSF
GM-CSF
c
G-CSF (ng/ml)
GM-CSF (ng/ml)
CXCL-2(ng/ml)
LPS
G-CSF
GM-CSF
Day 1
Day 4
d
TNFα (pg/ml)
30000
20000
10000
0
IL-10 (pg/ml)
500
400
300
200
100
0
G-CSF + - + -
LPS + + - -



Supplementary figure 1: Flow cytometric analysis of bone marrow cells in early differentiation stages with gating stratedy. Bone marrow cells from mice at different time points and from high fat fed and control mice were isolated and stained for the following surface markers: C-kit (CD117) , Sca-1, CD34 and Fc-□(DC16