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List of abbreviations

BM	bone marrow
BMDM	bone marrow derived macrophage
CSF1	colony stimulating factor 1
CSF1R	colony stimulating factor 1 receptor
DEX	dexamethasone
IGF1	insulin like growth factor 1
LBW	low birth weight
PBS	phosphate buffered saline

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Abstract

Signaling via the colony stimulating factor 1 receptor (CSF1R) controls the survival, differentiation and proliferation of macrophages. Mutations in CSF1, or CSF1R in mice and rats have pleiotropic effects on postnatal somatic growth. We tested the possible application of CSF1-Fc as a therapy for low birth weight (LBW) at term, using a model based upon maternal dexamethasone treatment in rats. Neonatal CSF1-Fc treatment did not alter somatic growth, and did not increase the blood monocyte count. Instead, there was a substantial increase in the size of liver in both control and LBW rats, and the treatment greatly exacerbated the lipid droplet accumulation seen in the dexamethasone LBW model. These effects were reversed upon cessation of treatment. Transcriptional profiling of the livers supported histochemical evidence of a large increase in macrophages with a resident Kupffer cell phenotype, and revealed increased expression of many genes implicated in lipid droplet formation. There was no further increase in hepatocyte proliferation over the already high rates in neonatal liver.

Conclusion: Treatment of neonatal rats with CSF1-Fc caused an increase in liver size and hepatic lipid accumulation, due to Kupffer cell expansion and/or activation rather than hepatocyte proliferation. Increased liver macrophage numbers and expression of endocytic receptors could mitigate defective clearance functions in neonates.

New and noteworthy

This study is based upon extensive studies in mice and pigs of the role of CSF1/CSF1R in macrophage development and postnatal growth. We extended the study to neonatal rats as a possible therapy for low birth weight. Unlike our previous studies in mice and pigs, there was no increase in hepatocyte proliferation, and no increase in monocyte numbers. Instead, neonatal rats treated with CSF1 displayed reversible hepatic steatosis and Kupffer cell expansion.

Introduction

Signals initiated following binding of macrophage colony-stimulating factor (CSF1) or interleukin 34 to a shared receptor (CSF1R) control the survival, differentiation and proliferation of cells of the mononuclear phagocyte lineage (6, 21, 24). Mutation of CSF1 in rats or mice produces a global deficiency of macrophage numbers in most tissues, whereas interleukin 34 appears to be required more specifically for macrophages of the brain (microglia) and skin (Langerhans cells) (55). Mutation of the receptor, CSF1R, which ablates the response to both ligands, has a more penetrant phenotype in mice (8) and rats (CP, DAH et al. Ms under review). The requirement for continuous CSF1R signalling is retained in adult mice, in that treatment with an anti-CSF1R antibody depletes tissue macrophages from the majority of organs (26). However, the availability of CSF1R ligands *in vivo* is not saturating. Administration of recombinant human CSF1 to mice expanded the circulating blood monocyte and tissue macrophage populations (22). These studies subsequently led to confirmation of biological efficacy in human patients (Reviewed in (21)). The circulating CSF1 concentration is controlled by CSF1R-mediated endocytic clearance by macrophages of the liver and spleen (3), providing a homeostatic loop in which tissue macrophages control monocyte production from the bone marrow (BM) (24). Accordingly, anti-CSF1R treatment or mutation of the receptor increases circulating CSF1 concentration (8, 26). Macrophages throughout the body occupy defined niches or territories (17) and the local availability of CSF1 in tissues may be one determinant of the size/boundary of those territories and local self-renewal of macrophages (24).

Mutation of CSF1 or CSF1R in mice or rats produces a severe postnatal growth retardation due, at least in part, to diminished production of the somatic growth factor, insulin like growth factor 1 (IGF1). Hence, CSF1/CSF1R could be considered part of the growth hormone/IGF1 axis (15). Consistent with that hypothesis, treatment of newborn mice with recombinant CSF1 produced an increase in somatic growth rate 30 days after birth, associated with increased *Igf1* expression (1). To increase the circulating half-life, and potentially efficacy of CSF1, we produced a pig CSF1-Fc fusion protein (14). Pig CSF1 is equally active in humans and mice (13), and the pig provides a pre-clinical model in which to evaluate therapeutic potential (9).

CSF1-Fc treatment of adult mice produced a rapid increase in the size of the liver associated with extensive hepatocyte proliferation (14). The *Csf1r* gene is expressed solely in cells of the macrophage lineage, and the transcriptional regulation has been studied in detail (39). A *Csf1r*-EGFP marker provides a marker for Kupffer

cells in the liver (44) and treatment of mice with a neutralising anti-CSF1R antibody completely depletes the transgene-positive Kupffer cell population (26). By contrast, hepatic parenchymal cells *in vivo,* or *in vitro*, do not possess any detectable CSF1 binding activity (3). Hence, the effects of CSF1-Fc on the liver must be mediated indirectly through interactions between macrophages and other liver cells. Subsequent studies confirmed that the liver controls circulating CSF1 concentration in humans and CSF1-Fc treatment can promote liver regeneration in mouse models (50). CSF1-Fc treatment also promoted liver growth when administered to pigs (46). Based on these findings, CSF1 was proposed to be a significant component of the so-called hepatostat (46), which controls the homeostatic size of the liver (30). Treatment of newborn piglets with CSF1-Fc did not produce the increase in body weight gain that had previously been observed in mice (46). Pigs are a precocial species, and commercial animals have been genetically selected for very rapid growth rate. We, therefore decided to investigate the impacts of CSF1-Fc in rats.

Our initial experiments suggested that CSF1-Fc treatment did not increase postnatal growth in rats, but we did not determine whether this was because the CSF1 was inactive, or because it was already saturating. We considered the possibility that CSF1-Fc might be more efficient in animals with LBW. To this end, we utilized a well-studied model of maternal stress in which pregnant rats are injected with glucocorticoids in the 3rd week of pregnancy (10, 11). This treatment produces lifelong impacts on several organ systems, notably predisposing to lipid accumulation in the liver (20, 53). Here we show that pig CSF1-Fc is active in neonatal rats, and produces a significant expansion of mononuclear phagocytes in the liver and spleen, associated with hepatosplenomegaly. However, the treatment also caused an unexpected, large, but reversible increase in fat deposition in the liver.

Experimental Procedures

Rats

The experiments were carried out under the authority of a UK Home Office Project Licence under the regulations of the Animals (Scientific Procedures) Act 1986, approved by The Roslin Institute and The University of Edinburgh Animal Welfare and Ethical Review Body. Rats were housed in Techniplast GM1500 Green Line individually ventilated cages lined with Eco pure aspen chip (2HK) and fed T.2914.12 irradiated 14% protein (Envigo, UK). Enrichment was provided with aspen chew sticks and sizzle pet (LBS Biotechnology, UK). Sprague Dawley rats were injected subcutaneously with 0.15mg/kg dexamethasone (Dexadreson) or PBS on days 14-21 of pregnancy. At parturition, pups were given to foster dams and injected subcutaneously with either 1 µg/g porcine CSF1-Fc (14) or PBS for 5 days. Injections occurred during the light cycle. Pups were weighed daily and blood was collected by cardiac puncture following sacrifice at day 6. A separate cohort was treated as above and culled on Day 32 (recovery experiment).

Bone marrow macrophage viability assay

Bone marrow (BM) was isolated from adult male Sprague Dawley rats. Cells were plated at 3×10^5cells/well of a 96-well plate in complete medium either without growth factors, with 10^4U/ml (100ng/ml) recombinant human CSF1 or various concentrations of porcine CSF1-Fc (pCSF1-Fc) and incubated at 37°C, 5% CO_2 for 7 days for macrophage differentiation. MTT was added directly to growth medium at a final concentration of 0.5mg/ml and the plate was incubated at 37°C for 3h. Solubilisation of tetrazolium salt was achieved with a solution of 10% SDS/50% isopropanol/0.01 M HCl at 37°C overnight. The plates were read at 570nm.

Histology and Immunohistochemistry

Spleen and livers were weighed then fixed in 10% buffered formalin and processed into paraffin by the Histopathology department at the Royal (Dick) School of Veterinary Studies using standard procedures. Slides were stained with Haematoxylin and Eosin (H&E). Immunohistochemistry (IHC) was performed with mouse anti-rat CD68 (Clone ED1, 1:500, Bio-Rad). For Oil Red O staining, formalin fixed livers were placed in 18% sucrose at 4°C overnight and cryosections prepared as described in (45). Staining was performed as described in (29). Sections were imaged using a Nanozoomer digital scanner and viewed using NDP.view 2

(Hamamatsu, Japan). Oil Red O staining was imaged with standard light microscopy using ZEN software (Zeiss).

Image Analysis

Image analysis was performed in ImageJ using 6 images per organ. CD68 staining was quantified in spleen and liver based on the threshold (1-150). The size and abundance of lipid particles in the liver were quantified using haematoxylin and eosin (H&E) stained sections by particle analysis (5μm- infinity and 0.45-1 circularity).

Blood analysis

All tests were performed by the Clinical Pathology department at the Royal (Dick) School of Veterinary Studies. Blood was collected into 0.5 ml EDTA tubes (Teklab). Total WBC, RBC, monocytes, lymphocytes and neutrophils were measured on the ABX Pentra 60 haematology analyser (Hariba Medical). EDTA-plasma was used for a Total Bile Acids detection kit (Diazyme) and measured on an ILab 650 Biochemistry Analyser.

Statistical analysis

Data were analysed using a Mann–Whitney test. Results are presented as box and whisker plots (the horizontal line within the box indicates the median, boundaries of the box indicate the 25^{th} and 75^{th} percentile, and the whiskers indicate the highest and lowest values of the results).Weights (days 0-6) were analysed using repeated measures two-way ANOVA. All analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc.,). A P value < 0.05 was considered statistically significant.

Microarray

RNA was extracted from rat liver using Trizol® (ThermoFisher Scientific) followed by purification using the Rneasy mini kit (Qiagen) according to the manufacturer's protocols. RNA integrity and quality were assessed using the RNA ScreenTape Kit on the Agilent 2200 TapeStation. Samples with a RNA integrity number greater than seven were used for microarray. Microarray was performed by Edinburgh Genomics (Edinburgh, UK) using Affymetrix Rat Gene 2.1 ST Array plates and Expression Console™ 1.4.1.46 was used for quality control following amplification.

The signal intensity of microarray results were summarized to probe sets and normalized using robust multi-array average (RMA) in R using the Affymetrix Transcription Analysis Console. The RMA normalized data was loaded into the network analysis tool Miru (Kajeka, UK) for further analysis alongside that of BM derived

macrophage (BMDM) data generated from Dark Agouti rats (unpublished). A Pearson correlation matrix of a gene-to-gene profile comparison was used to filter for expression correlation relationships of ≥ 0.96 across the microarray samples. Nodes within the network graph represent transcripts and the edges between them represent expression correlations above the set threshold. To identify groups of tightly co-expressed genes, the graph was clustered using the graph-based clustering algorithm MCL set at an inflation value (which determines the granularity of the clusters) of 1.8. Gene lists associated with the clusters were exported for GO annotation analysis (Biological and Metabolic Processes Level-FAT) using DAVID (Database for Annotation, Visualization and Integrated Discovery). GEO accession No. GSE104584. CIBERSORT analysis (http://cibersort.stanford.edu/, (32)) was performed using the LM22 expression matrix, which contains expression signatures for 22 human haematopoietic cell types/states, and default settings. The macrophage-lineage expression profiles in the LM22 matrix are derived from freshly isolated monocytes from peripheral blood mononuclear cells, unpolarized macrophages (M0, generated by differentiation of monocytes in human serum for 7 days), classically activated macrophages (M1, generated by differentiation of monocytes in CSF1 for 7 days followed by stimulation with LPS and IFNγ for 18 h) and alternatively activated macrophages (M2, generated by generated by differentiation of monocytes in CSF1 for 7 days followed by stimulation with IL-4 for 18 h) (32). Deconvolution of liver samples was associated with global p-values of 0.0 (for CSF1-Fc-treated samples) and 0.07-0.09 (for PBS-treated samples), indicating strong goodness of fit for the deconvolution.

Results

Pig CSF1-Fc is active on rat macrophages but does not affect post-natal growth

Prenatal dexamethasone (DEX) treatment of rats produces 8.5-25% lower birth weight in different studies (7, 33) when compared to vehicle injected rats. To establish the model, Sprague Dawley rats were injected on days 14-21 of pregnancy with either DEX or PBS. Injection of PBS was sufficient to reduce the average birth weight. DEX treatment lowered birth rate further, producing a 36% reduction relative to non-injected controls (Figure 1a). The activity of pig CSF1-Fc on rat BM was comparable to the human recombinant protein (Figure 1b). Newborn pups were injected with either PBS or 1mg/kg pCSF1-Fc subcutaneously from birth to postnatal day 5, weighed daily, and analysed on day 6. The smaller pups from PBS and dexamethasone-treated mothers showed a somewhat greater weight gain, apparently recovering their initial weight disadvantage. However, pCSF1-Fc treatment did not increase or decrease, weight gain in any of the treatment groups (Figure 1c).

Impact of CSF1-Fc treatment on circulating haematopoietic cells in neonatal rats

In mice and humans, there is a post-natal surge in serum CSF1 (40, 41). We considered the possibility that CSF1 levels in all rat pups may already be saturating, and/or that CSF1-Fc was not active in neonatal rats. Treatment of adult mice or weaner pigs with pCSF1-Fc greatly increased the number of circulating monocytes (14, 46), but we have not previously examined this in neonates. The blood monocyte numbers were marginally affected by the treatment (Figure 2). Changes in total blood leukocytes, lymphocytes and neutrophils were also marginal (Figure 2). However, CSF1-Fc was clearly active. In initial studies of infusion of CSF1 in human patients, the dose-limiting toxicity was thrombocytopenia (23), and we also observed reduced platelet numbers in mice and pigs treated with CSF1-Fc. In the CSF1-Fc-treated neonatal rats, there was a 70% reduced platelet count and a 20-30% reduction in red blood cells in all groups (Figure 2).

CSF1-Fc causes hepatosplenomegaly in neonatal rats

Treatment of adult mice with CSF1-Fc caused hepatosplenomegaly, associated with the proliferation of hepatocytes in the liver (14). Although the size of major organs did not differ between normal and LBW rats, CSF1-Fc caused a >30% increase in the relative size of the liver in each treated group (Figure 3). In adult mice, there is very little hepatocyte proliferation at steady state, and CSF1-Fc treatment caused a massive increase in staining with Ki67 or proliferating cell nuclear antigen (14). In weaner pigs, the baseline proliferation of hepatocytes was higher, but an increase in response to CSF1-Fc was still evident. In rat pups,

there was extensive staining for proliferating cell nuclear antigen, even in untreated animals, and no additional effect of CSF1-Fc was detected (not shown).

CSF1-Fc treatment increases lipid accumulation in neonatal rats

Examination of liver histology revealed the likely reason for the increased size of the liver in response to CSF1-Fc treatment, as a large accumulation of lipid droplets was evident in each group (Figure 4a). Consistent with published data (42), lipid accumulation was evident in untreated pups from DEX-treated mothers. The abundance and apparent size of these droplets were further exacerbated by CSF1-Fc treatment (Figure 4a-c). The impact of CSF1-Fc treatment was confirmed by Oil-Red O staining (Figure 4d). Lipid accumulation in the liver is commonly accompanied by accumulation of bile acids, which have themselves been attributed roles in fetal liver haematopoiesis (49). In all of the treated groups, there was a 2-3 fold increase in the circulating bile acids in response to CSF1-Fc treatment (Figure 4e).

CSF1-Fc increases hepatic and splenic macrophage numbers

In the light of the relative lack of impact of CSF1-Fc on blood monocyte counts, we investigated whether the treatment increased tissue macrophage numbers in the liver and spleen. As shown in Figure 5, there was a 3-4 fold increase in $CD68^+$ positive cells in the liver and a smaller increase, from a higher basal level, in the spleen. Low birth weight following maternal dexamethasone treatment did not affect macrophage numbers in either organ, or the response to CSF1-Fc.

CSF1-Fc-regulated gene expression in neonatal rat liver

In adult mice and weaner pigs, the mechanism(s) underlying the impact of CSF1-Fc treatment on liver growth was dissected by array profiling intact liver in parallel with BMDM (14, 46). Analysis of the data as a network graph, using Biolayout Express^{3D} (now Miru), enabled the identification of clusters of genes that were induced by CSF1, including those that are shared with BMDM and those induced specifically in the liver. The latter set would include genes that are expressed uniquely by the macrophages of the liver (28). We generated mRNA expression array profiles for rat BMDM and for the livers from control (PBS), and CSF1-Fc treated control rats (born to PBS treated dams). The complete primary dataset is provided in Table S1, with average expression of each transcript and fold-change between PBS and CSF1-Fc treated rat livers. The set of around 650 transcripts increased at least 2-fold in the liver by CSF1-Fc treatment (Table S1) is consistent with the 3-4 fold increase in macrophage content shown in Figure 5. It includes markers such as *Adgre1* (F4/80), *Fcgr1a* (Cd64) and *Cd14* and multiple transcription factor genes (*Fli1, Nr1h3, Tfec, Elf4, Irf8, Spi1, SpiC,*

Runx1, Runx3, Klf4, Etv1, Etv5, Irf5, Fos), implicated in regulated expression of Csf1r and/or macrophage differentiation (39). None of these transcription factors was enriched in the liver relative to BMDM. *Id3*, which was implicated in Kupffer cell differentiation in mice (28) was constitutively expressed in neonatal rat liver and not elevated further by CSF1-Fc suggesting it is not specifically associated with the macrophage population. To gain insight into the broad impact of CSF1-Fc treatment on liver macrophages, and other haematopoietic cell populations, we used CIBERSORT, a tool for characterizing cellular composition of complex tissues from gene expression profiles (32). Livers from control mice were predicted to contain a higher proportion of monocyte-like cells compare to treated mice, which may reflect liver macrophage immaturity in neonatal rats (Figure 6a). CSF1-Fc treatment led to a significant reduction in the monocyte expression signature, with a concomitant increase in mature macrophage (M0) and alternatively activated macrophage (M2) signatures (Figure 6a). No significant changes in other liver haematopoietic cell populations were observed, confirming the specific impact of CSF1-Fc on the macrophage lineage.

Gene set enrichment analysis (GSEA) (Figures 6b-d) revealed relative enrichment of cell cyle and inflammation-related terms in the CSF1-Fc treated livers, and of lipid and xenobiotic metabolism in control livers. A network graph created with Miru generated similar liver- and macrophage-associated clusters to conventional hierarchical clustering (Figures 7a-b). The average profiles of the 4 largest clusters and representative transcripts are shown. The complete transcript list for each of these clusters is provided in Table S2. The implications of the set of inducible genes and each of the clusters and their relationship to the macrophage recruitment and accumulation of lipids are discussed below.

CSF1-Fc-induced macrophage expansion, hepatosplenomegaly and lipid accumulation is reversible

Having observed the impact of CSF1-Fc treatment on the growth of the liver and fat deposition, we asked if the effect was reversible upon cessation of treatment. Control and treated animals were allowed to recover for an additional 27 days following treatment. As shown in Figure 8, at this time there was no longer any difference between the groups in terms of relative liver and spleen size, the content of $CD68^+$ cells in the spleen was normalized and fat deposition in the liver was no longer evident.

DISCUSSION

Concentrations of CSF1 were found to be higher in fetal than in maternal blood throughout mouse gestation and to peak around the time of birth, at which time there was also a peak of CSF1 protein in the liver (41). In humans, CSF1 is also elevated in the embryonic circulation relative to the maternal, and there was a two-threefold increase in the first few days after a full term birth (40). In the current study, we have examined the possible roles of that increase in available CSF1 by administering an exogenous source for the first 5 days of life in rats.

The FANTOM5 consortium produced detailed time courses of the mRNA expression profile of developing mouse organs based upon Cap Analysis of Gene Expression. Analysis of these data revealed a signature of increased macrophage content with time of development (51). The macrophages of the mouse liver display a unique gene expression profile including endocytic receptors (*Clec4f* and *Timd4*). Their expression in the liver escalated rapidly between neonatal day 0 and day 7, consistent with a role for the postnatal surge in the liver and circulating CSF1 in the expansion of this population. Correlation-based network analysis of gene expression profiles of CSF1-Fc-treated and control livers from neonatal rats, alongside BMDM, revealed 4 clear clusters of liver- and macrophage- associated genes. Cluster 3 (Figure 7b Table S2) contains transcripts that were relatively low or absent in BMDM, higher in liver from control mice, and elevated 4-5 fold further in response to CSF1-Fc. The cluster includes *Clec4f*, *Marco, Vsig4* and *Timd4* identified in mice as definitive markers of resident Kupffer cells, compared to monocytes and monocyte-derived macrophages (47, 54). The cluster also contains Kupffer cell-associated transcripts, *Cd163*, ferroportin (*Slc40a1*) the haem transporter, *Slc48a1*, and haem oxygenase (*Hmox1*), involved in the elimination of senescent red cells and recycling of haem iron (31). Two other macrophage-related clusters were identified in the data. Cluster 4 (Figure 7b) was expressed highly in BMDM, and strongly-inducible in the liver in response to CSF1-Fc. The cluster includes *Cd68* (consistent with Figure 5), *Adgre1* (F4/80), *Csf1r* and *Mpeg1*. Cluster 1 (Figure 7b) contains macrophage-related genes (e.g. *Gpnmb*) that were strongly expressed in BMDM and induced to a lesser extent by CSF1-Fc treatment. Cluster 2 contains transcripts that were not detected in BMDM, and were reduced in CSF1-Fc-treated livers. The reduction in their expression is not simply due to dilution by the macrophage-associated transcripts. Hepatocyte-specific genes such as *Alb* and *Afp* were unaffected (Table S1).

In adult mice, CSF1-Fc treatment promoted the proliferation of both resident Kupffer cells and infiltrating monocytes (50). Monocyte recruitment depends upon *Ccl2* signalling via *Ccr2*, but, unlike in mice, neither gene was induced by CSF1-Fc in rats. Monocyte markers such as *Spn* (*Cd43*) (19) and *Ly6C* were readily detected in the liver, but were not increased further by CSF1-Fc (Table S1). CIBERSORT analysis identified a reduced monocyte expression signature in CSF1-Fc-treated livers (Figure 6a). Taken together with the large increase in expression of liver macrophage-specific genes such as *Timd4* and *Vsig4,* and the lack of a substantial increase in circulating monocyte numbers (Figure 2), these findings suggest that most of the increase in liver macrophages arises from proliferation of resident Kupffer cells. Some of the regulated genes in Table S1, such as *Cd163, Cd206*, and *Msr1 (Cd204*) have been regarded as markers of "M2"-macrophage polarization in rats (52) (12), and the inducible gene profile also includes members of the *Tgfb* family (*Tgfb1, Tgfb3*), which are M2-associated cytokines (57). However, *Cd163* is known to be expressed constitutively by Kupffer cells and many resident macrophages in rats (37). These genes are all expressed constitutively in the naïve liver, and they are not increased to any greater extent than the Kupffer cell markers or generic macrophage-associated transcripts such as *Cd14, Cd68, Adgre1* and *Csf1r.* By contrast, IL4 target genes such as *Tgm2, Arg1* and *Retnla* (27) were not increased in the treated liver, suggesting the "M2" expression signature detected in CSF1-Fc treated liver by CIBERSORT deconvolution analysis relates to the Kupffer cell profile mentioned above rather than IL4-mediated alternative activation. Accordingly, CSF1-Fc treatment appears to act solely to promote expansion and maturation of the resident macrophage population of the neonatal liver, reflected by increased mature macrophage expression signatures (M0, M2).

CSF1-Fc treatment did not increase hepatocyte proliferation in neonatal rats. Hence, the increase in liver size was mainly due to the extensive lipid droplet formation. Genes such as *Pcna*, the transcription factor *FoxM1* and cyclins (e.g. *Ccna2*), were expressed constitutively in both rat BMDM, which are actively proliferating, and in the liver, regardless of CSF1-Fc treatment (Table S1). Nevertheless, the GSEA in Figure 6 indicates enrichment for cell-cycle-associated genes in response to CSF1-Fc, which is likely to be associated with proliferation of the macrophages (50). In adult mice, the proliferation of hepatocytes is dependent in part upon inflammatory cytokines such as IL6 and TNF produced by incoming Ccr2-dependent monocytes in response to exposure to portal blood (14, 50). The increased expression of classical LPS-responsive pro-inflammatory genes was detectable in expression profiles of liver from CSF1-Fc treated mice and pigs (14, 50). By contrast, no induction of mRNA encoding these inflammatory cytokines was seen in the

CSF1-Fc treated neonatal rat livers. We suggest that the lack of inflammatory cytokine induction in neonatal liver reflects the absence of monocyte recruitment.

CSF1-Fc treatment alone was sufficient to promote lipid droplet accumulation, and the comparative gene expression profiles in Table S1 may give clues as to the underlying mechanism. The biogenesis of lipid droplets has been reviewed by Pol et al. (36). Most genes involved (e.g. *Acscl1,3* and *4*, *Aup1*, *Dgat2, Spg20, Cct1, Pemt, Perilipins (Plin2, Plin3) and Bscl2 (aka seipin*)) were expressed in neonatal rat liver, but were not increased further by CSF1-Fc (Table S1). One possible site of regulation is the uptake of fatty acids. However, the two most highly-expressed hepatocyte fatty acid transporters, encoded by *Slc27a2* and *Slc27a5,* were each somewhat down-regulated in CSF1-Fc treated livers, as were the major fatty acid binding proteins, *Fabp1*, *Fabp5* and *Fabp 7.* The fatty acid translocase, *Cd36*, was elevated 4-5 fold in the CSF1-Fc treated livers. *Cd36* is highly expressed in macrophages, however it is also implicated in fatty acid uptake in hepatocytes in rats (5) and mice (56); suggesting some of this induction may occur in hepatocytes. Hepatocyte-specific conditional deletion of *Cd36* in mice was shown recently to greatly attenuate lipid accumulation in two models of hepatic steatosis (56). The active cholesterol transporter, *Abcg1* (34), less well-studied cholesterol-sensitive transporter, *Abca9* (35), phospholipid transfer protein (*Pltp*) (2) and the insulin-converting enzyme*, Pcsk1*, linked to regulated lipid droplet accumulation in adipocytes (43), were also up-regulated in response to CSF1. Downstream of lipid uptake, Acyl-CoA synthesis is an essential event in lipid droplet formation, in which the Acsl family are implicated (36). The long and short chain acyl-coA synthetases, *Acsbg1, Acsf2* and *Acss1,* and the remodeling enzyme *Lpcat2* (18) were each strongly increased by CSF1-Fc treatment. Ceramide kinase (*Cerk*), an upstream regulator of PLA2 activation and lipid droplet formation (16) was also increased >2-fold by CSF1-Fc. The set of genes down-regulated by CSF1-Fc (Cluster 2) may also contribute to lipid droplet formation through alterations in lipid metabolism. For example, *Cyp27a1* controls the generation of the cholesterol derivative, 27-hydroxycholesterol, and knockout of the gene is associated with hyper-triglyceridemia (38). Cluster 2 includes the master regulator, *Fgf21*, which is highly-expressed in neonatal liver, and reduced nearly 4-fold by CSF1-Fc. Fgf21 has been implicated in control of numerous lipid/obesity-related liver pathologies (25). The bile acid receptors, *Nr1h4 (Fxr) and* Gpbar1 *(Tgr5),* are also within this cluster, and might be subject to feedback regulation by elevated bile acids. Many of the genes in Cluster 2, notably the cytochrome P450 enzymes such as Cyp27a1 and Cyp2e1, have a centrilobular enrichment in adult mouse liver (4).

In adult mice, treatment with CSF1-Fc mimicked many effects of insulin, producing a marked down-regulation of genes encoding enzymes of gluconeogenesis, fatty acid oxidation and amino acid catabolism (14). This was not evident in the treated neonatal rats, where CSF1-Fc treatment did not regulate known targets of insulin repression, including the insulin receptor itself (*Insr*), the transcription factor *FoxO1* and downstream targets such as the signal transducer *Irs2*, gluconeogenic enzymes *Pck1* and *G6pase* tyrosine amino transferase (*Tat*), *Igfbp1* and *ApoC2* (58), indicating that insulin signaling is unaltered. We suggest that the difference between the effects of adult and neonatal CSF1-Fc treatments lies in the recruitment of monocytes and inflammatory cytokine production.

In overview, the transcriptional profiling of neonatal rat livers treated with CSF1-Fc highlights many genes that may control lipid droplet formation. Lipid droplet accumulation was not observed in adult mice, or pigs, in response to CSF1-Fc administration (14, 46, 50). In adult animals, CSF1-Fc caused a profound monocytosis and the expanded hepatic macrophage population was predominantly recruited monocytes. The response to CSF1-Fc in the neonates suggests that lipid accumulation is controlled at least in part by resident Kupffer cells. In that case, treatment with anti-CSF1R, which rapidly depletes Kupffer cells (26), could have some benefit in reversing lipid accumulation. The focus on lipid droplet formation, which we have shown is rapidly reversible, potentially neglects the potential benefit of such a treatment. In the context of models of acute liver failure, CSF1-Fc treatment rapidly expanded the Kupffer cell population and restored the capacity for removal of potential infections arriving in the portal blood (50). Neonatal sepsis is one of the main causes of morbidity and mortality, attributed in part to the immaturity of the neonatal immune system (48). We have shown that CSF1-Fc treatment of neonatal rats can produce a rapid increase in Kupffer cell numbers, independently of monocyte recruitment, and regardless of prior maternal stress and LBW. The inducible genes in Table S1 and S2 include numerous pattern recognition and endocytic receptors (e.g. *Siglec1, Siglec5, Nlrp3, Nlrc4, Mrc1, Tlr1,2,4,6,7, Cd163, Clec4a, Msr1, Marco, Timd4, Axl, Vsig4, Fcgr2a*), and the components of the phagocyte oxidase system (*Cyba, Cybb, Ncf2, Ncf1 and 4*). Accordingly, we suggest that CSF1-Fc treatment has potential to promote rapid maturation of innate immunity in neonates at high risk of infection.

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Figure Legends

Figure 1 – CSF1-Fc does not promote weight gain in neonatal rats.

(a) Rats were injected with PBS or Dexamethasone (DEX) on days 14-21 of pregnancy. Control dams were not injected. Box and whisker plots of birth weight. n=37-65. Results were analysed with a Mann-Whitney test. **** $p < 0.0001$. (b) Bone marrow from male rats was cultured in 100ng/ml recombinant human CSF1 (rhCSF1), porcine CSF1-Fc or without growth factor (cells only) for 7 days. MTT was used to assess cell viability. n=2 repeat experiments. Graphs show mean +SEM. (c) Offspring were injected with PBS or 1µg/g CSF1 on days 0 to 5 and weighed daily. Box and whisker plots of weight at day 6. n=7-19. Results were analysed by repeat measures two-way ANOVA (days 0-6).

Figure 2 – Effect of CSF1-Fc treatment on the blood of neonatal rats.

Rats were injected with PBS or Dexamethasone (DEX) on days 14-21 of pregnancy. Control dams were not injected. Offspring were treated with PBS or 1µg/g porcine CSF1-Fc on days 0 to 5. EDTA-blood was obtained from rats at day 6. n= 4-5 (control), 8 (PBS) and 11-15 (DEX). Results were analysed with a Mann Whitney test. P values are indicated on the box and whisker plots.

Figure 3 – Effect of CSF1-Fc treatment on organ weights of neonatal rats.

Rats were injected with PBS or Dexamethasone (DEX) on days 14-21 of pregnancy. Control dams were not injected. Offspring were treated with PBS or 1µg/g porcine CSF1-Fc on days 0 to 5. Organs were weighed at day 6. n= 7 (control), 8 (PBS) and 15-17 (DEX). Results were analysed with a Mann Whitney test. P values are indicated on the box and whisker plots.

Figure 4 – CSF1-Fc treatment causes lipid accumulation in livers of neonatal rats.

Rats were injected with PBS or Dexamethasone (DEX) on days 14-21 of pregnancy. Control dams were not injected. Offspring were treated with PBS or 1µg/g porcine CSF1-Fc on days 0 to 5.(a) Representative H&E sections of formalin-fixed paraffin embedded livers. ImageJ was used to quantify (b) the percentage area of lipids and (c) lipid size using 6 H&E images per liver. n= 7 (control), 11-12 (PBS) and 7 (DEX). (d) Representative Oil red O staining of frozen liver sections. (e) EDTA-plasma was tested for bile acids. n= 7-8

for all groups. All results were analysed with a Mann Whitney test. P values are indicated on the box and whisker plots.

Figure 5 – Treatment of neonatal rats with CSF1-Fc causes an increase in liver and splenic macrophages.

Rats were injected with PBS or Dexamethasone (DEX) on days 14-21 of pregnancy. Control dams were not injected. Offspring were treated with PBS or 1µg/g porcine CSF1-Fc on days 0 to 5. Organs were collected at day 6. Representative images of formalin-fixed, paraffin-embedded livers (a) and spleens (b) stained with an antibody against CD68. Sections shown are from neonatal rats born to PBS-injected dams. The percentage area of $CD68^+$ staining was calculated with ImageJ using 6 images per organ. n= 6-8 for all groups. All results were analysed with a Mann Whitney test. P values are indicated on the box and whisker plots.

Figure 6 – Gene Set Enrichment Analysis of neonatal rat livers.

Rats were injected with PBS on days 14-21 of pregnancy and offspring were treated with PBS or 1µg/g porcine CSF1-Fc on days 0 to 5. Livers were collected at day 6 for RNA isolation and subsequent microarray analysis. (a) Relative proportion of haematopoietic cell types in PBS- and CSF1-Fc-treated livers predicted using CIBERSORT. (b) Significantly enriched hallmark gene sets were determined by Gene Set Enrichment Analysis (FDR q-value <0.05). Example gene set enrichment plots for (c) CSF1-Fc and (d) PBS-treated neonatal rat livers. Expression data was ranked according to normalized expression in CSF1-Fc vs. PBS-treated liver (indicated by red-blue bars) and the gene set of interest was mapped onto this profile (black bars) to determine enrichment scores (green lines).

Figure 7 – CSF1-Fc –regulated gene expression in neonatal rat liver.

Rats were injected with PBS on days 14-21 of pregnancy and offspring were treated with PBS or 1µg/g porcine CSF1-Fc on days 0 to 5. Livers were collected at day 6 for RNA isolation and subsequent microarray analysis. (a) Normalized data were clustered with gene expression data from bone marrow derived macrophages (BMDM) by Spearman correlation using Miru. (b) Graphs show the average expression of clusters in BMDM, CSF1-Fc and PBS treated neonatal rats born to PBS-injected dams.

Figure 8 – Cessation of CSF1-Fc treatment reverts organ size, lipid accumulation and macrophage numbers to control levels.

Rats were injected with Dexamethasone on days 14-21 of pregnancy and offspring were treated with PBS or 1µg/g porcine CSF1-Fc on days 0 to 5. (a) Organs were collected and weighed 27 days following the last injection (b) Representative H&E sections of formalin-fixed, paraffin-embedded livers. (c) Box and whisker plots showing the percentage area of $CD68^+$ staining as calculated by ImageJ using 6 images per organ. n= 5 for all groups. (d) Representative CD68 immunohistochemistry of formalin-fixed, paraffin-embedded spleens.