The Expression of Inflammatory Genes in 3T3-L1 Adipocytes Exhibits a Memory to Stimulation by Macrophage Secretions

Dale Hancock, Luxi Meng, Mira Holliday, Nicole Yuwono, Ning Zhang, Gareth Denyer

School of Life and Environmental Sciences, University of Sydney, NSW 2006, Australia

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Corresponding Author:

Gareth Denyer

School of Molecular Bioscience

University of Sydney

NSW 2006

Australia

Phone: +61 2 9351 3466

Fax: +61 2 9351 4726

Email: gareth.denyer@sydney.edu.au

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ABSTRACT

Obesity is characterized by increased output of inflammatory compounds from adipose tissue. Whilst the relative contribution of adipocytes and resident macrophages to this phenomenon is debated, there is no doubt that the secretions of each cell type can stimulate the expression of inflammatory genes in the other. We hypothesized that mechanisms must exist to prevent an escalating positive feedback loop between the two cell types, so that after an initial exposure to macrophage secretions, adipocytes would become desensitized to subsequent inflammatory stimulation.

We used microarrays to investigate the response of 3T3-L1 adipocytes to macrophage secretions (macrophage conditioned medium, MCM). MCM caused a rapid (<4 hours) and high amplitude (over 100-fold) rise in the expression of several inflammatory genes. For some genes, generally cytokines, expression returned to basal levels within 24 h following removal of the MCM, but other transcripts, notably those for acute phase proteins and extracellular matrix remodeling proteins, remained highly expressed even during the washout period.

Unexpectedly, some cytokine genes (e.g., iNOS, IL-6) showed an enhanced expression to a second exposure of MCM, illustrating that the transcriptome response of 3T3-L1 adipocytes retains a memory to the first stimulus. We characterized the parameters that give rise to the memory phenomenon, finding that additional stimuli do not augment or abrogate the effect. The memory is preserved for several days after the initial exposure and it is not due to a change in sensitivity to the MCM but, rather, a change in the capacity of the signal-target system. The possible mechanisms of the memory are discussed, along with the physiological ramifications should the phenomenon be replicated *in vivo*.

INTRODUCTION

Two decades ago, the mechanistic relationship between elevated adipose tissue mass and obesity-related disease was unclear. It is now known that, in the obese state, adipose tissue secretes a wide range of inflammatory mediators, and that elevated systemic levels of these compounds contribute to both Type 2 Diabetes and cardiovascular disease, as recently reviewed by (1). A more complete understanding of how the production of inflammatory agents from adipose tissue is regulated is likely to lead to better therapeutic strategies for the treatment of obesity related disease.

Many different types of inflammatory secretions emanate from obese adipose tissue, and cytokines comprise one of the major groups. Both adipocytes and the macrophages resident in adipose tissue are potential sources of these cytokines and there is some controversy regarding which of these two cell types is mainly responsible for the release of most of the inflammatory hormones. This issue is especially difficult to unravel since, in the obese state, adipose tissue becomes infiltrated with macrophages (2)

The situation is further complicated by the fact that agents released from adipocytes can activate macrophages and *vice versa*. Incubation of adipocytes with the culture medium collected from M1-macrophages (macrophage conditioned medium, MCM) causes a rapid and high-amplitude rise in inflammatory gene expression in both human and mouse adipocyte cell lines, and that this rise is accompanied by increased secretion of cytokines (3) (4) (5) (6) and at least one of these cytokines (TNF α) independently induces similar responses (7) (8) (9). ‘Inflamed’ adipocytes not only secrete agonists that increase the expression and production of cytokines in macrophages, they even express proteins (like MCP-1) which attract even more macrophages (10). Clearly, these interactions have the potential to form a positive feedback loop that could exacerbate systemic inflammation.

Microarray studies have revealed that the response of adipocytes to MCM does not just involve the expression of cytokine mRNAs but is also characterized by rapid and sizeable rises in the levels of extra-cellular matrix remodeling transcripts and repression of adipocyte-specific genes (11) (12). Interestingly, despite the fact that these rises in mRNA levels are often >100-fold over basal, the changes are generally transient, even if the stimulatory milieu is maintained. This waxing and waning of gene expression is a common property in immune cells in response to inflammatory stimuli, and is caused by compensatory and counter-regulatory responses, with prolonged or prior exposure of many types of cells to hormones results in a dampening and loss of sensitivity to subsequent exposures. This is due, in part, to decreased receptor levels (13) (14) (15) and persistence of the post-stimulation dampening responses (16) (17).

Since adipocytes exhibit this kind of response to insulin, showing reduced insulin sensitivity after exposure to high insulin concentrations (13) (14) (18), we reasoned that fat cells might show a similar repression to further inflammatory stimuli after prior exposure to macrophage secretions. It is intuitive that such a mechanism should exist to prevent a rampant positive feedback loop between adipocytes and macrophages. In sharp contrast to these expectations, we describe here experiments that demonstrate quite the reverse: namely, that exposure to inflammatory stimuli leaves an imprint on fat cells that actually renders them more responsive to subsequent stimulation by macrophage secretions.

METHODS

3T3-L1 Cell Culture

Low passage number 3T3-L1 cells were cultured in DMEM (Life Technologies, Australia) which contained 25 mM glucose, 4 mM L-glutamine and 1 mM sodium pyruvate supplemented with 10% (v/v) fetal bovine serum (referred to as DMEM/FBS). Cells were cultured in 6 or 12 well plates in a humidified incubator at 37°C supplemented with 5% (v/v) CO₂.

Two days post-confluence, differentiation was induced with a cocktail of stimulants; 2 µg mL⁻¹ insulin, 0.5 mM isobutylmethylxanthine, 0.25 µM dexamethasone and 2 µM rosiglitazone (all purchased from Sigma) in DMEM/FBS. After three days, the differentiation medium was removed and cells were maintained in post-differentiation medium (DMEM/FBS with 2 µg mL⁻¹ insulin) for a further 6–9 days, with media changes every two days or as required. Differentiation was monitored both by microscopy and Oil-Red O staining.

Production of MCM

The murine monocyte cell line, RAW264.7 was employed to obtain macrophage-conditioned medium (MCM). Cells were cultured until confluence in DMEM/FBS in a CO₂ supplemented humidified incubator at 37°C. To stimulate an inflammatory response, the cells were treated with lipopolysaccharide (LPS, 50 ng/mL) for 4 h. At the end of this time the incubation medium was collected and filtered through a 0.22 µm filter (Millipore) to remove any debris and cells. The MCM was stored at -80°C until required. Any variations from this procedure are described in the relevant figure or table legend.

Memory Experiment setup

For the initial stimulation, 9-day differentiated 3T3-L1 cells were treated for 4 h with media containing MCM diluted 1:1 with DMEM/FBS. Following this, the medium was removed, and the cells were washed with PBS and then fresh DMEM/FBS was added to the wells for a further 24 h “washout” period. The medium was then removed and these cells were exposed to a second challenge with MCM diluted 1:10 with DMEM/FBS for 4 h. Total RNA was isolated from cells at 0, 1, 2 and 4 h after the addition of the second MCM challenge (28, 29, 30 and 32 h after the initiation of the first MCM stimulation).

Control cells, which had only been exposed to MCM for one challenge, were also prepared. These were age matched (10-day differentiated) 3T3-L1 cells from the same batch and passage number as above, and there were incubated with MCM diluted 1:10 with DMEM/FBS for 4 h. Total RNA was isolated at 0, 1, 2 and 4 h after the addition of the MCM. Any variations to this scheme are described in the relevant figure or table legend.

RNA Isolation

Total RNA was isolated by Trizol (Sigma-Aldrich, Australia), using a variation of the method of Chomczynski and Sacchi (19). Essentially the media was removed from each well and the adherent cells were washed once with PBS followed by lysis in Trizol reagent. The lysate was then extracted with chloroform (5:1 vol:vol) at room temperature. Following centrifugation at $12,000 \times g$ for 15 min the upper aqueous phase containing the RNA was removed and the RNA precipitated overnight at 20°C with 1.25 volumes of isopropanol. The pellet, collected after centrifugation at $12,000 \times g$ for 30 min, was washed twice with 75% (v/v) ethanol then re-suspended in nuclease-free water. The yield and purity of the RNA was estimated by UV spectrophotometry. The integrity of the RNA was further assessed by

denaturing agarose gel electrophoresis (1% (w/v) agarose in 2.2 M formaldehyde MOPS) (20) followed by ethidium bromide staining and visualization using a UV trans-illuminator.

Microarray analysis

Microarray analysis was performed on RNA samples in order to get a complete perspective of gene expression changes after MCM stimulation. Isolated RNA was treated for application onto Affymetrix GeneChip Gene Mouse 2.0 ST arrays by the Ramacotti Centre at the University of NSW, according to the manufacturers instructions. Microarray data was analyzed using an in-house designed package as has been employed in several other studies (21) (22) (23) (24) (25) (26) (27) (28) (29) (30).

cDNA synthesis and qPCR

Complementary DNA from each RNA preparation was synthesized with Reverse Transcriptase (Bioline, Australia) according to the manufacturer's protocol. 500 ng samples of total RNA were primed using random hexamers in 20 µL reaction mixtures containing 500 µM dNTP, 50 mM Tris HCl pH 8.6, 40 mM KCl, 5 mM MgCl₂, 1 mM MnSO₄, 1 mM DTT and 0.5 U/µL RNase inhibitor. Primers were annealed at 25°C for 10 min, cDNA synthesis was then carried out at 45°C for 30 min with 10 U/ µL reverse transcriptase followed by enzyme inactivation at 85°C for 5 min.

qPCR was performed in 20 µL reactions using SybrGreen (FAST SybrGreen master mix, Applied Biosystems, Australia) amplifying two different amounts of each cDNA preparation (10 ng/ reaction and 1.25 ng/ reaction) for each primer set. A two-step thermocycler program was employed using an Applied Biosystems 7500 Fast instrument. This was 10 min at 50 °C pre-incubation to remove any contaminating amplified product from previous PCR assays, then 95°C for 20 s followed by 40 cycles of 3 s at 95 °C then 30 s at 60 °C. A melt curve was performed on all reaction products. and each *Ct* normalized to its respective 18S value. As a *Ct* difference between the 10 ng reaction value and its corresponding 1:8 (1.25

164 ng) value of 3 indicates a quantitative, consistent amplification only samples which showed this *Ct*
 165 difference between dilutions were used in subsequent analysis. Primers were designed using a
 166 combination of the UCSC Genome browser (<https://genome.ucsc.edu/>) and Primer3 Plus
 167 (www.bioinformatics.nl/primer3plus) with at least one primer in each pair hybridizing across an exon-
 168 splice sit, thus restricting amplification products to processed transcripts.

RESULTS

A subset of adipocyte-expressed genes display a memory effect after the first of two challenges with MCM

To assess the effect of MCM on adipocytes in culture, we exposed mature 3T3-L1 cells to MCM for up to 24 hours, either with or without a washout of the stimulus at 4 h. Figure 1 shows that the transcriptional output of inflammatory targets in 3T3-L1 adipocytes responds to macrophage secretions in the same way as reported by others (31) (3) (11). The MCM caused a rapid (within 2 h) and high amplitude (>100-fold) rise in the levels of both IL-6 and iNOS transcripts, two key indicators of the adipocyte inflammatory response. Moreover, despite the continued presence of the MCM stimulus, the level of both these mRNAs decreased after 4 h of exposure. In neither case did transcript levels return to basal values, even after a further 24 h incubation. In contrast, when the MCM stimulus was removed and the cells incubated in fresh culture medium, the expression of both genes declined to pre-exposure levels within 8–12 h.

Although washout of the MCM stimulus appears to reverse the effects of the inflammatory exposure (at least with respect to iNOS and IL-6 expression), we were curious to know if the previously-exposed cells would behave differently to naive cells when exposed to MCM again. Figure 2 shows that cells previously exposed to MCM show an enhanced response to a second challenge. These results indicate that the first exposure to MCM leaves some imprint on the gene expression apparatus of iNOS and IL-6 in adipocytes.

We next asked whether other transcripts also exhibit this transcriptional memory. We performed microarrays on mRNA isolated from cells exposed to MCM for 0–4 hours, either as a primary stimulation (i.e., cells that had never seen MCM before) or as a secondary stimulation (i.e., after a previous 4-hour

exposure followed by a 24-hour washout). As observed by others (11) (31), the expression of several hundred genes was stimulated after MCM exposure (original data available in NCBI Gene Expression Omnibus). Of these, about 20 targets show a greater response to MCM after a previous exposure. Table 1 lists the genes that exhibited: a) a rise in response to the first MCM exposure of >4-fold at least one time point, b) a return to basal (or near-basal) levels of expression after washout, and c) a secondary response to MCM that was at least 2-fold greater than the first exposure at least one time point. In every case, the targets listed in Table 1 have at least one inflammatory ontological classification.

Whilst it was encouraging that iNOS and IL-6 were amongst the genes identified as displaying transcriptional memory in the arrays, only a single microarray was performed for each sample and so it was necessary to confirm these observations by more quantitative methods using a greater number of preparations. To this end, the experiment was repeated multiple times (as shown in the legends to individual tables and figures) and qPCR used to measure transcript levels. The behavior of all the targets measured (over half of those shown in Table 1) was verified using this approach. Figure 3 shows four representative genes (CCL2, IL-1a, TNF-a and PDH11A). In general, the secondary response shows the same kinetics as the primary response, following the same pattern of increase and decay, but the rise in expression either occurs sooner or is more exaggerated in amplitude.

Microarray analysis revealed a further class of targets whose expression does not decrease after removal of the MCM stimulus (Table 2). For these genes, the transcript levels remain elevated (and often even keep increasing) even after 24 hours of washout. In most, but not all cases, expression levels increased even more on secondary exposure. As before, we confirmed these patterns using qPCR in new samples; Figure 4 shows data for one example: SAA3. As with the genes that showed transcriptional memory in Table 1, the genes that show persistence of gene expression elevation even after removal of the MCM stimulus all have at least one ontology which classifies them as inflammatory.

Genes exhibiting Transcriptional Memory share only loose commonality in Promoter Properties

The promoters associated with the sequences in Table 1 and Table 2 were interrogated using the module in the Genomatix Software Suite (<https://www.genomatix.de>) that identifies common transcription factor binding sites. Analysis included all the members of Tables 1 (20 sequences) and Table 2 (21 sequences) except Masp1 for which information was not available.

There were 87 transcription factor binding sites (TFBs) that were common between at least 17 of the 20 genes in Table 1. Of these, a subset of 16 TFBs were characterised as being selectively concentrated in these genes in comparison to the mouse genome as a whole ($P < 0.0001$). Those with the most extensive commonality included AP4R (transcription factor AP4), HUB1 (HTLV-I U5 repressive element-binding protein 1), GFI1 (Growth factor independence transcriptional repressor), IKRS (Ikaros zinc finger family), BHLH (bHLH transcription factors) and ZF12 (C2H2 zinc finger transcription factor family).

Similarly, the promoters of the 20 genes from Table 2 showed a total of 64 types of TFB, of which 10 were identified as being distinct for this set. The three with the most penetration across the 20 genes were NRSF (Neuron-restrictive silencer factor), SF1F (Vertebrate steroidogenic factor) and STAF (Selenocysteine tRNA activating factor). There was some commonality in TFBs between the two tables, but not in any of the afore-mentioned genes.

Genes repressed by MCM exposure do not show Transcriptional Memory

Exposure of adipocytes to MCM reduced the expression of several genes, and these are shown in Table 3, with selected qPCR confirmation from separate biological preparations shown in Figure 5. The horizontal divider in Table 3 delineates transcripts that recover to pre-stimulation levels after the washout from those that remain repressed after removal of the first stimulus. The targets in Table 3 are generally associated with ontological groupings related to adipocyte biology. Interestingly, even if there was full

recovery of the transcript level during the washout period, the secondary response never showed an overtly faster or high amplitude response than the initial exposure.

Adipocytes retain but do not magnify the memory effect following multiple MCM challenges

Figure 6 shows the results of several rounds of MCM stimulation and washout on genes that show either an enhanced secondary response (iNOS) or persistent high expression after the first stimulus (SAA3). In the case of iNOS, the memory effect was retained after a second and third washout and so gave rise to accentuated responses (compared to the first exposure) on the third and fourth challenges. However, the extra exposures did not result in a greater level in expression relative to the second challenge. The first period of exposure and washout is therefore sufficient to elicit the full memory effect. In the case of SAA3, the first washout period was, as before, characterised by a continued rise in levels of this transcript, and the expression level remained highly elevated regardless of the removal or re-instigation of the stimulus.

Transcriptional memory is retained for at least one week

To determine the longevity of the memory effect after the initial stimulus, we extended the time of the washout period between MCM challenges to up to 12 days. Because the adipocytes were already 12-days post-differentiation at this stage, and because the transcriptome profile of 3T3-L1 cells varies over such a long period (32), age-matched naive cells were included as controls at each time point. Figure 7 shows the results of this experiment on iNOS expression. Because the level of this transcript in naive cells and in cells after washout is always close to undetectable, these values are omitted for clarity. As before, after 1 day of washout, there is a pronounced memory effect, with the secondary stimulus giving rise to a 3-fold greater response than the primary exposure. Despite the fact that the absolute magnitude of the MCM-stimulation decreased with cell age, a clear difference between the primary and secondary responses was

still seen after three and six days post-washout of the initial stimulus. However, after a washout period of 12 days, the response of previously exposed cells was not different to that of naive (never-exposed) cells. It should be noted however, that the responsiveness of cells to MCM stimulus at this stage (some 24 days post-differentiation) was only about 50% of that of younger cells. Therefore, at least in the case of iNOS, the effects of the primary MCM exposure persist for at least six days post-washout.

Transcriptional Memory does not involve a change in the sensitivity to MCM

In order to determine if the primary MCM exposure alters the sensitivity or responsiveness to subsequent MCM exposure, the dose-response relationship for naive and pre-stimulated cells was established (Figure 8). In both groups of cells, iNOS expression was not raised by MCM if it was diluted by more than 100-fold. However, a 1:50 dilution of MCM was sufficient to cause a change in the level of iNOS transcript in both groups. At higher concentrations of MCM, the iNOS expression was always greater in cells that had been pre-exposed to MCM. Whereas iNOS expression appeared to plateau when naive cells were incubated with undiluted MCM, in pre-exposed cells the level of iNOS expression appeared to be proportional to MCM concentration. Since more concentrated MCM was not available, it was not possible to determine the MCM concentration at which the response of pre-exposed cells would plateau. Regardless, these data show that the effect of prior MCM exposure is to increase the responsiveness of the iNOS expression system to subsequent MCM exposure, rather than increasing the sensitivity of the process.

DISCUSSION

Recent research (33) (34) (35) (36) (37) (38) suggests that cells of the innate immune system exhibit a form of transcriptional memory in that a priming event alters the cells so that later re-exposure to a similar stimulus causes an exaggerated response. Our results indicate that adipocytes possess a similar transcriptional memory to macrophage secretions and that it is manifested in two ways. Firstly, elevated expression of some genes persists long after removal of the stimulus. Secondly, and more intriguingly, the response of a subset of genes is reproducibly enhanced following a second challenge, despite the fact that their expression levels return to baseline between exposures. It is important to appreciate that although we have reported detailed data for a limited number of targets (e.g., iNOS, IL-6, SAA3, etc.) we have confirmed the existence of the memory response for more than 10 of the genes identified by microarray analyses.

In trying to dissect out the molecular mechanisms that might underlie this phenomenon, it is important to reflect on the nature of the stimulus used, and the normal processes that operate to control the waxing and waning of the resulting transcriptional response. It is likely that most of the stimulants in the MCM are cytokines and residual lipopolysaccharide. After an initial surge in expression, caused by stimulation of genes such as Toll-like receptors and interleukin receptors, braking mechanisms are rapidly initiated (for example the concomitant activation of the counter-regulatory SOCS pathway (39) as recently reviewed by (17). Rapid clearance of the initially produced transcripts is aided by the fact that most of the responsive mRNAs have a very short half-life. Therefore, the ‘transcriptional memory’ may be a consequence of increased initiation factor or RNA polymerase activity, an impaired dampening response or even a change in the turnover of transcripts.

We do know that a single exposure to MCM is sufficient to prime the memory, and that multiple cycles of washout and re-exposure do not give rise to lesser or greater memory effects. Another important clue is

that, in the case of genes like iNOS, after each cycle of washout, the expression of every memory-enabled transcript returns to basal levels. Therefore, whatever imprint on the transcriptional system is responsible for the memory requires a second stimulus for it to be revealed. In contrast, in the case of genes like SAA3, a single exposure appears to render the transcriptional apparatus continually active, even in the absence of the stimulus. Significantly, it does not increase further upon subsequent stimulation, which implies that the initial exposure is sufficient to set in train transcriptional processes that are hard to switch off.

We did not delineate precisely which components of MCM are required to give rise to the transcriptional memory phenomenon. MCM will comprise of tens, if not hundreds, of inflammogens and, furthermore, although always prepared and used in the same way, each preparation of MCM will likely have had a slightly different composition. We were, however, able to establish that the LPS residual in MCM preparations was not responsible for the memory effect. Thus, although LPS is effective at raising the expression of genes like iNOS and IL-6, it does so at a much lower magnitude than MCM and subsequent cycles of washout and re-exposure do not result in a different response (results not shown).

It was established that the primary exposure does not increase the sensitivity of adipocytes to MCM but, rather, the total capacity of the inflammatory gene response seems to be accentuated by a single four-hour exposure to MCM. Changes in responsiveness are usually the result of a physical increase in the number of components in a signaling system – either at the receptor level (40) (41) (42), the intermediate signaling molecules (43) (44) (45) or, more usually, in the total amount of the final target (46) (47). In the context of the current system, the final target could be envisaged to be active RNA polymerase bound to the promoter region of the genes. It is becoming increasingly clear that initiation of transcription is only partly responsible for the regulation of the rate of transcript production (48). After the assembly of RNA polymerase and relevant transcription factors on open chromatin in the promoter area, the efficiency of elongation can be differentially regulated, and this is especially true of genes that are highly

regulated (49). Indeed, it is possible to convert a relatively unstable transcription complex into a unit with enhanced processivity (50). In other cases in which there are enhanced secondary responses, termination of the primary stimulus leaves the RNA polymerase still associated with the activated genes. Thus the RNA polymerase is poised, ready for the arrival of the next stimulus (51). Although we have not directly measured the location of RNA polymerase after the first stimulus, our data is entirely consistent with this mechanism operating for the targets that show TM. Clearly, measuring the presence of RNA pol II around the promoter area will be crucial in determining if the first stimulus leaves RNA pol II poised in this manner.

At a more general level, if the chromatin around the promoter region of a gene is open then it is easier for gene expression to be initiated than if the nucleosomes in same area of chromatin are highly organized and densely packed. The observations that the first exposure seems to increase the total responsiveness of the system and that multiple exposures do not incrementally increase the TM effect, are both consistent with the primary exposure simply making the chromatin maximally accessible. Techniques now exist to determine the extent to which particular sections of chromatin are accessible (e.g., FAIRE (52) (53) (54) and mono nuclease digestion (55) (52) (56)) and these approaches will be important in trying to elucidate the molecular mechanisms involved. Although we were not successful in identifying specific promoter modules that might be responsible for the two types of TM, there was enough consistency in the groups to inform the choice of probes in future experiments aimed at determining if particular transcription factors are involved. However, although the nearly 60 transcription factor modules present on the genes in question also appear with high frequency on all inflammatory genes, the fact that not all inflammatory genes show the memory response indicates that other factors must be involved. Accordingly, and consistent with the memory in the innate immune response, the priming event may lay down epigenetic markers in particular areas of the genome and it is also possible that long non-coding RNAs and micro-RNAs are involved. These are obviously areas for further investigation.

Another mechanistic clue may be in the fact that the memory effect is preserved for at least six days after the initial exposure, especially as this is despite the inclination of differentiated 3T3-L1 adipocytes to become less sensitive to MCM with increasing time in culture. Therefore whatever mechanisms operate to reduce MCM responsiveness during extended culture are not abrogated by an previous MCM exposure but, equally, the mechanisms responsible for the memory effect are clearly still operational and can impose themselves in long-term cultured cells.

The ability of MCM to reduce the expression of adipocyte-characteristic genes was of interest and, in particular, it was noted that targets coding for enzymes with a critical role in lipogenesis (eg, ACC and PEPCK) were not only reduced in expression by MCM but largely failed to recover during the 24 hour washout period, even undergoing further reductions in expression level on subsequent MCM exposure. Commensurate with this, we have observed that prolonged incubation of mature 3T3-L1 cells with MCM does elicit substantial de-differentiation and blunts the differentiation process (results not shown), and this is supported by other studies that implicate MCM in reducing markers of the adipocyte phenotype (57) (58) (59) (60) (61) (7) (8) (62).

The existence of adipocyte transcriptional memory introduces an exciting complexity into our understanding of the relationship between adipocytes and macrophages. Although this behavior should be confirmed *in vivo* before extrapolating the potentially far-reaching physiological consequences, it does emphasize the importance of better understanding the mechanisms which exist in adipose tissue to prevent an escalating positive feedback loop between the adipocyte and macrophage inflammatory secretions. If proven to be physiologically relevant, the phenomenon has implications for long-term health in diet cycles.

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FIGURE LEGENDS

Figure 1. Expression of inflammatory genes in 3T3-L1 adipocytes that received a 4h MCM stimulation followed by a washout or continuous stimulation. Mature 3T3-L1 adipocytes were initially stimulated with MCM for 4h. Medium was either replaced with fresh normal growth medium (washout [?](#)) or was unchanged (unchanged medium [?](#)) for 24h. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using students t-test, $p < 0.05^*$, $<0.01^{**}$, $<0.001^{***}$

Figure 2. Expression of genes in 3T3-L1 adipocytes during primary and secondary MCM exposures. 3T3-L1 adipocytes were exposed to MCM for 4h (primary stimulation) and then exposed again for another 4h (secondary stimulation) after receiving a 24h washout in normal complete growth medium. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using students t-test, $p < 0.05^*$, $<0.01^{**}$, $<0.001^{***}$, $<0.0001^{****}$

Figure 3. Expression of inflammatory genes in 3T3-L1 adipocytes during primary and secondary MCM exposures that show transcriptional memory. 3T3-L1 adipocytes were exposed to MCM for 4h (primary stimulation) and then exposed again for another 4h (secondary stimulation) after receiving a 24h washout in normal complete growth medium. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using students t-test, $p < 0.05^*$, $<0.01^{**}$, $<0.001^{***}$, $<0.0001^{****}$

Figure 4. Expression of SAA3 in 3T3-L1 adipocytes during primary and secondary MCM exposures. 3T3-L1 adipocytes were exposed to MCM for 4h (primary stimulation) and then exposed again for another 4h (secondary stimulation) after receiving a 24h washout in normal complete growth medium. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using students t-test, $p < 0.05^*$, $<0.01^{**}$

Figure 5. Expression of genes in 3T3-L1 adipocytes during primary and secondary MCM exposures. 3T3-L1 adipocytes were exposed to MCM for 4h (primary stimulation) and then exposed again for another 4h (secondary stimulation) after receiving a 24h washout in normal complete growth medium. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using students t-test, $p < 0.05^*$, $<0.01^{**}$, $<0.001^{***}$, $<0.0001^{****}$

Figure 6. Expression of genes in 3T3-L1 adipocytes during primary, secondary, third and fourth MCM exposures. 3T3-L1 adipocytes were stimulated with MCM for 2h (primary) after receiving either one (secondary), two (third) or three (fourth) previous exposures with MCM for 4h followed by a 24h washout in normal growth medium. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using students t-test, $p < 0.05^*$, $<0.01^{**}$, $<0.001^{***}$

Figure 7. Expression of iNOS in 3T3-L1 adipocytes during primary and secondary MCM exposures with extended washout times. 3T3-L1 adipocytes were either grown in normal growth medium (naïve) or exposed to MCM for 4h (pre-stimulated) and then both cell groups were subsequently exposed to MCM for 4h after receiving a 1, 3, 6 or 12 day washout in normal complete growth medium. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using students t-test, $p < 0.05^*$, $<0.01^{**}$, $<0.001^{***}$ naïve compared to pre-stimulated.

Figure 8. Expression of iNOS in 3T3-L1 cells during primary and secondary stimulation with varying concentrations of MCM. 3T3-L1 adipocytes were either grown in normal growth medium (naïve) or exposed to 1:1 MCM for 4h (pre-stimulated) and then both cell groups were subsequently exposed to varying concentrations of MCM for 4h after receiving a 24h washout in normal complete growth medium. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using students t-test, $p < 0.05^*$, $<0.01^{**}$

Table 1. Genes that show transcriptional memory

Gene Name	Gene	Primary Stimulation (h)				Secondary Stimulation (h)				Expression Enhancement (Fold change)
		0	1	2	4	0	1	2	4	
Chemokine (C-C motif) ligand 2	CCL2	709	128	18055	19394	6587	17347	18561	19700	135.5 (1h)
PHD finger protein 11	PHDF11A	231	149	174	521	282	333	955	2513	4.8 (4h)
Interleukin 1 alpha	IL1 α	30	49	153	317	33	535	763	1277	4.0 (4h)
Serpin peptidase inhibitor, clade B, member 2	SERPINB2	41	61	337	761	94	986	1858	2994	3.9 (4h)
Immunoresponsive gene 1	IRG1	27	36	205	792	38	353	1353	2735	3.4 (4h)
Tumour necrosis factor alpha	TNF α	87	181	465	153	113	617	495	192	3.4 (1h)
Interferon regulatory factor 5	IRF5	89	79	122	299	113	141	397	774	2.5 (4h)
Chemokine (C-C motif) receptor-like 2	CCRL2	33	133	705	1328	53	906	2426	3300	2.4 (4h)
Interleukin 33	IL33	90	95	680	1345	831	970	2097	2722	2.0 (4h)
2'-5' oligoadenylate synthetase 1G	OASG1	11	63	507	1721	505	1780	3154	3470	2.0 (4h)
Prostaglandin-endoperoxide synthase 2	PTGS2	487	1732	5709	5684	931	6005	10348	7872	1.8 (2h)
2'-5' oligoadenylate synthetase-like 1	OASL1	71	119	161	647	127	181	585	1143	1.7 (4h)
Inducible nitric oxide synthase	iNOS	93	102	1311	5340	213	419	4248	9198	1.7 (4h)
Bradykinin receptor B1	BDKRB1	174	259	1066	1131	254	693	1191	1892	1.6 (4h)
Tumour necrosis factor (ligand) superfamily 10	TNFSF10	50	35	196	774	68	289	979	1273	1.6 (4h)
Interleukin 15	IL15	278	312	1230	2077	338	646	2346	3386	1.6 (4h)
Interleukin 15 receptor alpha	IL15R α	565	523	1056	1747	453	714	1658	2807	1.6 (4h)
Leukaemia inhibitory factor	LIF	156	199	831	949	142	552	1382	1458	1.5 (4h)
Chemokine (C-C motif) ligand 3	CCL3	62	93	1704	3909	47	899	4415	5760	1.4 (4h)
Interleukin 6	IL6	71	6355	9174	8744	435	8831	12379	10962	1.3 (2h)
T-cell specific GTPase 2	TGTP2	174	347	663	964	179	677	1274	1263	1.3 (4h)

Table 2. Genes with persistent expression after primary MCM stimulation.

Gene Name	Gene	Primary Stimulation (h)				Secondary Stimulation (h)			
		0	1	2	4	0	1	2	4
2'-5' oligoadenylate synthetase 2	OAS2	122	130	475	1413	2072	2402	3057	4978
Complement component 4b	C4B	437	420	468	701	1641	1677	1684	2200
Matrix metalloproteinase 3	MMP3	102	113	562	1079	1378	1472	1385	2442
Mannan-binding lectin serine protease 1	MASP1	322	289	324	250	671	564	597	557
Orosomucoid 2	ORM2	1935	2621	2552	2306	4508	3956	3518	3415
Complement component 1, R subcomponent B	C1RB	1649	1391	2232	2684	3038	3184	3583	3831
Complement component 3	C3	1114	11743	11002	13692	14443	14293	14743	15173
Proteoglycan 4	PRG4	643	586	1125	1326	1825	1688	1939	2185
2'-5' oligoadenylate synthetase 3	OAS3	83	117	252	645	570	581	1039	2270
Chemokine (C-C motif) ligand 8	CCL8	84	343	1561	1655	1618	2005	3015	4235
Apolipoprotein L 9b	APOL9B	48	187	486	1024	1193	1114	2070	2392
Serpin peptidase inhibitor, clade G, member 1	SERPINC1	116	81	259	473	540	479	560	889
Z-DNA binding protein 1	ZBP1	251	652	2036	3888	2748	3240	4654	6669
Chemokine (C-C motif) ligand 9	CCL9	1159	1753	2811	3539	2889	4324	5266	5955
Complement factor B	CFB	97	498	1946	3770	3885	4121	5036	5936
Histocompatibility 2, M region locus 3	H2-M3	438	472	778	1054	921	1384	1410	1589
Histocompatibility 2, T region locus 23	H2-T23	204	226	368	606	630	653	644	860
2'-5' oligoadenylate synthetase-like 2	OASL2	576	1210	4605	8764	6405	6544	8939	11763
Interleukin 13 receptor, alpha	IL13RA	855	1666	4501	6428	6506	5931	7825	8434
Serum amyloid A3	SAA3	82	2161	7771	8752	7287	8689	10088	11430
Chemokine (C-C motif) ligand 5	CCL5	692	1450	6812	14716	10799	11675	16050	18829

Table 3. Genes with decreased expression upon MCM stimulation

Gene Name	Gene	Primary Stimulation (h)				Secondary Stimulation (h)			
		0	1	2	4	0	1	2	4
RAS, dexamethasone-induced 1	RASD1	2214	330	243	460	1603	285	180	327
Insulin receptor substrate 1	IRS1	1427	1066	548	433	1164	1055	716	557
CCAAT/enhancer-binding protein alpha	CEBPα	1739	1628	932	574	1501	1149	962	616
Lipin 1	LPIN1	2906	3162	2014	1006	1726	1667	1416	829
Low-density lipoprotein receptor-related protein 6	LRP6	5850	5034	3848	2342	5204	4989	4102	2235
Retinoic acid receptor, alpha	RARA	808	729	513	375	1160	934	673	472
Insulin receptor substrate 2	IRS2	1315	2695	1904	619	1190	1083	667	709
Peroxisomal biogenesis factor 11 alpha	PEX11A	935	758	673	458	1179	987	721	527
Peroxisome proliferator-activated receptor gamma	PPARγ	7849	7766	5926	4476	8103	7823	7279	5442
Laminin, alpha 4	LAMA4	5465	4521	4416	3619	5026	5306	5061	4406
Hormone-sensitive lipase	HSL	1738	1752	1552	1201	1830	1795	1490	966
Perilipin 4	PLIN4	4427	4141	3778	3302	4564	4536	3495	2642
Phosphoenolpyruvate carboxylase kinase 1	PCK1	1923	1160	665	328	326	300	168	177
Insulin-induced gene 1	INSIG1	8718	9154	6401	4711	5500	5698	5388	3281
Adipogenin	ADIG	821	552	591	449	483	347	295	227
Leptin	LEP	155	132	113	91	85	69	77	53
Glucose transporter type 4	GLUT4	3889	3820	3472	2783	2476	2375	2138	1786
Fatty acid synthase	FAS	12293	12552	10305	9779	8274	8569	7367	5277
Fatty acid binding protein 5	FABP5	1116	1037	1055	963	897	824	770	673
Lipoprotein lipase	LPL	18124	16207	15929	16211	15839	15772	14698	13811
Diglyceride O-acyltransferase 1	DGAT1	6898	10381	10032	8948	8700	8118	7718	6771

Figure 1. Expression of inflammatory genes in 3T3-L1 adipocytes that received a 4-h MCM stimulation followed by a washout or continuous stimulation. Mature 3T3-L1 adipocytes were initially stimulated with MCM for 4 h. Medium was either replaced with fresh normal growth medium (washout ●) or was unchanged (unchanged medium ■) for 24 h. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using Student's t-test, $p < 0.05^*$, $< 0.01^{**}$, $< 0.001^{***}$.

Figure 2. Expression of genes in 3T3-L1 adipocytes during primary and secondary MCM exposures. 3T3-L1 adipocytes were exposed to MCM for 4 h (primary stimulation) and then exposed again for another 4 h (secondary stimulation) after receiving a 24 h washout in normal growth medium. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using students t-test, $p<0.05^*$, $<0.01^{**}$, $<0.001^{***}$, $<0.0001^{****}$

Figure 3. Expression of inflammatory genes that show transcriptional memory in 3T3-L1 adipocytes following primary and secondary MCM exposures. 3T3-L1 adipocytes were exposed to MCM for 4 h (primary stimulation) and then exposed again for another 4 h (secondary stimulation) after receiving a 24 h washout in normal complete growth medium. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using students t-test, $p < 0.05^*$, $<0.01^{**}$, $<0.001^{***}$, $<0.0001^{****}$

Figure 4. Expression of SAA3 in 3T3-L1 adipocytes during primary and secondary MCM exposures. 3T3-L1 adipocytes were exposed to MCM for 4 h (primary stimulation) and then exposed again for another 4 h (secondary stimulation) after receiving a 24 h washout in normal complete growth medium. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using students t-test, $p < 0.05^*$, $<0.01^{**}$

Figure 5. Expression of genes in 3T3-L1 adipocytes during primary and secondary MCM exposures. 3T3-L1 adipocytes were exposed to MCM for 4 h (primary stimulation) and then exposed again for another 4 h (secondary stimulation) after receiving a 24 h washout in normal complete growth medium. cDNA was synthesised from RNA extracts and gene expression levels were analysed

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Figure 7. Expression of iNOS in 3T3-L1 adipocytes during primary and secondary MCM exposures with extended washout times. 3T3-L1 adipocytes were either grown in normal growth medium (naïve) or exposed to MCM for 4 h (pre-stimulated) and then both cell groups were subsequently exposed to MCM for 4 h after receiving a 1, 3, 6 or 12 day washout in normal complete growth medium. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using students t-test, $p < 0.05^*$, $< 0.01^{**}$, $< 0.001^{***}$ naïve compared to pre-stimulated.

Figure 8. Expression of iNOS in 3T3-L1 cells during primary and secondary stimulation with varying concentrations of MCM. 3T3-L1 adipocytes were either grown in normal growth medium (naïve) or exposed to 1:1 MCM for 4 h (pre-stimulated) and then both cell groups were subsequently exposed to varying concentrations of MCM for 4 h after receiving a 24 h washout in normal complete growth medium. cDNA was synthesised from RNA extracts and gene expression levels were analysed using qPCR. C_T results were normalised to 18S rRNA and expressed as transcript copies per 10^6 18S copies. Data are shown as mean $\pm$ SEM ($n=3$). Statistical analysis was performed using students t-test, $p < 0.05^*$, $< 0.01^{**}$

IL-6

iNOS