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

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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*.

45 **INTRODUCTION**

46

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

53

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

59

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

69

70 Microarray studies have revealed that the response of adipocytes to MCM does not just involve the
71 expression of cytokine mRNAs but is also characterized by rapid and sizeable rises in the levels of extra-
72 cellular matrix remodeling transcripts and repression of adipocyte-specific genes (11) (12). Interestingly,
73 despite the fact that these rises in mRNA levels are often >100-fold over basal, the changes are generally
74 transient, even if the stimulatory milieu is maintained. This waxing and waning of gene expression is a
75 common property in immune cells in response to inflammatory stimuli, and is caused by compensatory
76 and counter-regulatory responses, with prolonged or prior exposure of many types of cells to hormones
77 results in a dampening and loss of sensitivity to subsequent exposures. This is due, in part, to decreased
78 receptor levels (13) (14) (15) and persistence of the post-stimulation dampening responses (16) (17).
79
80 Since adipocytes exhibit this kind of response to insulin, showing reduced insulin sensitivity after
81 exposure to high insulin concentrations (13) (14) (18), we reasoned that fat cells might show a similar
82 repression to further inflammatory stimuli after prior exposure to macrophage secretions. It is intuitive
83 that such a mechanism should exist to prevent a rampant positive feedback loop between adipocytes and
84 macrophages. In sharp contrast to these expectations, we describe here experiments that demonstrate
85 quite the reverse: namely, that exposure to inflammatory stimuli leaves an imprint on fat cells that
86 actually renders them more responsive to subsequent stimulation by macrophage secretions.
87

88 **METHODS**

89

90 ***3T3-L1 Cell Culture***

91

92 Low passage number 3T3-L1 cells were cultured in DMEM (Life Technologies, Australia) which
93 contained 25 mM glucose, 4 mM L-glutamine and 1 mM sodium pyruvate supplemented with 10% (v/v)
94 fetal bovine serum (referred to as DMEM/FBS). Cells were cultured in 6 or 12 well plates in a humidified
95 incubator at 37°C supplemented with 5% (v/v) CO_2.

96

97 Two days post-confluence, differentiation was induced with a cocktail of stimulants; 2 µg mL^{-1} insulin,
98 0.5 mM isobutylmethylxanthine, 0.25 µM dexamethasone and 2 µM rosiglitazone (all purchased from
99 Sigma) in DMEM/FBS. After three days, the differentiation medium was removed and cells were
100 maintained in post-differentiation medium (DMEM/FBS with 2 µg mL^{-1} insulin) for a further 6–9 days,
101 with media changes every two days or as required. Differentiation was monitored both by microscopy
102 and Oil-Red O staining.

103

104 ***Production of MCM***

105

106 The murine monocyte cell line, RAW264.7 was employed to obtain macrophage-conditioned medium
107 (MCM). Cells were cultured until confluence in DMEM/FBS in a CO_2 supplemented humidified
108 incubator at 37°C. To stimulate an inflammatory response, the cells were treated with lipopolysaccharide
109 (LPS, 50 ng/mL) for 4 h. At the end of this time the incubation medium was collected and filtered through
110 a 0.22 µm filter (Millipore) to remove any debris and cells. The MCM was stored at -80°C until required.
111 Any variations from this procedure are described in the relevant figure or table legend.

112

113 ***Memory Experiment setup***

114

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

121

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

127

128 ***RNA Isolation***

129

130 Total RNA was isolated by Trizol (Sigma-Aldrich, Australia), using a variation of the method of
131 Chomczynski and Sacchi (19). Essentially the media was removed from each well and the adherent cells
132 were washed once with PBS followed by lysis in Trizol reagent. The lysate was then extracted with
133 chloroform (5:1 vol:vol) at room temperature. Following centrifugation at 12,000 × *g* for 15 min the
134 upper aqueous phase containing the RNA was removed and the RNA precipitated overnight at 20°C with
135 1.25 volumes of isopropanol. The pellet, collected after centrifugation at 12,000 x *g* for 30 min, was
136 washed twice with 75% (v/v) ethanol then re-suspended in nuclease-free water. The yield and purity of
137 the RNA was estimated by UV spectrophotometry. The integrity of the RNA was further assessed by

138 denaturing agarose gel electrophoresis (1% (w/v) agarose in 2.2 M formaldehyde MOPS) (20) followed
139 by ethidium bromide staining and visualization using a UV trans-illuminator.
140 ***Microarray analysis***
141
142 Microarray analysis was performed on RNA samples in order to get a complete perspective of gene
143 expression changes after MCM stimulation. Isolated RNA was treated for application onto Affymetrix
144 GeneChip Gene Mouse 2.0 ST arrays by the Ramacotti Centre at the University of NSW, according to the
145 manufacturers instructions. Microarray data was analyzed using an in-house designed package as has
146 been employed in several other studies (21) (22) (23) (24) (25) (26) (27) (28) (29) (30).
147
148 ***cDNA synthesis and qPCR***
149
150 Complementary DNA from each RNA preparation was synthesized with Reverse Transcriptase (Bioline,
151 Australia) according to the manufacturer's protocol. 500 ng samples of total RNA were primed using
152 random hexamers in 20 µL reaction mixtures containing 500 µM dNTP, 50 mM Tris HCl pH 8.6, 40 mM
153 KCl, 5 mM $MgCl_2$, 1 mM $MnSO_4$, 1 mM DTT and 0.5 U/µL RNase inhibitor. Primers were annealed at
154 25°C for 10 min, cDNA synthesis was then carried out at 45°C for 30 min with 10 U/ µL reverse
155 transcriptase followed by enzyme inactivation at 85°C for 5 min.
156
157 qPCR was performed in 20 µL reactions using SybrGreen (FAST SybrGreen master mix, Applied
158 Biosystems, Australia) amplifying two different amounts of each cDNA preparation (10 ng/ reaction and
159 1.25 ng/ reaction) for each primer set. A two-step thermocycler program was employed using an Applied
160 Biosystems 7500 Fast instrument. This was 10 min at 50 ^{o}C pre-incubation to remove any contaminating
161 amplified product from previous PCR assays, then 95°C for 20 s followed by 40 cycles of 3 s at 95 ^{o}C
162 then 30 s at 60 ^{o}C. A melt curve was performed on all reaction products. and each *Ct* normalized to its
163 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.

169 **RESULTS**

170

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

172 **MCM**

173

174 To assess the effect of MCM on adipocytes in culture, we exposed mature 3T3-L1 cells to MCM for up to

175 24 hours, either with or without a washout of the stimulus at 4 h. Figure 1 shows that the transcriptional

176 output of inflammatory targets in 3T3-L1 adipocytes responds to macrophage secretions in the same way

177 as reported by others (31) (3) (11). The MCM caused a rapid (within 2 h) and high amplitude (>100-fold)

178 rise in the levels of both IL-6 and iNOS transcripts, two key indicators of the adipocyte inflammatory

179 response. Moreover, despite the continued presence of the MCM stimulus, the level of both these

180 mRNAs decreased after 4 h of exposure. In neither case did transcript levels return to basal values, even

181 after a further 24 h incubation. In contrast, when the MCM stimulus was removed and the cells

182 incubated in fresh culture medium, the expression of both genes declined to pre-exposure levels within 8–

183 12 h.

184

185 Although washout of the MCM stimulus appears to reverse the effects of the inflammatory exposure (at

186 least with respect to iNOS and IL-6 expression), we were curious to know if the previously-exposed cells

187 would behave differently to naive cells when exposed to MCM again. Figure 2 shows that cells

188 previously exposed to MCM show an enhanced response to a second challenge. These results indicate

189 that the first exposure to MCM leaves some imprint on the gene expression apparatus of iNOS and IL-6

190 in adipocytes.

191

192 We next asked whether other transcripts also exhibit this transcriptional memory. We performed

193 microarrays on mRNA isolated from cells exposed to MCM for 0–4 hours, either as a primary stimulation

194 (i.e., cells that had never seen MCM before) or as a secondary stimulation (i.e., after a previous 4-hour

195 exposure followed by a 24-hour washout). As observed by others (11) (31), the expression of several
196 hundred genes was stimulated after MCM exposure (original data available in NCBI Gene Expression
197 Omnibus). Of these, about 20 targets show a greater response to MCM after a previous exposure. Table 1
198 lists the genes that exhibited: a) a rise in response to the first MCM exposure of >4-fold at least one time
199 point, b) a return to basal (or near-basal) levels of expression after washout, and c) a secondary response
200 to MCM that was at least 2-fold greater than the first exposure at least one time point. In every case, the
201 targets listed in Table 1 have at least one inflammatory ontological classification.
202
203 Whilst it was encouraging that iNOS and IL-6 were amongst the genes identified as displaying
204 transcriptional memory in the arrays, only a single microarray was performed for each sample and so it
205 was necessary to confirm these observations by more quantitative methods using a greater number of
206 preparations. To this end, the experiment was repeated multiple times and qPCR used to measure
207 transcript levels. The behavior of all the targets measured (over half of those shown in Table 1) was
208 verified using this approach. Figure 3 shows four representative genes (CCL2, IL-1a, TNF-a and
209 PDHF11A). In general, the secondary response shows the same kinetics as the primary response,
210 following the same pattern of increase and decay, but the rise in expression either occurs sooner or is
211 more exaggerated in amplitude.
212
213 Microarray analysis revealed a further class of targets whose expression does not decrease after removal
214 of the MCM stimulus (Table 2). For these genes, the transcript levels remain elevated (and often even
215 keep increasing) even after 24 hours of washout. In most, but not all cases, expression levels increased
216 even more on secondary exposure. As before, we confirmed these patterns using qPCR in new samples;
217 Figure 4 shows data for one example: SAA3. As with the genes that showed transcriptional memory in
218 Table 1, the genes that show persistence of gene expression elevation even after removal of the MCM
219 stimulus all have at least one ontology which classifies them as inflammatory.
220

221 **Genes exhibiting Transcriptional Memory share only loose commonality in Promoter Properties**

222

223 The promoters associated with the sequences in Table 1 and Table 2 were interrogated using the module
224 in the Genomatix Software Suite (https://www.genomatix.de) that identifies common transcription factor
225 binding sites. Analysis included all the members of Tables 1 (20 sequences) and Table 2 (21 sequences)
226 except Masp1 for which information was not available.
227 There were 87 transcription factor binding sites (TFBs) that were common between at least 17 of the 20
228 genes in Table 1. Of these, a subset of 16 TFBs were characterised as being selectively concentrated in
229 these genes in comparison to the mouse genome as a whole ($P<0.0001$). Those with the most extensive
230 commonality included AP4R (transcription factor AP4), HUB1 (HTLV-I U5 repressive element-binding
231 protein 1), GFI1 (Growth factor independence transcriptional repressor), IKRS (Ikaros zinc finger
232 family), BHLH (bHLH transcription factors) and ZF12 (C2H2 zinc finger transcription factor family).
233 Similarly, the promoters of the 20 genes from Table 2 showed a total of 64 types of TFB, of which 10
234 were identified as being distinct for this set. The three with the most penetration across the 20 genes were
235 NRSF (Neuron-restrictive silencer factor), SF1F (Vertebrate steroidogenic factor) and STAF
236 (Selenocysteine tRNA activating factor). There was some commonality in TFBs between the two tables,
237 but not in any of the afore-mentioned genes.

238

239 **Genes repressed by MCM exposure do not show Transcriptional Memory**

240

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

246 recovery of the transcript level during the washout period, the secondary response never showed an
247 overtly faster or high amplitude response than the initial exposure.
248
249 **Adipocytes retain but do not magnify the memory effect following multiple MCM challenges**
250
251 Figure 6 shows the results of several rounds of MCM stimulation and washout on genes that show either
252 an enhanced secondary response (iNOS) or persistent high expression after the first stimulus (SAA3). In
253 the case of iNOS, the memory effect was retained after a second and third washout and so gave rise to
254 accentuated responses (compared to the first exposure) on the third and fourth challenges. However, the
255 extra exposures did not result in a greater level in expression relative to the second challenge. The first
256 period of exposure and washout is therefore sufficient to elicit the full memory effect. In the case of
257 SAA3, the first washout period was, as before, characterised by a continued rise in levels of this
258 transcript, and the expression level remained highly elevated regardless of the removal or re-instigation of
259 the stimulus.
260
261 **Transcriptional memory is retained for at least one week**
262
263 To determine the longevity of the memory effect after the initial stimulus, we extended the time of the
264 washout period between MCM challenges to up to 12 days. Because the adipocytes were already 12-days
265 post-differentiation at this stage, and because the transcriptome profile of 3T3-L1 cells varies over such a
266 long period (32), age-matched naive cells were included as controls at each time point. Figure 7 shows
267 the results of this experiment on iNOS expression. Because the level of this transcript in naive cells and in
268 cells after washout is always close to undetectable, these values are omitted for clarity. As before, after 1
269 day of washout, there is a pronounced memory effect, with the secondary stimulus giving rise to a 3-fold
270 greater response than the primary exposure. Despite the fact that the absolute magnitude of the MCM-
271 stimulation decreased with cell age, a clear difference between the primary and secondary responses was

272 still seen after three and six days post-washout of the initial stimulus. However, after a washout period of
273 12 days, the response of previously exposed cells was not different to that of naive (never-exposed) cells.
274 It should be noted however, that the responsiveness of cells to MCM stimulus at this stage (some 24 days
275 post-differentiation) was only about 50% of that of younger cells. Therefore, at least in the case of iNOS,
276 the effects of the primary MCM exposure persist for at least six days post-washout.
277
278 **Transcriptional Memory does not involve a change in the sensitivity to MCM**
279
280 In order to determine if the primary MCM exposure alters the sensitivity or responsiveness to subsequent
281 MCM exposure, the dose-response relationship for naive and pre-stimulated cells was established (Figure
282 8). In both groups of cells, iNOS expression was not raised by MCM if it was diluted by more than 100-
283 fold. However, a 1:50 dilution of MCM was sufficient to cause a change in the level of iNOS transcript
284 in both groups. At higher concentrations of MCM, the iNOS expression was always greater in cells that
285 had been pre-exposed to MCM. Whereas iNOS expression appeared to plateau when naive cells were
286 incubated with undiluted MCM, in pre-exposed cells the level of iNOS expression appeared to be
287 proportional to MCM concentration. Since more concentrated MCM was not available, it was not
288 possible to determine the MCM concentration at which the response of pre-exposed cells would plateau.
289 Regardless, these data show that the effect of prior MCM exposure is to increase the responsiveness of the
290 iNOS expression system to subsequent MCM exposure, rather than increasing the sensitivity of the
291 process.
292
293

294 **DISCUSSION**

295

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

306

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

317

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

320 that, in the case of genes like iNOS, after each cycle of washout, the expression of every memory-enabled
321 transcript returns to basal levels. Therefore, whatever imprint on the transcriptional system is responsible
322 for the memory requires a second stimulus for it to be revealed. In contrast, in the case of genes like
323 SAA3, a single exposure appears to render the transcriptional apparatus continually active, even in the
324 absence of the stimulus. Significantly, it does not increase further upon subsequent stimulation, which
325 implies that the initial exposure is sufficient to set in train transcriptional processes that are hard to switch
326 off.
327
328 We did not delineate precisely which components of MCM are required to give rise to the transcriptional
329 memory phenomenon. MCM will comprise of tens, if not hundreds, of inflammogens and, furthermore,
330 although always prepared and used in the same way, each preparation of MCM will likely have had a
331 slightly different composition. We were, however, able to establish that the LPS residual in MCM
332 preparations was not responsible for the memory effect (results not shown). Thus, although LPS is
333 effective at raising the expression of genes like iNOS and IL-6, it does so at a much lower magnitude than
334 MCM and subsequent cycles of washout and re-exposure do not result in a different response.
335
336 It was established that the primary exposure does not increase the sensitivity of adipocytes to MCM but,
337 rather, the total capacity of the inflammatory gene response seems to be accentuated by a single four-hour
338 exposure to MCM. Changes in responsiveness are usually the result of a physical increase in the number
339 of components in a signaling system – either at the receptor level (40) (41) (42), the intermediate
340 signaling molecules (43) (12) (44) (45) or, more usually, in the total amount of the final target (46) (47).
341 In the context of the current system, the final target could be envisaged to be active RNA polymerase
342 bound to the promoter region of the genes. It is becoming increasingly clear that initiation of transcription
343 is only partly responsible for the regulation of the rate of transcript production (48). After the assembly
344 of RNA polymerase and relevant transcription factors on open chromatin in the promoter area, the
345 efficiency of elongation can be differentially regulated, and this is especially true of genes that are highly

346 regulated (49). Indeed, it is possible to convert a relatively unstable transcription complex into a unit with
347 enhanced processivity (50). In other cases in which there are enhanced secondary responses, termination
348 of the primary stimulus leaves the RNA polymerase still associated with the activated genes. Thus the
349 RNA polymerase is poised, ready for the arrival of the next stimulus (51). Although we have not directly
350 measured the location of RNA polymerase after the first stimulus, our data is entirely consistent with this
351 mechanism operating for the targets that show TM. Clearly, measuring the presence of RNA pol II
352 around the promoter area will be crucial in determining if the first stimulus leaves RNA pol II poised in
353 this manner.
354
355 At a more general level, if the chromatin around the promoter region of a gene is open then it is easier for
356 gene expression to be initiated than if the nucleosomes in same area of chromatin are highly organized
357 and densely packed. The observations that the first exposure seems to increase the total responsiveness of
358 the system and that multiple exposures do not incrementally increase the TM effect, are both consistent
359 with the primary exposure simply making the chromatin maximally accessible. Techniques now exist to
360 determine the extent to which particular sections of chromatin are accessible (e.g., FAIRE (52) (53)
361 (54)and mono nuclease digestion (55) (52) (56)) and these approaches will be important in trying to
362 elucidate the molecular mechanisms involved. Although we were not successful in identifying specific
363 promoter modules that might be responsible for the two types of TM, there was enough consistency in the
364 groups to inform the choice of probes in future experiments aimed at determining if particular
365 transcription factors are involved. However, although the nearly 60 transcription factor modules present
366 on the genes in question also appear with high frequency on all inflammatory genes, the fact that not all
367 inflammatory genes show the memory response indicates that other factors must be involved.
368 Accordingly, and consistent with the memory in the innate immune response, the priming event may lay
369 down epigenetic markers in particular areas of the genome and it is also possible that long non-coding
370 RNAs and micro-RNAs are involved. These are obviously areas for further investigation.
371

372 Another mechanistic clue may be in the fact that the memory effect is preserved for at least six days after
373 the initial exposure, especially as this is despite the inclination of differentiated 3T3-L1 adipocytes to
374 become less sensitive to MCM with increasing time in culture. Therefore whatever mechanisms operate
375 to reduce MCM responsiveness during extended culture are not abrogated by an previous MCM exposure
376 but, equally, the mechanisms responsible for the memory effect are clearly still operational and can
377 impose themselves in long-term cultured cells.
378
379 The ability of MCM to reduce the expression of adipocyte-characteristic genes was of interest and, in
380 particular, it was noted that targets coding for enzymes with a critical role in lipogenesis (eg, ACC and
381 PEPCK) were not only reduced in expression by MCM but largely failed to recover during the 24 hour
382 washout period, even undergoing further reductions in expression level on subsequent MCM exposure.
383 Commensurate with this, we have observed that prolonged incubation of mature 3T3-L1 cells with MCM
384 does elicit substantial de-differentiation and blunts the differentiation process (results not shown), and this
385 is supported by other studies that implicate MCM in reducing markers of the adipocyte phenotype (57)
386 (58) (59) (60) (61) (7) (8) (62).
387
388 The existence of adipocyte transcriptional memory introduces an exciting complexity into our
389 understanding of the relationship between adipocytes and macrophages. Although this behavior should
390 be confirmed *in vivo* before extrapolating the potentially far-reaching physiological consequences, it does
391 emphasize the importance of better understanding the mechanisms which exist in adipose tissue to
392 prevent an escalating positive feedback loop between the adipocyte and macrophage inflammatory
393 secretions. If proven to be physiologically relevant, the phenomenon has implications for long-term
394 health in diet cycles.
395

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Table 1. Genes that show transcriptional memory

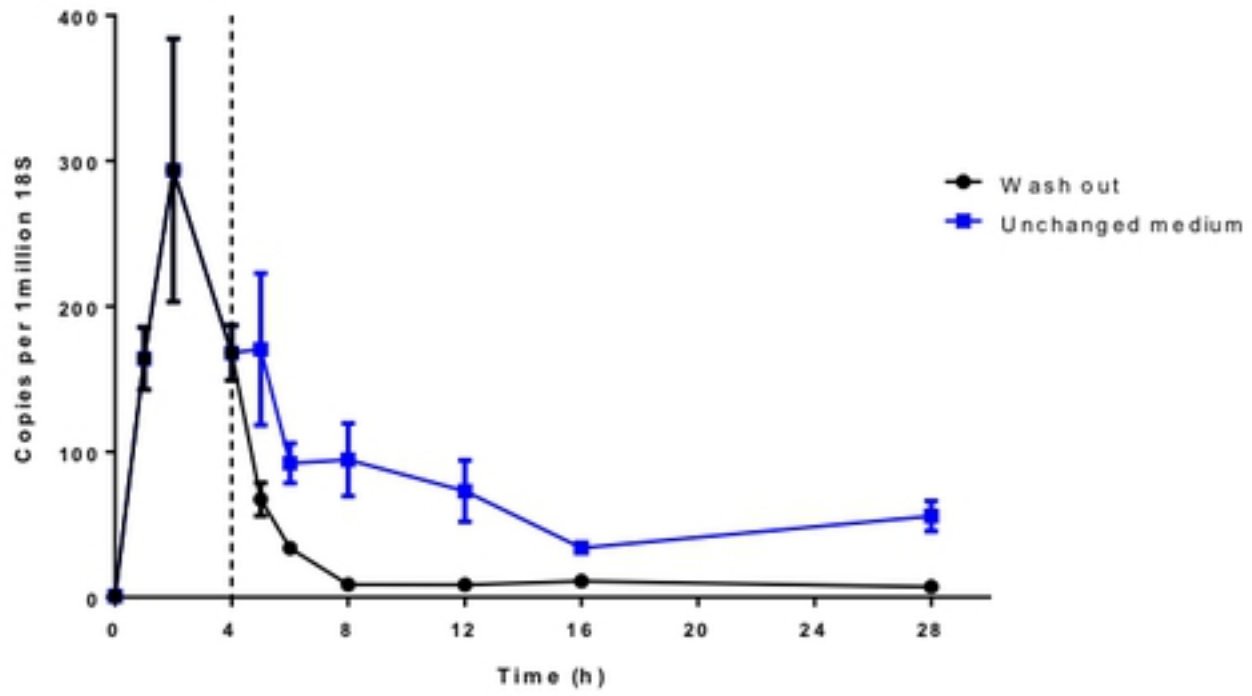
Gene Name	Gene	Primary Stimulation (h)				Secondary Stimulation (h)				Expression Enhancement (Fold change)
		0	1	2	4	0	1	2	4	
Chemokine (C-C motif) ligand 2	CCL2	709	128	18055	19394	6587	17347	18561	19700	135.5 (1h)
PHD finger protein 11	PHDF11A	231	149	174	521	282	333	955	2513	4.8 (4h)
Interleukin 1 alpha	IL1α	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	TNFα	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	IL15Rα	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 metallopeptidase 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	SERPING1	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



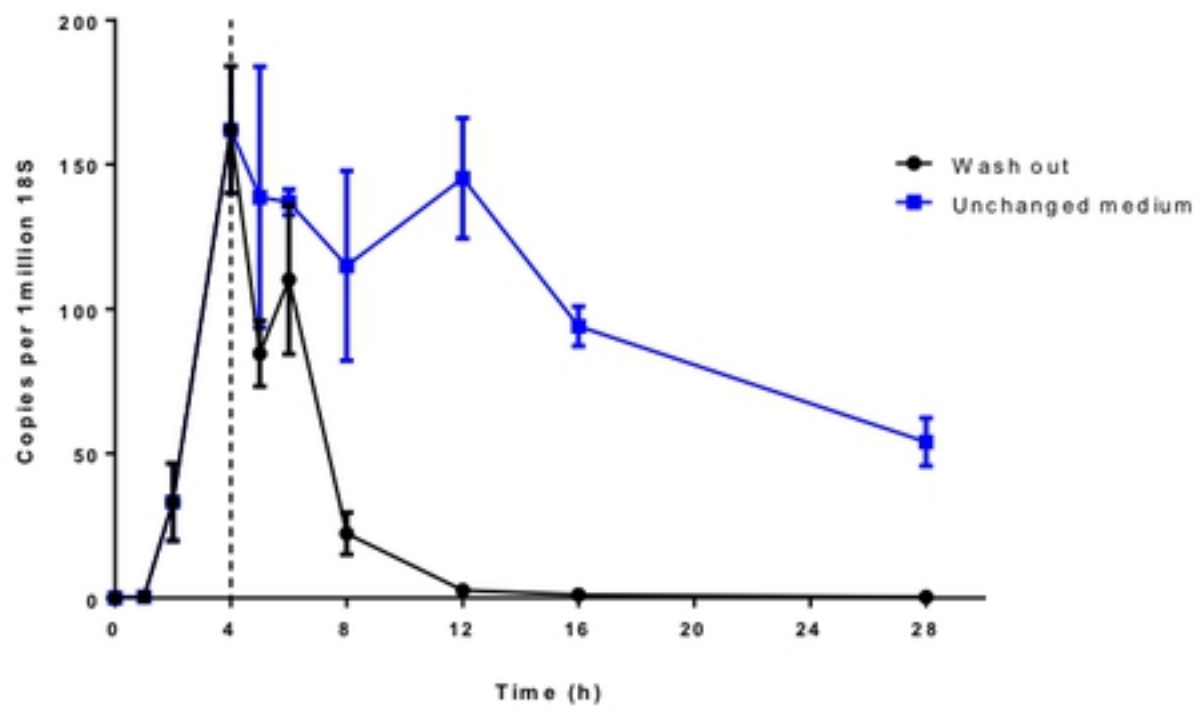


Figure 1. Expression of inflammatory genes in 3T3-L1 adipocytes that received a 4h MCM stimulation followed by a washout or continuous stimulation.

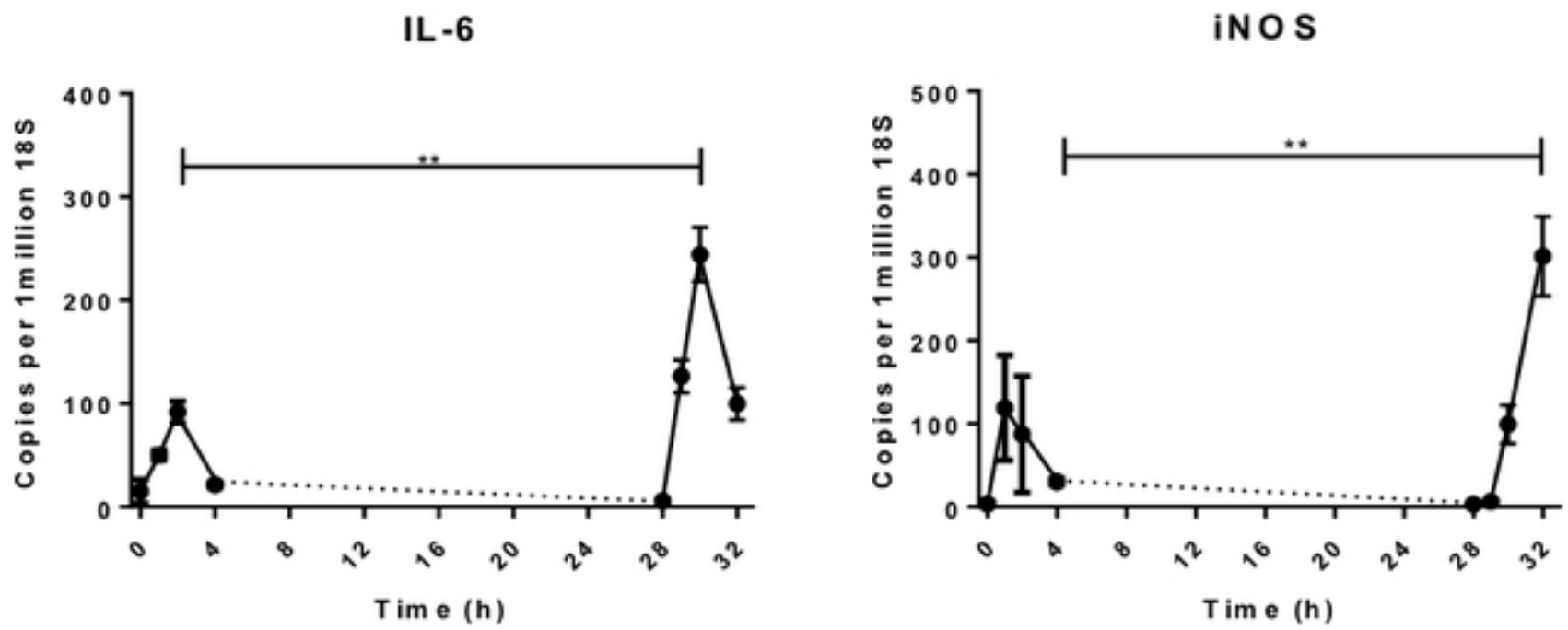


Figure 2. Expression of genes in 3T3-L1 adipocytes during primary and secondary MCM exposures.

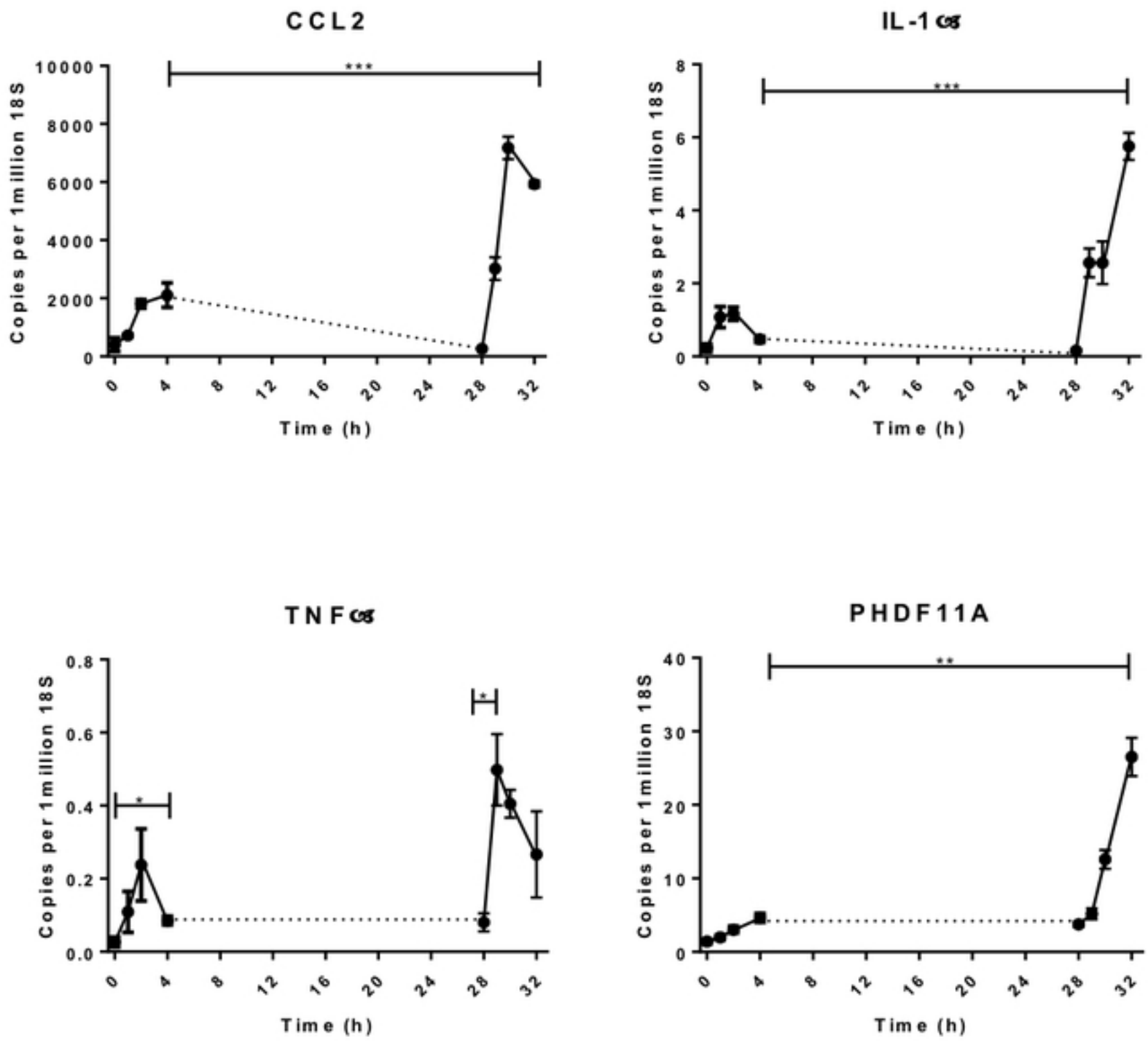


Figure 3. Expression of inflammatory genes in 3T3-L1 adipocytes during primary and secondary MCM exposures that show transcriptional memory.

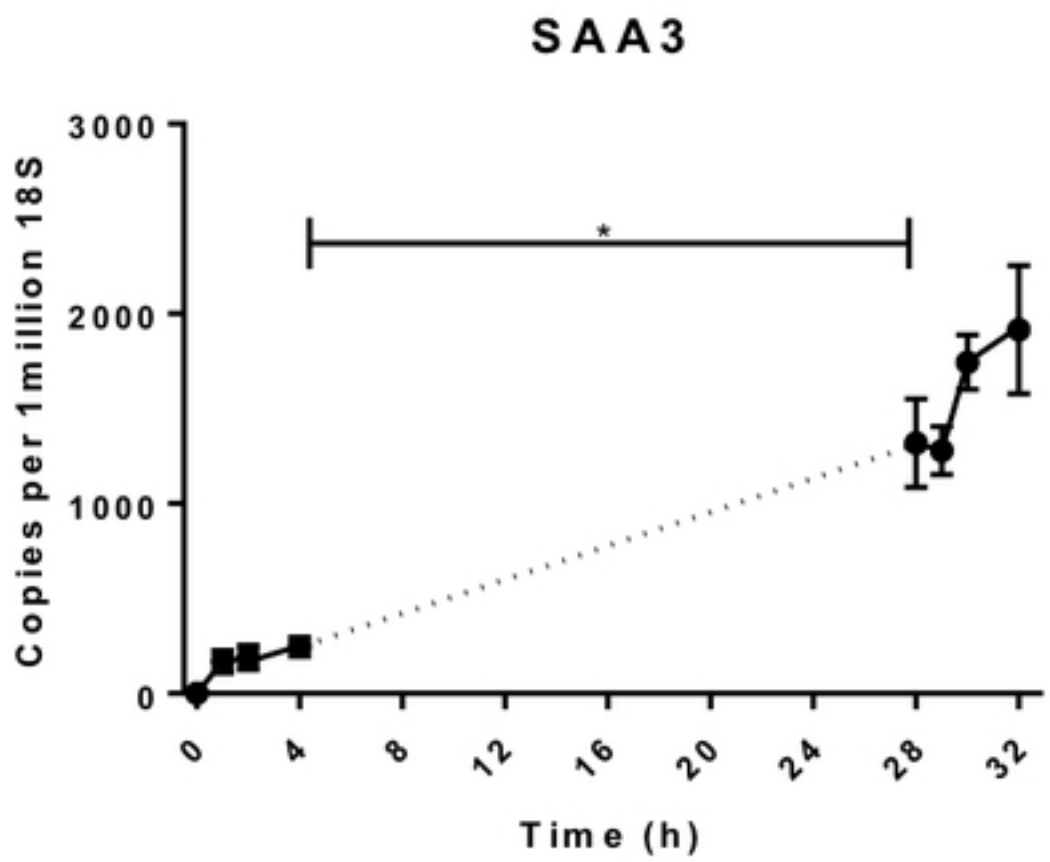


Figure 4. Expression of SAA3 in 3T3-L1 adipocytes during primary and secondary MCM exposures.

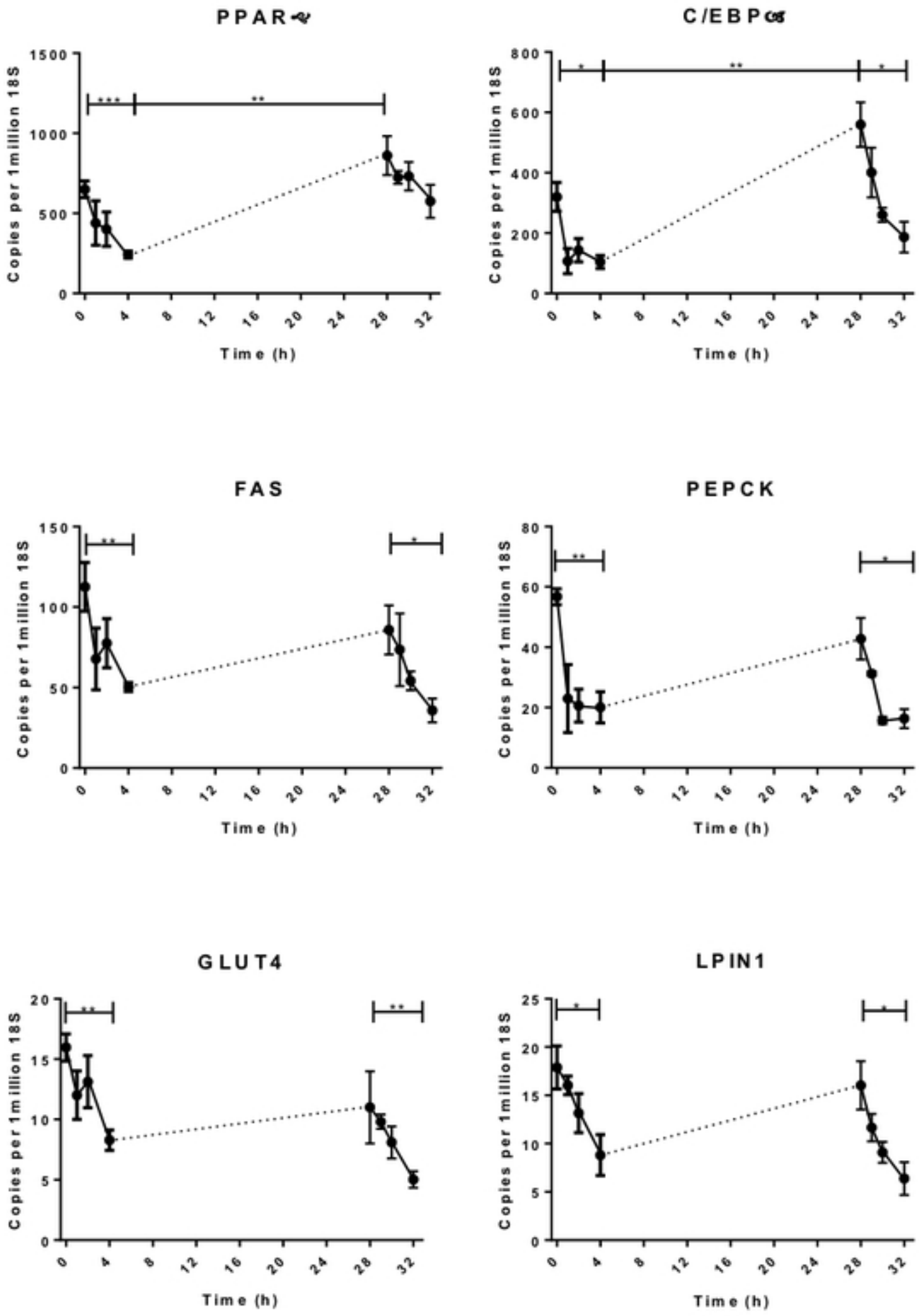


Figure 5. Expression of genes in 3T3-L1 adipocytes during primary and secondary MCM exposures.

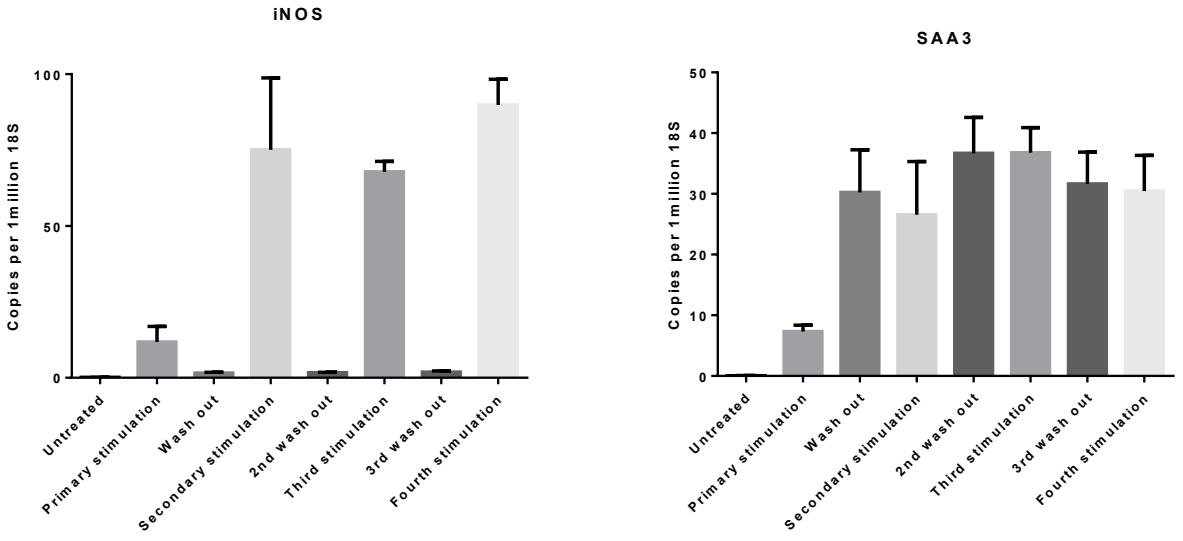


Figure 6. Expression of genes in 3T3-L1 adipocytes during primary, secondary, third and fourth MCM exposures.

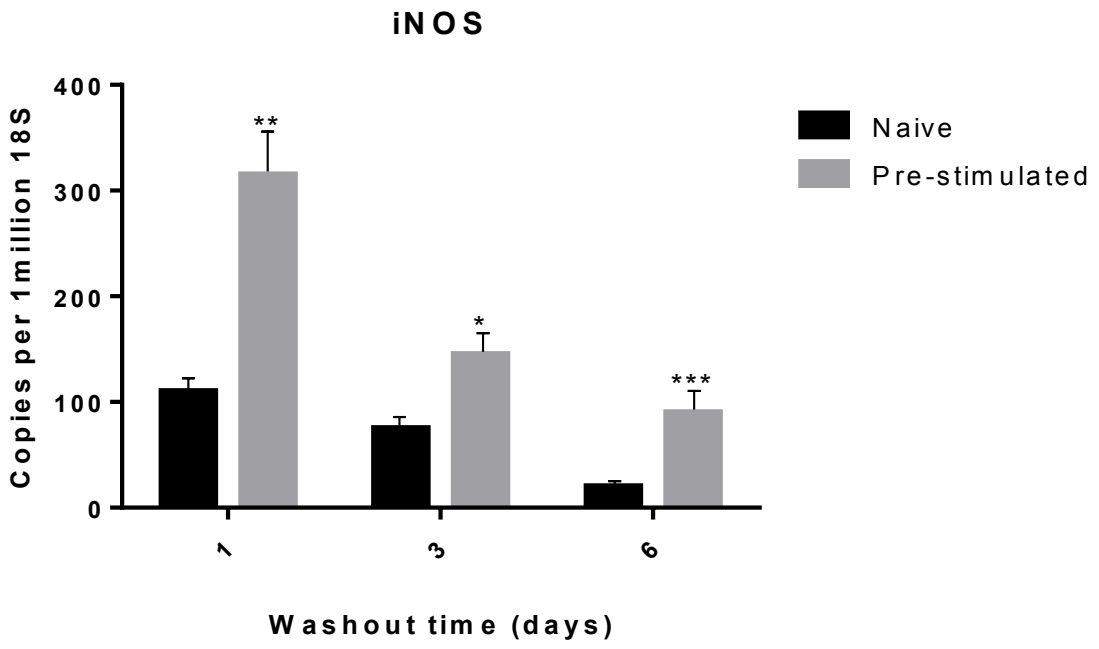


Figure 7. Expression of iNOS in 3T3-L1 adipocytes during primary and secondary MCM exposures with extended washout times.

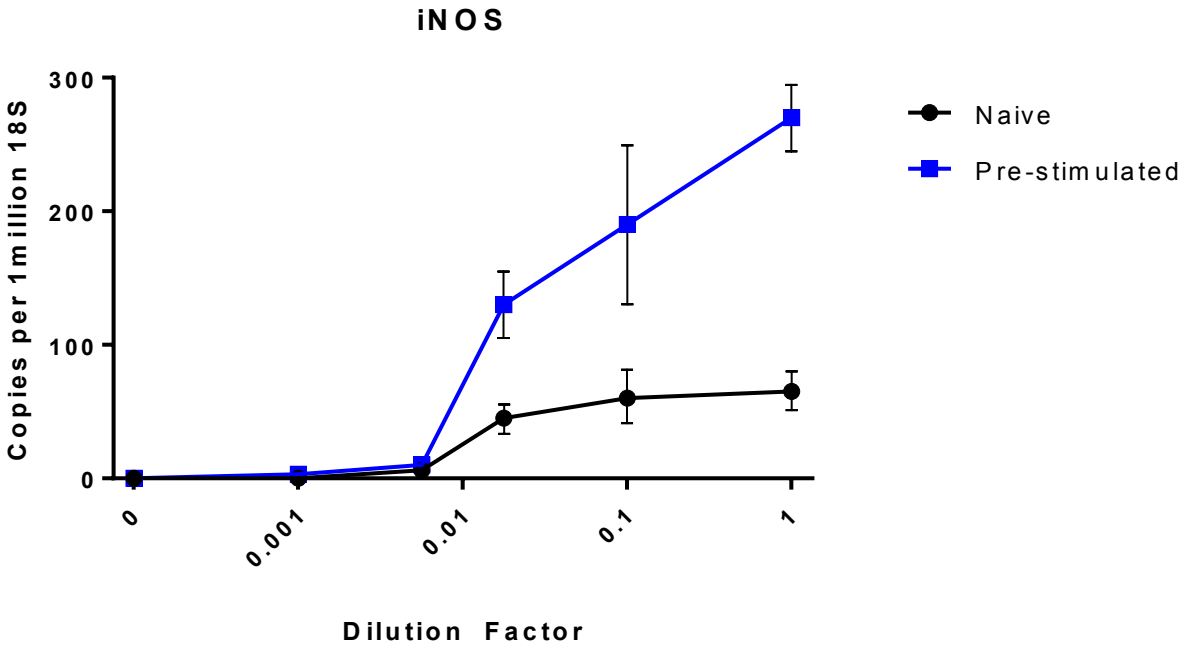


Figure 8. Expression of iNOS in 3T3-L1 cells during primary and secondary stimulation with varying concentrations of MCM.