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RESEARCH NOTE

Additive effects of β-adrenergic and cytokine signaling on lipolytic activation [version 1; referees: 2 approved with reservations]

Nicholas Card, William S. Garver, Robert A. Orlando

University of New Mexico, School of Medicine Department of Biochemistry and Molecular Biology, University of New Mexico, Albuquerque, NM 87131-0001, USA

v1 **First published:** 24 Jun 2014, **3**:134 (doi: 10.12688/f1000research.4151.1)
Latest published: 24 Jun 2014, **3**:134 (doi: 10.12688/f1000research.4151.1)

Abstract

Obesity often leads to increased systemic inflammation which is now thought to play a causative role in the development of atherosclerotic disease and insulin resistance. This inflammatory response originates within large adipose tissue depots and is initiated by classically activated macrophages that infiltrate the tissue from the circulation. The large number of macrophages residing in obese adipose tissue leads to significant increases in interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) secretion; achieving levels sufficient to elevate circulating plasma concentrations. These cytokines activate potent signals to initiate lipolysis, to release free fatty acids from triacylglycerol stores and contribute to hyperlipidemia in obese individuals. Obese adipose tissue responds to normal β-adrenergic and glucagon stimuli to recover from negative energy balance by inducing lipolysis. However, it is not clear what quantitative influence additional lipolytic stimulation by IL-6 and TNFα has on normal β-adrenergic activity. Although, β-adrenergic and cytokine signaling activate separate pathways for lipolytic activation, it is undefined whether the effects of multiple signaling events on lipolysis are additive or coincident. To clarify this issue, we measured lipolytic activity in 3T3-L1-derived adipocytes stimulated by a β-adrenergic agonist (isoproterenol), IL-6 or TNFα individually and in combinations as co- and tri-stimulation. Treatment of adipocytes with isoproterenol and either IL-6 or TNFα as co-stimulants increased lipolytic activation by approximately the sum of the individual ligands, suggesting contributions from two independent pathways. Co-stimulation with IL-6 and TNFα provided slightly more than an additive response indicating signaling contributions from independent and common pathways. Tri-stimulation resulted in the largest level of lipolytic activation with a value approximate to adding isoproterenol stimulation to a combined treatment of IL-6 and TNFα. The additive nature of cytokine signaling to β-adrenergic activity suggests its therapeutic inhibition will prevent excessive lipolysis, yet minimally interfere with maintaining normal responses to varying energy demands.

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Referee Status: ? ?

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version 1 published 24 Jun 2014	? report	? report

1 **Marleen van Baak**, Maastricht University Netherlands

2 **Guy Laureys**, University Hospital Brussels Belgium

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Corresponding author: Robert A. Orlando (rorlando@salud.unm.edu)

How to cite this article: Card N, Garver WS and Orlando RA. **Additive effects of β-adrenergic and cytokine signaling on lipolytic activation [version 1; referees: 2 approved with reservations]** *F1000Research* 2014, **3**:134 (doi: 10.12688/f1000research.4151.1)

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Grant information: We thank the University of New Mexico, School of Medicine, Office of Research for providing generous funding for this work.

Competing interests: No competing interests were disclosed.

First published: 24 Jun 2014, **3**:134 (doi: 10.12688/f1000research.4151.1)

Introduction

Obesity often leads to increased systemic inflammation which is now thought to play a causative role in the development of atherosclerotic disease and insulin resistance[1–4]. Increasing adiposity due to excessive weight gain sets up a chronic inflammatory response within adipose tissue which is promoted by recruitment and infiltration of classically activated, type-1 macrophages from the circulation[5]. Although the reasons for sustained adipose inflammation remain unclear, the large number of macrophages residing in obese adipose tissue leads to significant increases in secretion of interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα); achieving levels sufficient to elevate circulating plasma concentrations[6]. IL-6[7] and TNFα[8,9] are multifunctional cytokines that have well-established roles in inflammatory responses. In adipose tissue, these cytokines share a common activity in delivering potent signals for stimulation of lipolysis[10–13]. Increased lipolytic activity in obese adipose tissue increases free fatty acid (FFA) flux into the circulation. From a lipocentric view, elevated plasma levels of non-esterified fatty acids (NEFA) leads to increased amounts of atherogenic lipoproteins in the circulation resulting in unmanaged hyperlipidemia[14], often accompanied by reduced systemic insulin responsiveness[15,16].

The metabolic actions of IL-6 are diverse. Determination of which actions take precedence is largely dictated by tissue and metabolic context[17]. For example, IL-6 is an important mediator of the acute phase response which includes hepatic effects to increase glucose output and elevate CRP levels[17,18]. IL-6 secretion is also increased as a result of exercise[19,20], which in turn increases glucose oxidation[21] and insulin sensitivity[22] in skeletal muscle. IL-6 infusion in humans increased circulating FFA levels[11,23], and when adipose tissue or isolated adipocytes were treated with IL-6, lipolytic activity was increased[17]. In adipocytes, IL-6 binds to a cell surface heterodimer composed of the IL-6 receptor and gp130[24,25], and activates two intracellular signaling pathways; the Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway, and the p44/42 Mitogen-activated protein kinase (MAPK) pathway[17,26].

TNFα is a potent metabolic effector that, in adipocytes, signals primarily through TNFα receptor-1[27]. Intracellular signaling in adipocytes is mediated by p44/42 MAPK and Jun N-terminal kinase (JNK)[12,28]. Once activated, these pathways induce phosphorylation of perilipin to recruit hormone sensitive lipase for triacylglycerol hydrolysis and the release of FFA, and to downregulate perilipin expression[29,30]. Perilipin is a phosphoprotein that coats intracellular lipid droplets in adipocytes to maintain minimal lipolytic activity. Phosphorylation of perilipin serves a dual purpose: to release bound CGI-58 to activate adipose triglyceride lipase and to relocate perilipin away from the lipid droplet permitting catalytic access for activated lipases[31]. However, signaling the sequence of events leading to lipolytic activation through JAK/STAT, p44/42 MAPK and JNK pathways is not the normal physiologic response to increased energy demands requiring the release of fatty acid fuel stores from adipocytes. Normal physiologic activation of lipolysis by β-adrenergic and glucagon signaling during periods of increased systemic energy demands is mediated through heterotrimeric G-protein activation followed by increased intracellular cAMP and protein kinase-A (PKA) activation. Obese adipose tissue is subject to normal β-adrenergic and glucagon stimuli to regulate energy balance; however, this tissue is also subject to additional lipolytic stimuli by IL-6 and TNFα. With the presence of multiple pathways for lipolytic activation, i.e. the normal endocrine/neural pathway and cytokine-mediated pathways, it is unclear whether the effects of multiple signaling events on lipolysis are additive or coincident; that is, do IL-6 and TNFα stimulate lipolytic activities that are in excess of that provided by maximal β-adrenergic activation, or do the pathways activated by these cytokines merge into the downstream β-adrenergic pathway and are they unable to add significantly beyond maximal β-adrenergic activation. To address this question, we have measured lipolytic activity in 3T3-L1-derived adipocytes activated by a β-adrenergic agonist, IL-6 or TNFα, both individually and in combination as co- and tri-stimulation experiments.

Materials and methods

Cell culture and treatment

Mouse 3T3-L1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and grown and differentiated in complete Dulbecco's Modified Eagle Medium (DMEM; Irvine Scientific, Santa Ana, CA) with high-glucose and supplemented with 10% fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA) and incubated at 37°C in a 5% CO_2 environment. Twelve-well culture plates (Costar Tissue-culture Treated; Corning, Tewksbury MA) were coated with 1% gelatin (Sigma-Aldrich, St. Louis, MO) prior to cell seeding. After reaching confluency, cells were differentiated into mature adipocytes by incubating with 450 µM 3-isobutyl-1-methylxanthine, 250 nM dexamethasone and 167 nM insulin (Sigma-Aldrich, St. Louis, MO) diluted into DMEM for 3 days, followed by 4 days supplementation with 167 nM insulin alone. Differentiation was confirmed by visual examination (MicrosOpics, IV900 Series inverted microscope) of cells to assess for lipid droplet formation and morphological changes. Prior to each experiment, cells were incubated with complete DMEM without insulin supplementation for a period of 24 hours. Cells were then treated either with isoproterenol (25 nM to 2.5 µM) (Sigma-Aldrich, St. Louis, MO), TNFα (0.1 nM to 25 nM) (Cell Signaling, Danvers, MA) or IL-6 (0.5 nM to 8 nM) (eBioscience, San Diego, CA) for 24 hours to identify an optimal concentration of effector necessary for induction of fatty acid hydrolysis and glycerol release. Once this concentration was determined, subsequent experiments were performed by incubating cells with this identified concentration for 24 hours.

Glycerol release assays

Lipolytic determinations were made by quantifying glycerol released into the culture medium following treatments. Culture media were collected and processed using Free Glycerol Reagent according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Following the prescribed incubation, the resulting quinoneimine dye in samples was measured by spectrophotometry at 540 nm (Molecular Devices, Spectramax 384 Plus, Sunnyvale, CA). Glycerol amounts in the culture media were determined through comparison with standard curves that were generated by parallel quantification assays using known concentrations of glycerol.

Statistical analysis

Mean values obtained for each experimental treatment group were compared by a one-way analysis of variance (ANOVA) using SigmaPlot 2001 software. Statistical significance is reported when P-values < 0.05.

Results and discussion

Our primary objective for this study was to determine if cytokine stimulation (IL-6 or TNFα) heightens lipolytic activity (as measured by glycerol release) over and above what is achieved by normal β-adrenergic signaling. To obtain this quantitative evaluation, we first incubated mature 3T3-L1-derived adipocytes with varying concentrations of either isoproterenol (a β-adrenergic agonist), IL-6 or TNFα individually in order to determine the concentration of ligand that provided maximal lipolytic stimulation. This will ensure that individual ligands will be used at concentrations that maximally activate their respective signaling pathways in co- and tri-stimulation experiments. From our titration study, we have determined that maximal lipolytic stimulation for each ligand is achieved with the following concentrations: 1 μM for isoproterenol, 2 nM for IL-6 and 0.58 nM for TNFα (data not shown).

Co- and tri-stimulation experiments were performed using the ligand concentrations determined above. When adipocytes were incubated with the individual ligands, different levels of lipolytic activation were noted with IL-6 < isoproterenol < TNFα (Figure 1. compare bar 2 < bar 1 < bar 5). These differences are likely due to activation of different signaling pathways which have varying quantitative effects on lipolytic activation. Co-stimulation of adipocytes with isoproterenol and IL-6 resulted in lipolytic activity that was greater than that achieved by stimulation with the individual ligands (Figure 1. compare bar 3 with bars 1 and 2), suggesting parallel activation of different signaling pathways that merge into downstream lipolytic activation. The level of increased lipolytic activation from isoproterenol and IL-6 co-stimulation is approximately the sum of the individual ligands, suggesting an additive effect of contributions from two independent pathways, likely cAMP/PKA and p44/42-JAK/STAT, respectively. When isoproterenol and TNFα were combined, again an additive effect on lipolytic activation was observed (Figure 1. compare bar 6 with bars 4 and 5), similarly suggesting a summing of the effects caused by the activation of two separate signaling pathways, cAMP/PKA and p44/42-JNK, respectively.

In vivo, obese adipose tissues express both IL-6 and TNFα when they are inflamed and susceptible to concurrent stimulation by both cytokines. To determine the effects of dual cytokine stimulation on lipolysis, adipocytes were incubated with both IL-6 and TNFα. In this case, lipolytic activation was somewhat more than an additive response (Figure 1. compare bar 7 with bars 2 and 5), suggesting that independent, as well as overlapping, signaling pathways were activated. The independent pathways include JAK/STAT for IL-6 and JNK for TNFα, while both cytokines are capable of activating the p44/42 pathway. The greater than additive response is likely due to dual stimulation of the common p44/42 pathway. Finally, in order to simulate a physiological environment where obese, inflamed adipose tissue undergoes normal β-adrenergic stimulation during fasting and/or exercise, we treated adipocytes with a triple combination of isoproterenol, IL-6 and TNFα. This condition resulted in the highest level of lipolytic activation with a value slightly greater

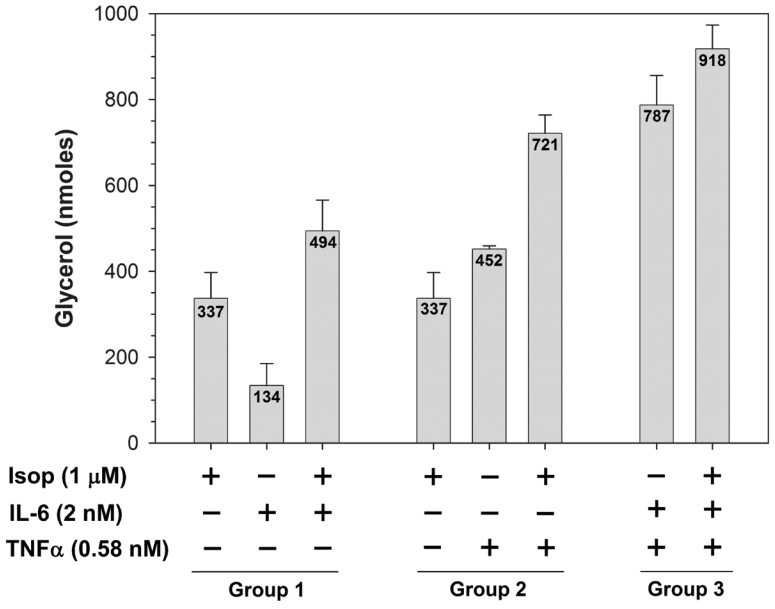

Figure 1. Lipolytic activation of 3T3-L1 adipocytes. 3T3-L1 cells were differentiated into mature adipocytes and incubated for 24 hours in the absence of insulin to diminish insulin-dependent anabolic signals. Cells were then incubated for an additional 24 hours in the presence (+) or absence (-) of the indicated concentrations of β-adrenergic agonist (isoproterenol) or cytokine (IL-6 or TNFα) for individual, co- and tri-stimulations. Medium was removed from cells and glycerol was quantified as described in the Materials and methods section. Experimental points were measured in triplicate to determine mean values (shown within bars) and standard deviations (error bars). Data shown are mean values determined using replicate experiments (n = 3). Mean values obtained for each experimental treatment group (1, 2 or 3) were compared by a one-way analysis of variance (ANOVA). Statistical significance is reported when P-values were < 0.05.

than adding isoproterenol stimulation to a combined treatment of IL-6 and TNFα (Figure 1. compare bar 8 with bars 1 and 7).

Dataset 1. Glycerol release following isoproterenol, IL-6 and TNFα stimulation.

Card, *et al.*, individual data sets.csv
Raw spectrophotometric values obtained for glycerol release following single, double and triple stimulation of 3T3-L1-derived adipocytes using isoproterenol, IL-6 and TNFα. Each treatment was measured in triplicate. Blank values (determined without addition of reagents to measure glycerol amounts) were subtracted from raw data. Final glycerol concentrations for each experimental point were determined from a standard curve obtained used known glycerol concentrations. Glycerol concentrations for experimental points were calculated based on the standard curve and converted from µg/µL to ng/µL.

Card, *et al.*, final data compilation for Fig.csv
Means were calculated for triplicate values for each experimental treatment as well as standard deviations (SD). Glycerol concentrations (ng/µL) were converted to nmoles using molecular weight of glycerol (92.1 g/mole) for construction of Figure 1.

Click here to access the data.

http://dx.doi.org/10.5256/f1000research.4151.d27711

Conclusion

The level of lipolytic activation from the triple stimulation with isoproterenol, IL-6 and TNFα far surpassed that of normal β-adrenergic stimulation alone, and provides mechanistic evidence for the cause of hyperlipidemia in obese individuals. Under normal circumstances (in lean individuals), β-adrenergic signaling is activated during fasting and exercise to mobilize fatty acids from adipose tissue and compensate for a negative systemic energy balance. Once the energy balance has returned to homeostasis, β-adrenergic stimulation is inactivated and the release of fatty acids is halted to prevent excessive plasma lipid levels. Obese individuals are also subject to normal β-adrenergic, epinephrine and norepinephrine stimulation of adipose tissue due to stress responses or negative energy balance, and this stimulation signals through the heterotrimeric G-protein, adenylyl cyclase, cAMP, PKA network[32]. In addition to this signaling, cytokines produced in inflamed obese adipose tissue also activate additional pathways that make a cumulative addition to the normal lipolytic response. Evidence provided here suggests that IL-6- and TNFα-activated pathways in adipose contribute to increased lipolysis through both independent and common signaling pathways (Figure 2). In considering therapeutic options for obese individuals, maintaining normal β-adrenergic signaling is vital to manage routine changes in energy balance that occur due

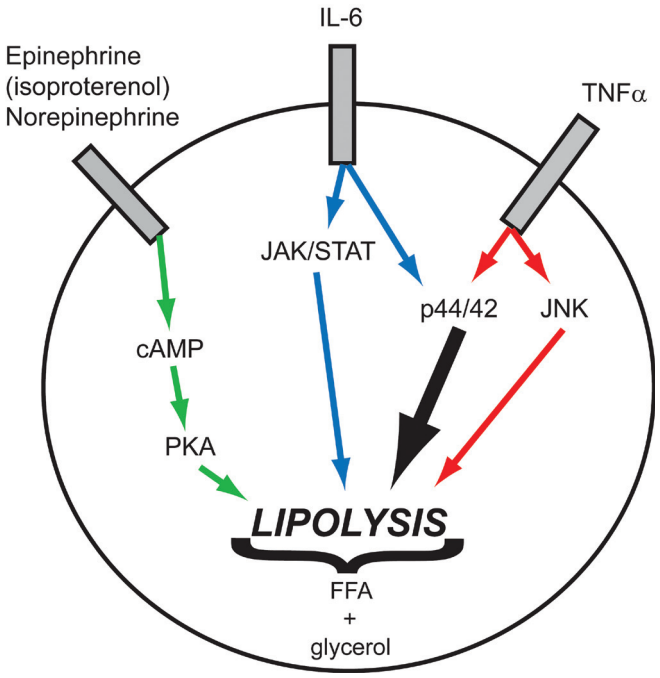

Figure 2. Proposed model of β-adrenergic and cytokine lipolytic activation pathways. β-adrenergic (epinephrine and norepinephrine) stimulation of adipose tissue to induce activation of lipolysis proceeds through heterotrimeric G-protein and adenylyl cyclase activation, which elevates cytosolic cAMP levels (green arrows). Cyclic-AMP-dependent protein kinase-A (PKA) then phosphorylates downstream regulatory proteins and lipases to initiate release of fatty acids from triacylglycerol stores. Inflamed obese adipose tissue is also subject to additional stimuli originating from secreted cytokines. In adipocytes, IL-6 activates the JAK/STAT and p44/42 pathways (blue/black arrows), while TNFα activates the JNK and p44/42 pathways (red/black arrows), which are independently sufficient to stimulate lipolysis. Based on the magnitude of fatty acid release when cytokine stimulation is concurrent with normal adrenergic signaling, it appears that the activation of combined pathways provides an additive response to lipolytic activation leading to an excess of fatty acids released.

to cyclical variations in physical activity. However, the contributions of IL-6 and TNFα to increased lipolytic activity being additive to normal β-adrenergic stimulation indicates that these pathways (p44/42, JAK/STAT and JNK) represent excellent therapeutic targets that will prevent excessive lipolysis, yet minimally interfere with maintaining normal responses to varying energy demands.

Data availability

F1000Research: Dataset 1. Glycerol release following isoproterenol, IL-6 and TNFα stimulation, 10.5256/f1000research.4151.d27711[33]

Author contributions

NC and RO conceived the study. NC and RO designed the experiments. NC carried out the research. NC and RO prepared the first draft of the manuscript. NC, WG and RO contributed to the experimental interpretations and preparation of the manuscript. All authors were involved in revising the draft manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed.

Grant information

We thank the University of New Mexico, School of Medicine, Office of Research for providing generous funding for this work.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We thank Drs. Carolina Franco Nitta and Yijuan Sun for their thoughtful contributions to this study.

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Current Referee Status: ? ?

Version 1

Referee Report 21 July 2014

doi:10.5256/f1000research.4444.r5475

? **Guy Laureys**
Department of Neurology, University Hospital Brussels, Brussels, Belgium

The authors evaluate the β-adrenergic pathwaywhich is an interesting target for modulation of inflammatory and metabolic pathways. This paper investigates the potential (additive) effects of β-adrenergic and cytokine stimulation on lipolysis in mouse adipose cells. Additive effects of isoproterenol, TNF-α and IL-6 on lipolysis in 3T3-L1 cells are demonstrated in vitro.

A major question concerns the applied statistics:

1. The n value seems insufficient to assume normal distribution, a non-parametric test seems more appropriate.

2. The post-hoc test after ANOVA is not mentioned. If performed the (non)significance between different experimental groups should be indicated.

3. Does the n=3 mean technical replicates? If so they should be averaged but not evaluated as separate experiments. If not, why does group 1 to 3 not cover all possible experimental strategies? This is confusing and should be clearly stated.

4. Are there data on a sham experiment? If so they should be included.

Other questions concerning this paper:

1. As for most in-vitro studies: what is the relevance for the in-vivo and/or human situation?

2. A good part of the discussion is based on assumptions - e.g. additive effects are suggested to be the consequence of the activation of different signaling pathways, however this is not experimentally verified.

These questions should be addressed in an updated version of the paper.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Referee Report 09 July 2014

doi:10.5256/f1000research.4444.r5232

Marleen van Baak
Department of Human Biology, Maastricht University, Maastricht, Netherlands

The paper investigates the additive effects of isoproterenol, interleukin-6 and TNF-alpha on lipolysis in mature mouse 3T3-L1 adipocytes. For this, the maximal lipolytic doses of each of the three agonists were established and 3T3-L1 adipocytes were incubated for 24h with these doses, alone or in combination. Lipolysis was assessed as glycerol release into the medium over the 24h incubation period.

The main conclusion to be drawn from the study is that IL-6 and TNF-alpha signaling are additive to the beta-adrenergic signaling with respect to 3T3-L1 adipocyte lipolysis. The investigators furthermore conclude "*that this suggests that therapeutic inhibition of cytokine signaling will prevent excessive lipolysis, yet minimally interfere with maintaining normal responses to varying energy demands*". This addition seems to be too far-fetched for several reasons: 1. confirmation in human adipocytes is required, because there may be species differences; 2. the study tested maximal lipolytic doses of the three lipolytic agents and under physiological conditions such a combination is unlikely to occur; 3. it is well-known that in obesity responsiveness to beta-adrenergic stimulation is blunted, therefore it could also be that the increased lipolysis due to increased cytokine levels in obesity helps to maintain normal lipolytic responsiveness to increased energy demand rather than result in excessive lipolysis.

Other comments:

1. Glucagon is mentioned several times as a stimulant of lipolysis without appropriate reference. There are few and mostly inconsistent data on a potential lipolysis-stimulating effect of glucagon in humans. Since the studies do not address glucagon, I would suggest not to include glucagon in the introduction and abstract.

2. I find the term 'coincident' confusing. Although it is explained in the introduction what the authors mean by coincident, its use is not necessary. The question can simply be whether cytokines and isoproterenol are additive in their effects on lipolysis or not.

3. In the Methods it is stated that the optimal dose to induce 'fatty acid hydrolysis' is identified. This should probably be 'triglyceride hydrolysis'.

4. Figure 1 represents the results of three groups. In the Methods these three groups are not mentioned. In the legend to figure 1 it is mentioned twice that mean values from three replicate experiments are shown. Both in Figure 1 as well as in the methods there is reference to statistical testing. However, the results of these tests are not reported.

5. The text in the Results section refers to bar numbers in Figure 1, but these are not indicated in the figure.

6. Why do the authors conclude that the more than additive effect of Il-6 and TNF-alpha suggests that independent, as well as overlapping, signaling pathways were activated? Please explain more clearly.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.