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Studying The Nadph Oxidase Enzyme And The Different Mutations Resulting From Macrophage Responses

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Abstract

The study aimed at assessing whether different mutations in the subunits of NADPH oxidase (gp91^{phox}, p22^{phox}, p40^{phox}, p47^{phox}, p67^{phox}) result in an altered macrophage response to infection.The study also examines the production of important inflammatory cytokines in human macrophages in which the expression of different NADPH oxidase subunits have been mutated. Several methods were employed in this study. Mutations in the various subunits of the NADPH enzyme may lead to the manifestation of CGD. In this study, we theorise that different mutations in the subunits of NADPH oxidase result in an altered macrophage response to infection. LPS was found to decrease macrophage viability but also cause increased production of TNFa and IL-1b. The build-up of dead macrophages may also contribute to the inflammatory respo[1]nse, characterised by TNFα and IL-1β release from the remaining active cells. PMA; used to differentiate U937 cells into macrophages, becomes cytotoxic to macrophages in a time and dose-dependent fashion. The results of the study add to the body of scientific literature that exists on the structure of the NADPH oxidase and the mutagenic approach devised is designed to further the understanding of how the structure of this important enzyme impacts on the activity of the immune system in general, and macrophages in particular. In the longer term, it is hoped that outcomes from this work may inform future therapeutic intervention strategies to combat chronic granulomatous disease.

***Keywords**: CGD, X-linked disorders, NADPH oxidase enzyme, RNA Interference and Mutations in genes.*

1. Introduction

The host immune system employs a plethora of defence mechanisms to protect against infection by pathogenic microorganisms. The result is a multifaceted defence mechanism, employing a myriad of immune cells, signalling proteins and small molecules that forms an intrinsic part of both the innate and adaptive immune systems. The following literature review aims to provide background information on these host defences, with a focus on phagocytosis by macrophages and the subsequent oxidative burst, mediated by respiration by NADPH oxidase enzyme. This review will outline the physiological and biochemical mechanisms underpinning this critical defence mechanism and will concentrate on one well-characterised condition arising from mutations in the NADPH oxidase system: chronic granulomatous disease. The decreased (or absent) of the oxidative burst in leukocytes seen in CGD is due to one or more inherited mutations in genes encoding

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subunits of the enzyme complex. This review will detail the impact of these mutations on NADPH oxidase formation and activity and highlight existing therapeutic intervention strategies to treat CGD.

Genetic diseases can result from either a single mutated gene (single gene disorder) or they can be 'polygenic', involving biological systems linked to environmental factors. It has been reported that there are more than 4,000 human diseases caused by single gene defects, many of which are heritable (Kumar, 2008). One type of heritable genetic disorder is 'autosomal inheritance', in which genetic traits located on the one of the twenty-two non-sex determining chromosomes (autosomes). Autosomal inheritance can be either dominant or recessive, referring to whether or not traits are expressed when two alleles are present. Recessive traits are only expressed phenotypically when two copies of the same allele are inherited (homozygous). In cases where both parents are carriers of an autosomal recessive trait the offspring have a 25% chance of the disease being inherited and a 50% chance of the offspring becoming carrier for the disease. The probability of an offspring inheriting an autosomal recessive trait is less than when the condition is autosomal dominant, where only one mutated copy of a gene is required to express the allele.

Another form single gene disorder is X-linked inheritance, where the mutated gene causing the disorder is located on the X-chromosome. Because females have two X-chromosomes, disorders on this chromosome are less likely to manifest, compared to males. However, females with one copy of a mutated gene on an X-chromosome can become carriers of the genetic condition. In contrast, males with only one copy of the X-chromosome are susceptible to the effects of mutated genes that cause an estimated 651 X-linked disorders (OMIM Statistics, 2013).

In addition to single gene disorders, there are a growing number of multifactorial disorders (e.g. heart disease, diabetes and cancer), which may have a genetic element, but the incidence rate also depends on environmental factors and there is no definitive pattern of inheritance. Furthermore, complex disorders are more difficult to diagnose and treat due to the lack of knowledge of the causes and the impact of contributing factors.

This dissertation will focus on mechanisms underpinning the single gene disorder chronic granulomatous disease (CGD), According to Kang et al. (2011) the majority of cases of CGD are X-linked; the condition is referred to as an 'X-linked trait', although, as will be discussed, the condition can also be transmitted in an autosomal recessive manner. Epidemiological data from van den Berg et al. (2009) is representative of CGD studies, finding that 67% of CGD cases were X-linked i.e a mutation in the $gp91^{phox}$ subunit. In accordance with heritability traits for other X-linked disorders, the authors reported that 98% of patients with X-linked CGD were males (n=290).

According to the available scientific literature, CGD-related pathogens are usually isolated from the lungs via biopsy or bronchial lavage and the type of pathogen tends to vary between studies. However, there is some degree of inter-study consistency for some pathogens, such as the fungi Aspergillus spp., which is represents a 'marker' strain for cases of CGD. In North America, the five microbial pathogens most associated with CGD are Staphylococcus aureus, Serratia marcescens, Burkholderia cepacia, Nocardia spp. and Aspergillus spp. (Kang et al., 2011). In Europe, Aspergillus infections made up the vast majority of clinical isolates from patients with CGD and infection with Burkholderia cepacia was relatively rare patients (van den Berg et al., 2009).

2. Materials and Methods:

All chemicals used were of laboratory grade; unless otherwise stated; and were primarily supplied by Invitrogen Life Sciences (Paisley; UK). When other suppliers were used; they have been noted in this section. All reagents and buffers were stored at 2-8°C for a maximum of one week to ensure full activity. All plastic and glassware were of laboratory standard and were sterilised prior to use.

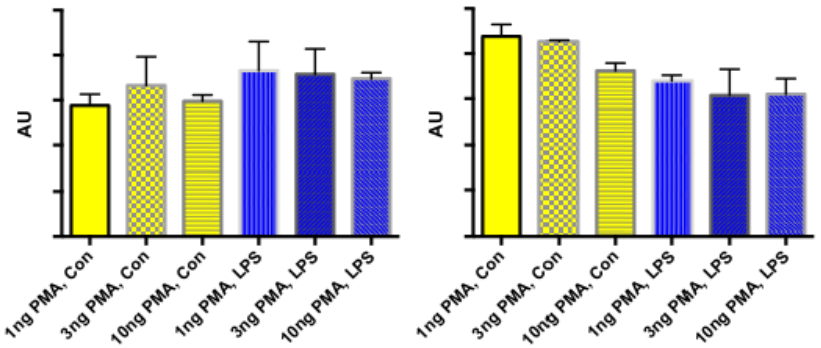
In this study, the researcher used several lab techniques and performed various experiments: Cell culture and in vitro treatment, ELISA tests, Quantifying the concentration of TNFα and of IL-1β protein, MTT assay, and certain statistical analyses. All of these are outlined in the analysis and discussion section.

3. Results and Discussion

3.1. MTT Assay

Colony A (treated for 4 hours with LPS)

24hours 72hours

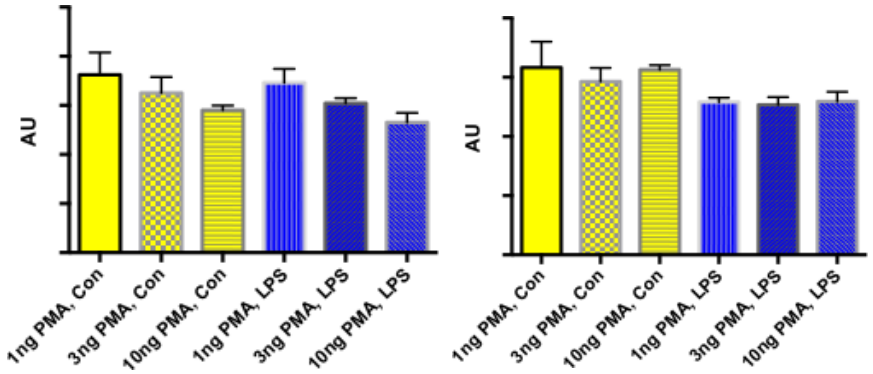


(Fig 2.10) MTT assay on PMA Differentiated U937 (Colony A). U937 cells were differentiated for 24 hours/72 hours with indicated concentrations of PMA (1; 3;10ng/ml); washed and treated for 4 hours with medium (Con) or LPS (100U/ml). At 24 hour differentiation; the two-way ANOVA shows that both the row factors (differences between the x values/effect of LPS and PMA concentration) and column factors (differences between the y values/AU measurements) are not significant; meaning that the data cannot be analyzed.

At 72 hour differentiation; the two–way ANOVA shows that the row factor is not significant but the column factor is significant ($p<0.0001$); meaning that the AU values obtained are statistically significant. In the LPS-activated group; the mean AU values obtained were higher than the control group. A small drop in the AU levels can be seen in the LPS-treated group in response to an increasing PMA concentration. This trend is not apparent in the control group.

Colony A (treated for 24 hours with LPS)

24hours 72hours

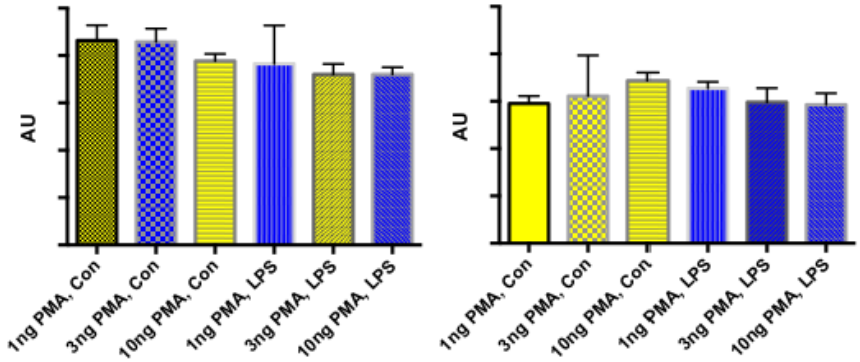


(Fig 2.11) MTT assay on PMA Differentiated U937 (Colony A)

At 24 hour differentiation; the two–way ANOVA concludes that the row factor is not significant but the column factor is significant ($p<0.0022$). A general trend is evident in both the control and LPS-treated groups- as the PMA concentration increases; the AU reading decreases in a step-wise manner. A small difference in AU levels exists between the control and LPS-treated groups; with slightly higher levels witnessed in the control group. At 72 hour differentiation; the two-way ANOVA shows that both the row factor and column factor are not significant therefore the data cannot be analysed.

Colony B (treated for 4 hours with LPS)

24hours 72hours

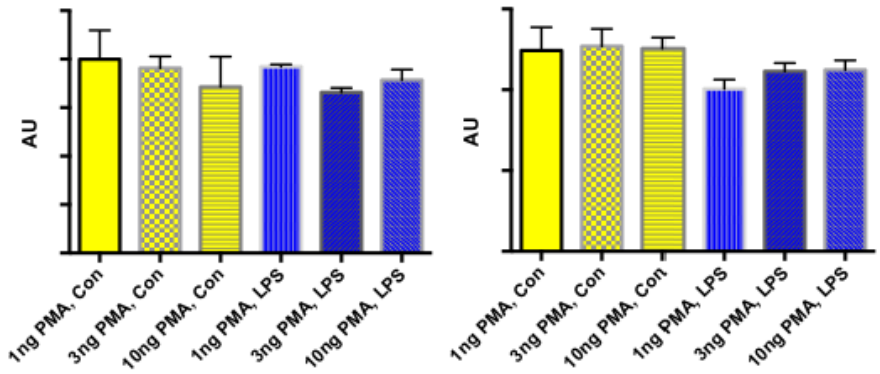


(Fig 2.12) MTT assay on PMA Differentiated U937 (Colony B)

At both 24 and 72-hour differentiations; the two-way ANOVAs indicate that the row factors and column factors were not significant for either group; meaning the data cannot be accurately interpreted.

Colony B (treated for 24 hours with LPS)

24hours 72hours



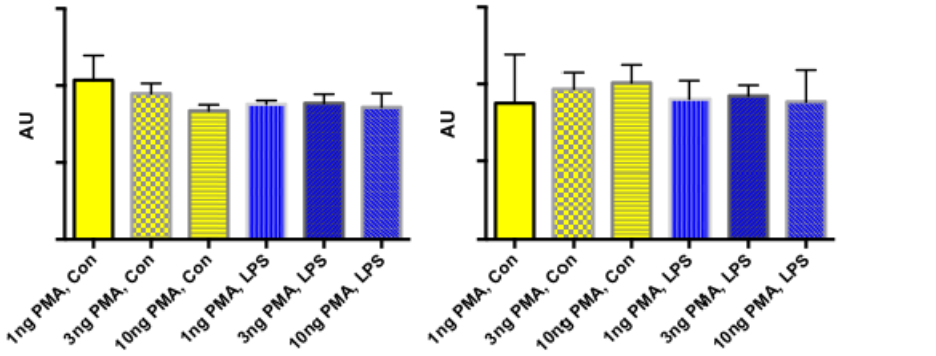
(Fig 2.13) MTT assay on PMA Differentiated U937 (Colony B)

At 24 hour differentiation; the two–way ANOVA shows that the row factor is not significant but column factor is highly significant ($p<0.0006$). Minor differences in the AU levels are apparent between the control and LPS-stimulated groups. A similar trend to that seen in Figure 2.11 can also be observed here; as the concentration of PMA rises in the control and LPS-treated groups; the AU values decrease accordingly.

At 72 hour differentiation; the two–way ANOVA shows that both the row factor ($p<0.0024$) and the column factor ($p<0.0001$) are significant. From the graph we can see that AU readings are lowest in the LPS-treated group. Minimal differences in the AU readings exist between each concentration of PMA in the LPS-treated group and also in the control group.

Colony C (treated for 4 hours with LPS)

24hours 72hours

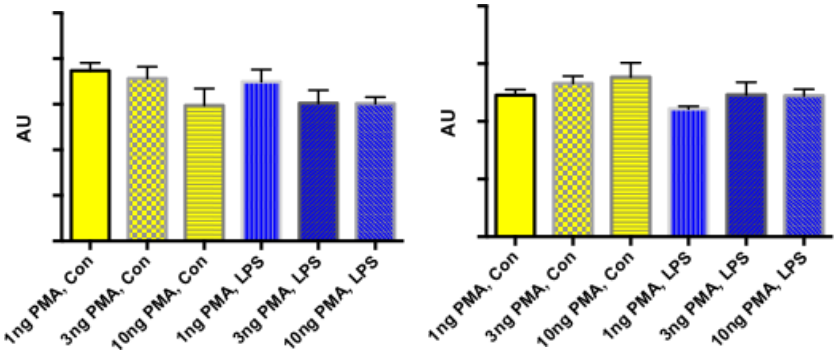


(Fig 2.14) MTT assay on PMA Differentiated U937 (Colony C).

In both the 24 and 72 hour differentiation groups; the two-way ANOVAs show that the row factors and column factors were not significant in either group meaning the data cannot be evaluated.

Colony C (treated for 24 hours with LPS)

24 hours 72 hours

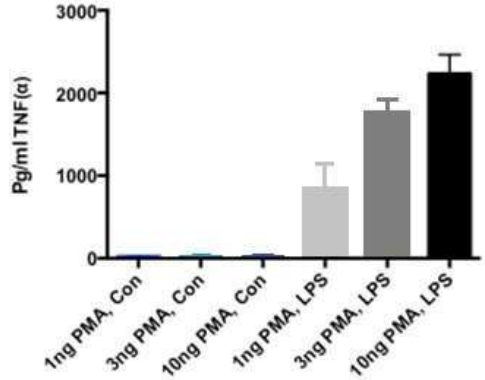


(Fig 2.15) MTT assay on PMA Differentiated U937 (Colony C)

At 24-hour differentiation; the two–way ANOVA shows that the row factor is not significant and the column factor is significant ($p<0.0038$). The general trend appearing in both the control and LPS-treated groups is that as the concentration of PMA rises; the AU value lowers in turn. Comparable levels are observed between the two experimental groups. At 72 hour differentiation; the two–way ANOVA shows that the row factor is not significant but the column factor is significant ($p<0.0017$). As the PMA concentration increases; the AU values increase; contrasting with previous findings. The AU values were highest in the control group in comparison to the LPS-treated group.

5.2. Colony A; TNFα and IL-1β analysis

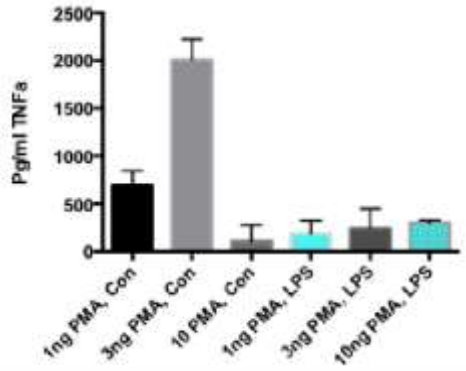
TNFα; 24 hours differentiation



(Fig 3.10) TNFα assay on 1 day PMA Differentiated U937 (Colony A). U937 cells were differentiated for 24 hours with indicated concentrations of PMA (1; 3;10ng/ml); washed and treated for 4 hours with medium (Con) or LPS (100U/ml).

None of the control readings were statistically significantly. However; significant results were obtained in the LPS-stimulated cells; a dose-related increase in TNFα production was seen in accordance with the PMA concentration.

Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = yes; 0.0104	1ng x 1ng = yes; 0.0130
1ng x 10ng= ns	1ng x 10ng= yes; 0.0037	3ng x 3ng= yes; 0.0001
3ng x 10ng= ns	3ng x 10ng= ns	10ng x 10ng= yes; 0.0001

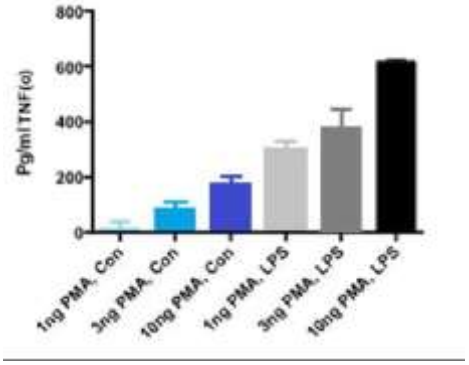


(Fig 3.11) TNFα assay on 1 day PMA Differentiated U937 (Colony A).

In the LPS-treated group; a dose-related increase in TNFα production can be observed; corresponding to the PMA concentration. The results of the control groups were significant; with an extremely high TNFα production at 3ng PMA. The levels of TNFα produced by the LPS-treated group were significantly less than the levels induced by the control group (with the possible exception of the 10ng PMA control group).

Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0001	1ng x 3ng = ns	1ng x 1ng = yes; 0.0025
1ng x 10ng= yes; 0.0022	1ng x 10ng= ns	3ng x 3ng= yes; 0.0001
3ng x 10ng= yes; 0.0001	3ng x 10ng=ns	10ng x 10ng= ns

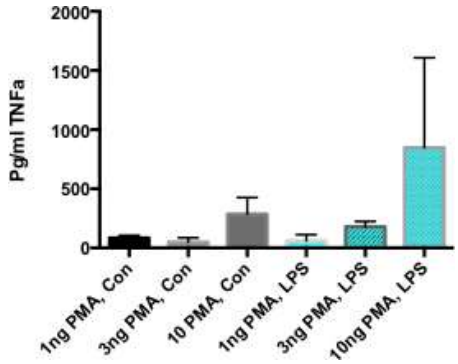
TNFα; 72 hours differentiation



(Fig 3.12) TNFα assay on 3 days PMA Differentiated U937 (Colony A).

The readings obtained for LPS stimulation at each concentration of PMA were statistically significant; versus the control. The apparent trend is that as the PMA concentration increases; the corresponding levels of TNFα increase in response. A similar trend was seen in the control cells. However; higher TNFα levels were observed in the LPS-treated groups in comparison with the controls. Compared to 24 hour differentiation; the longer the exposure to PMA; the greater amounts of TNFα generated.

Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = ns	1ng x 1ng = yes; 0.0002
1ng x 10ng= yes; 0.0012	1ng x 10ng= yes; 0.0010	3ng x 3ng= yes; 0.0024
3ng x 10ng= yes; 0.0126	3ng x 10ng= yes; 0.0180	10ng x 10ng= yes; 0.0002

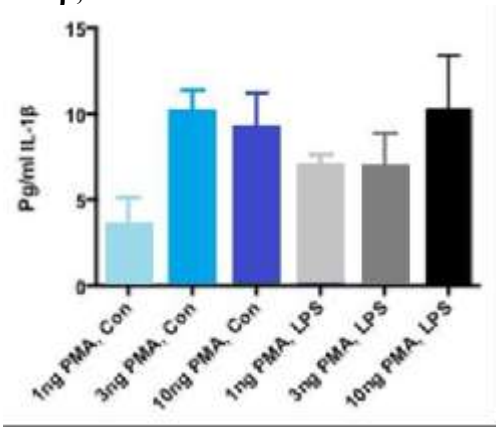


(Fig 3.13) TNFα assay on 3 days PMA Differentiated U937 (Colony A)

In the control group; significant readings were obtained for the 3ng and 10ng PMA groups; with a step-wise increase in TNFα production seen alongside an increase in PMA concentration. This confirms the findings of figure 3.12. With reference to the TNFα readings generated by the LPS-stimulated cells; the only significant reading obtained was at 3ng PMA and in comparison to the control group we can see that higher levels of TNFα are generated following LPS stimulation.

Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = yes; 0.0127	1ng x 1ng = ns
1ng x 10ng= yes; 0.0291	1ng x 10ng= ns	3ng x 3ng= yes; 0.0035
3ng x 10ng= yes; 0.0163	3ng x 10ng= ns	10ng x 10ng= ns

IL-1β; 24 hours differentiation

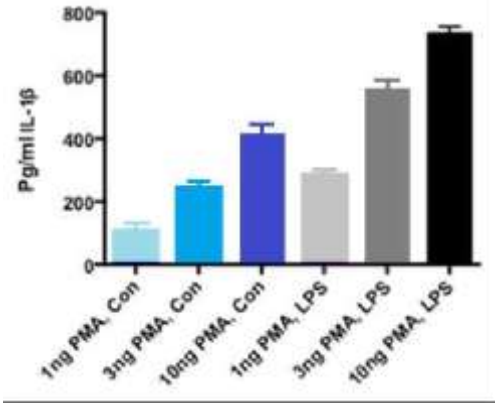


(Fig 3.14) IL-1β assay on 1 day PMA Differentiated U937 (Colony A).

This graph illustrates that higher concentrations of PMA induce higher levels of IL-1β. This is also demonstrated in the control group. Higher levels of IL-1β are seen in the LPS-treated groups (cells treated with 1ng PMA produced the only significant results) when compared to the controls.

Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0006	1ng x 3ng = ns	1ng x 1ng = yes; 0.0066
1ng x 10ng= yes; 0.0061	1ng x 10ng= ns	3ng x 3ng= ns
3ng x 10ng= ns	3ng x 10ng= ns	10ng x 10ng= ns

IL-1β; 72 hour differentiation

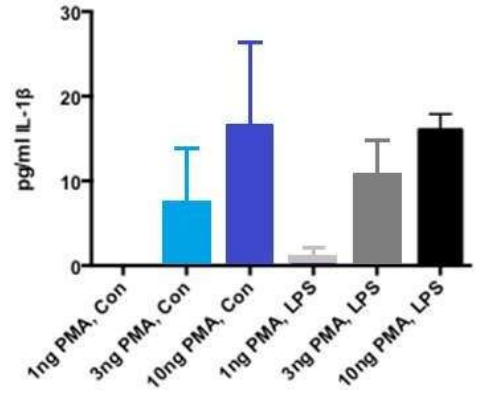


(Fig 3.15) IL-1β assay on 3 days PMA Differentiated U937 (Colony A).

Significant readings were obtained for the controls and LPS-stimulated cells at each concentration of PMA. The observed trend is that as PMA concentration increases; the IL-1β levels increase in accordance. Levels of IL-1β were highest in cells left to differentiate for 72 hours when compared to a 24 hour differentiation. A similar trend was seen in the control cells where LPS was not used. However; higher IL-1β levels were observed in the LPS-treated groups in comparison with the controls.

Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0002	1ng x 3ng = yes; 0.0001	1ng x 1ng = yes; 0.0001
1ng x 10ng= yes; 0.0001	1ng x 10ng= yes; 0.0001	3ng x 3ng= yes; 0.0001
3ng x 10ng= yes; 0.0001	3ng x 10ng= yes; 0.0001	10ng x 10ng= yes; 0.0001

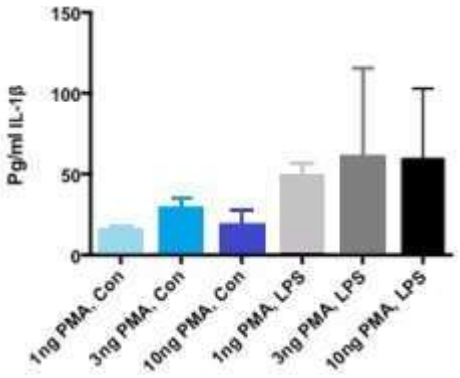
IL-1β; 18 hours differentiation; no treatment with MSU



(Fig 3.16) IL-1β assay on 3 days PMA Differentiated U937 (Colony A).

The readings obtained at 1ng and 3ng PMA with LPS showed statistical significance. Comparing them with each other; we can see that there is a jump in IL-1β production when the concentration of PMA rises to 3ng.

Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = yes; 0.0044	1ng x 1ng = ns
1ng x 10ng= yes; 0.0153	1ng x 10ng= yes; 0.0001	3ng x 3ng= ns
3ng x 10ng= ns	3ng x 10ng= ns	10ng x 10ng= ns

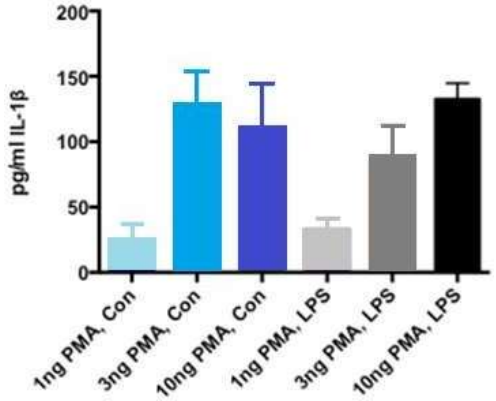


(Fig 3.17) IL-1β assay on 3 days PMA Differentiated U937 (Colony A).

P values calculated from this experiment were above the significance threshold; indicating that the results observed cannot be analysed and interpreted.

Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = ns	1ng x 1ng = yes; 0.0024
1ng x 10ng= ns	1ng x 10ng= ns	3ng x 3ng= ns
3ng x 10ng= ns	3ng x 10ng= ns	10ng x 10ng= ns

IL-1β; 18 hour differentiation with 4 hours treatment with MSU



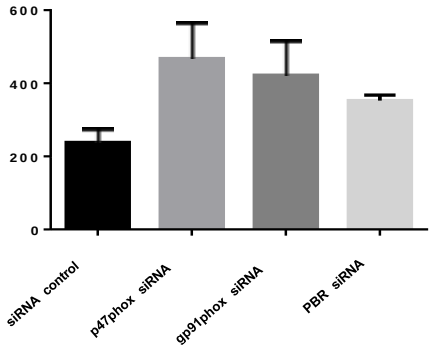
(Fig 3.18) IL-1β assay on 3 days PMA Differentiated U937 (Colony A) (MSU)

We can see a large jump in IL-1β production between the 1ng and 3ng PMA control groups; the only findings that proved to have statistical significance in the controls. We can also see the same trend in the LPS-treated group (all p values were significant). However; comparison with the MSU-untreated group is difficult due to statistically insignificant results being obtained.

Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0004	1ng x 3ng = yes; 0.0058	1ng x 1ng = ns
1ng x 10ng= yes; 0.0027	1ng x 10ng= yes; 0.0001	3ng x 3ng= ns
3ng x 10ng= ns	3ng x 10ng= yes; 0.0180	10ng x 10ng= ns

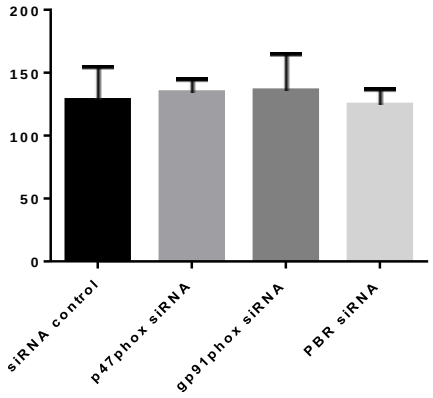
5.3. Colony A; RNA Interference

18 hours LPS stimulation; TNFα



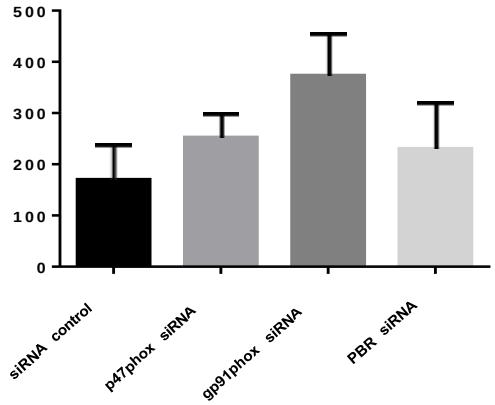
Statistically significant results were obtained for p47phoxsiRNA (p value 0.0045); gp91phoxsiRNA (p value 0.0102) and PBRsiRNA (p value 0.010) in comparison to the control group. P47phoxsiRNA generated the highest levels of TNFα relative to the control; followed by gp91phoxsiRNA and PBRsiRNA. The PBRsiRNA-treated cells had the lowest release of TNFα; however; levels were higher than that of the control. The column factor was significant (p value 0.0054)

18 hours LPS stimulation; IL-1β



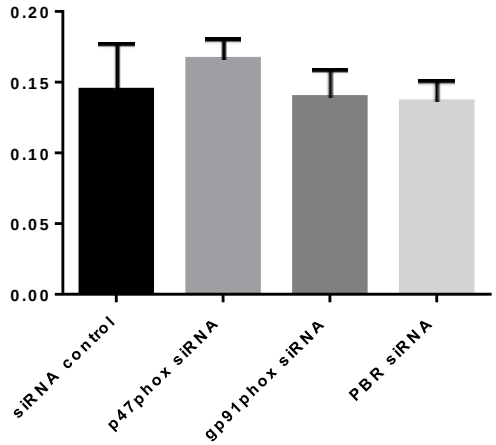
No statistically significant data was obtained from the two-way ANOVA. Both the row and column factors also proved to be insignificant.

18 hours LPS stimulation plus 4 hours MSU stimulation; IL-1β



Statistically significant results were obtained when gp91phoxsiRNA was compared with siRNA control (p value 0.0081) and when it was compared with p47phoxsiRNA (p value 0.0403). The column factor can also be considered statistically significant; with a p value of 0.0382. Knock out of the gp91 subunit resulted in a substantial increase in IL-1β release when compared to the control. However; the effect of MSU on IL-1β production cannot be determined, as a comparison cannot be made with the previous graph due to the statistically insignificant results.

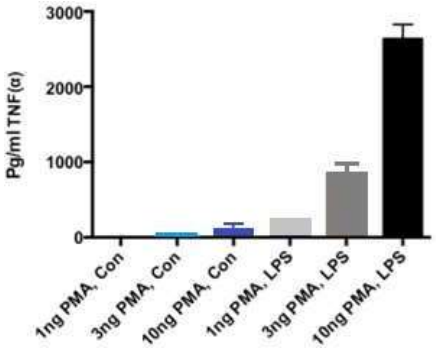
MTT graph



No significant results obtained.

5.4. Colony B; TNFα and IL-1β analysis

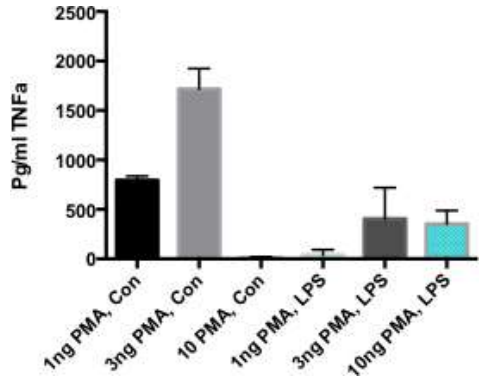
TNFα; 24 hours differentiation



(Fig 4.10) TNFα assay on 1 day PMA Differentiated U937 (Colony B). U937 cells were differentiated for 24 hours with indicated concentrations of PMA (1; 3;10ng/ml); washed and treated for 4 hours with medium (Con) or LPS (100U/ml).

The readings obtained for the control group were not significant. However; this is not the case in the LPS-stimulated group and a concentration-related increase in TNFα production can be seen with reference to the PMA concentration.

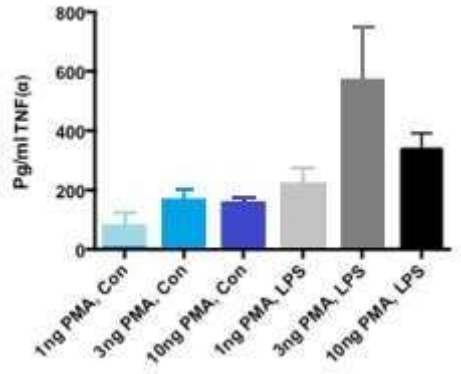
Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = yes; 0.0008	1ng x 1ng = yes; 0.0001
1ng x 10ng= ns	1ng x 10ng= yes; 0.0001	3ng x 3ng= yes; 0.0002
3ng x 10ng= ns	3ng x 10ng= yes; 0.0002	10ng x 10ng= yes; 0.0001



(Fig 4.11) TNFα assay on 1 day PMA Differentiated U937 (Colony B)

Both the control and LPS treated groups (relative to the control) yielded significant results. In the control group; a high level of TNFα was produced at the 3ng PMA concentration. Possibly related; a high level of TNFα was seen in the LPS-treated group at this PMA concentration but the reading was still lower than the control group. This was also observed with the 1ng group. The 3ng PMA LPS-stimulated group induced the highest levels of TNFα in the LPS treated group.

Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0001	1ng x 3ng = ns	1ng x 1ng = yes; 0.0001
1ng x 10ng= yes; 0.0001	1ng x 10ng= yes; 0.0050	3ng x 3ng= yes; 0.0004
3ng x 10ng= yes; 0.0001	3ng x 10ng= ns	10ng x 10ng= yes; 0.0022

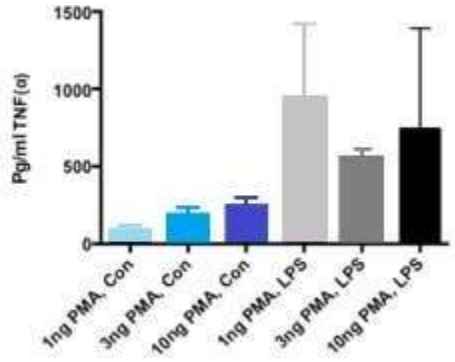


(Fig 4.12) TNFα assay on 1 day PMA Differentiated U937 (Colony B).

The readings of the control group were not statistically significant and will be discounted. The trend in the LPS-stimulated cells matches that seen in Figure 4.11; with the highest TNFα level produced in the cells treated with 3ng PMA.

Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = yes; 0.0140	1ng x 1ng = yes; 0.0065
1ng x 10ng= ns	1ng x 10ng= yes; 0.0331	3ng x 3ng= yes; 0.0205
3ng x 10ng= ns	3ng x 10ng= ns	10ng x 10ng= yes; 0.0048

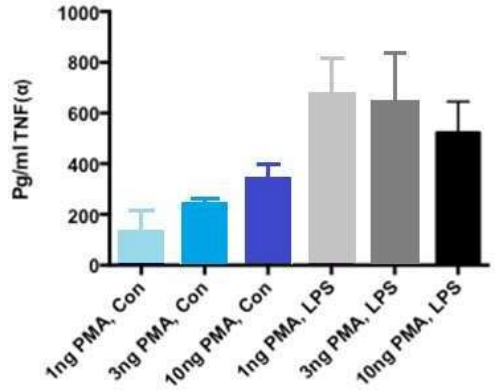
TNFα; 72 hour differentiation



(Fig 4.13) TNFα assay on 3 days PMA Differentiated U937 (Colony B)

The LPS-treated; 3ng PMA group obtained a significant TNFα reading relative to the control.

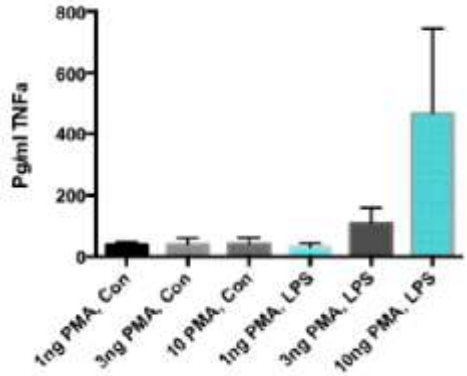
Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = ns	1ng x 1ng = ns
1ng x 10ng= yes; 0.0064	1ng x 10ng= ns	3ng x 3ng= yes; 0.0019
3ng x 10ng= ns	3ng x 10ng= ns	10ng x 10ng= ns



(Fig 4.14) TNFα assay on 3 days PMA Differentiated U937 (Colony B)

The LPS-treated; 3ng PMA group obtained a significant TNFα reading which was higher than its corresponding control group; as seen in figure 4.13. Similar levels of TNFα were seen in both the 1ng and 3ng PMA groups treated with LPS.

Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = ns	1ng x 1ng = yes; 0.0011
1ng x 10ng= yes; 0.0172	1ng x 10ng= ns	3ng x 3ng= yes; 0.0155
3ng x 10ng= ns	3ng x 10ng= ns	10ng x 10ng= ns

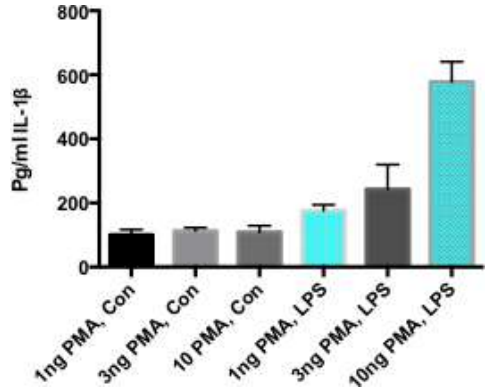


(Fig 4.15) TNFα assay on 3 days PMA Differentiated U937 (Colony B)

Statistically significant TNFα readings were obtained in the LPS-treated groups; and an increase in TNFα can be seen when there is a corresponding increase in PMA.

Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = yes; 0.0249	1ng x 1ng = ns
1ng x 10ng= ns	1ng x 10ng= yes; 0.0199	3ng x 3ng= yes; 0.0435
3ng x 10ng= ns	3ng x 10ng= yes; 0.0435	10ng x 10ng= yes; 0.0218

IL-1β; 24 hours differentiation

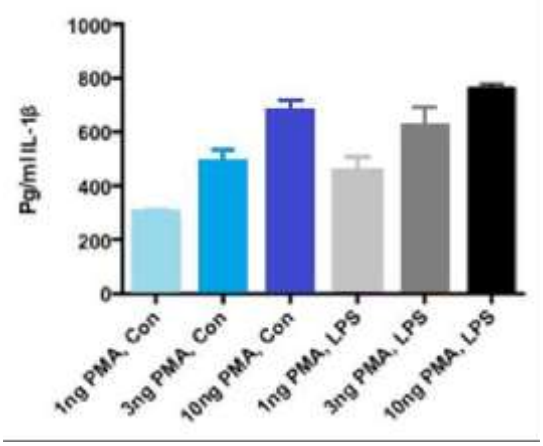


(Fig 4.16) IL-1β assay on 1 day PMA Differentiated U937 (Colony B)

The p values of the control groups are not considered to be significant. A general trend in IL-1β production; however; can be seen in the LPS-treated cells- as the PMA concentration increases the levels of IL-1β increase proportionately.

Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = ns	1ng x 1ng = yes; 0.0009
1ng x 10ng= ns	1ng x 10ng= yes; 0.0001	3ng x 3ng= yes; 0.0155
3ng x 10ng= ns	3ng x 10ng= yes; 0.0005	10ng x 10ng= yes; 0.0001

IL-1β; 72 hour differentiation

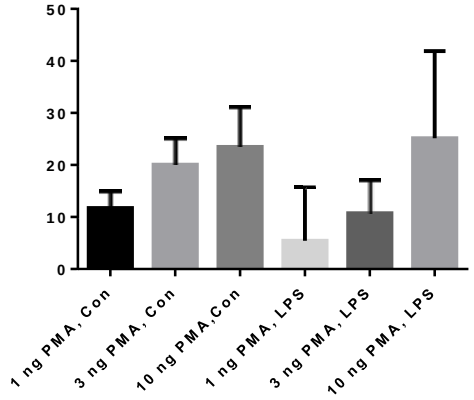


(Fig 4.17) IL-1β assay on 3 days PMA Differentiated U937 (Colony B)

P values indicate that all control group readings are significant; along with readings for the LPS-treated groups. However; a comparison cannot be made between the two groups; attributing to insignificant p values. The relationship demonstrated with colonies A and C is also apparent here- that PMA has a dose-related effect on the levels of TNFα generated in the U937 cells.

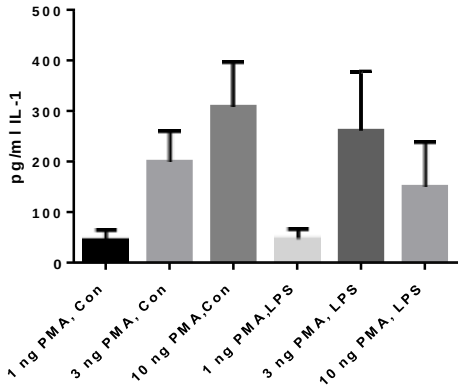
Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0001	1ng x 3ng = yes; 0.0116	1ng x 1ng = yes; 0.0013
1ng x 10ng= yes; 0.0001	1ng x 10ng= yes; 0.0001	3ng x 3ng= ns
3ng x 10ng= yes; 0.0005	3ng x 10ng=yes; 0.0084	10ng x 10ng= yes; 0.0071

IL-1β; 18 hour differentiation; no treatment with MSU



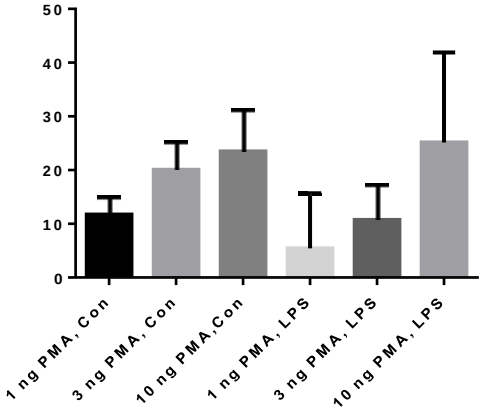
(Fig 4.19) IL-1β assay on 3 days PMA Differentiated U937 (Colony B)
Results from the graph proved to be insignificant (owing to their relative p values).

IL-1β; 18 hour differentiation with 4 hour treatment with MSU



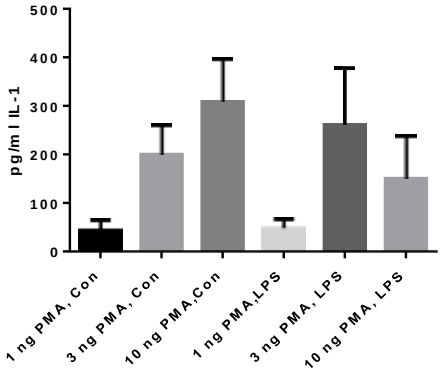
(Fig 4.20) IL-1β assay on 3 days PMA Differentiated U937 (Colony B)(MSU)
From the graph we can see an increase in IL-1β production when the concentration of PMA increases to 3ng. Unfortunately; further analysis is not possible due to the rest of the results being insignificant.

Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0027	1ng x 3ng = ns	1ng x 1ng = ns
1ng x 10ng= yes; 0.0011	1ng x 10ng= ns	3ng x 3ng= ns
3ng x 10ng=ns	3ng x 10ng= ns	10ng x 10ng= ns



(Fig 4.21) IL-1β assay on 3 days PMA Differentiated U937 (Colony B)(MSU)

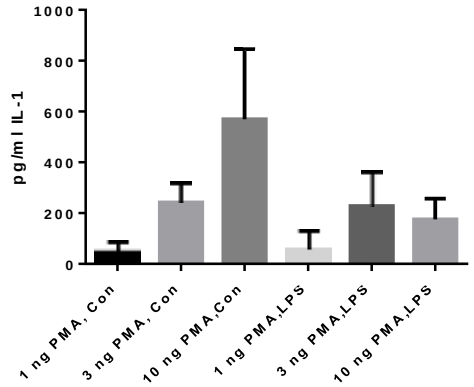
The two-way ANOVA concludes that the findings of this experiment are not statistically significant.



(Fig 4.22) IL-1β assay on 3 days PMA Differentiated U937 (Colony B)(MSU)

The column and row factors were found to be significant; along with the IL-1β readings attained by the 1ng and 3ng PMA control groups. There is a significant increase in IL-1β production in the controls when the concentration of PMA increases from 1ng to 3ng. The relative amount of IL-1β produced is larger in the group treated with MSU in comparison to the MSU-untreated group.

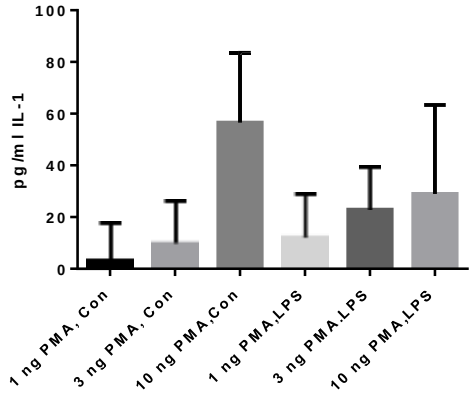
Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0027	1ng x 3ng = ns	1ng x 1ng = ns
1ng x 10ng= yes; 0.0011	1ng x 10ng= ns	3ng x 3ng= ns
3ng x 10ng= ns	3ng x 10ng= ns	10ng x 10ng= ns



(Fig 4.23) IL-1β assay on 3 days PMA Differentiated U937 (Colony B)(MSU)

The column factor plus the 1ng and 3ng PMA control group measurements have been calculated to be statistically significant. As demonstrated in the previous graph; a significant increase in IL-1β production is evident when the concentration of PMA increases from 1ng to 3ng. In addition; IL-1β production appears to be higher in the groups treated with MSU than those groups left untreated.

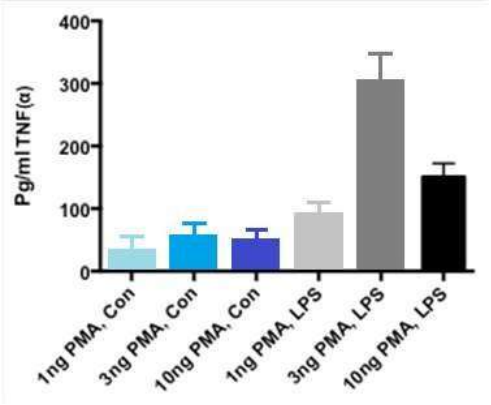
Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0036	1ng x 3ng = ns	1ng x 1ng = ns
1ng x 10ng= yes; 0.0097	1ng x 10ng= ns	3ng x 3ng= ns
3ng x 10ng= ns	3ng x 10ng= ns	10ng x 10ng= ns



(Fig 4.24) IL-1β assay on 3 days PMA Differentiated U937 (Colony B)(MSU)

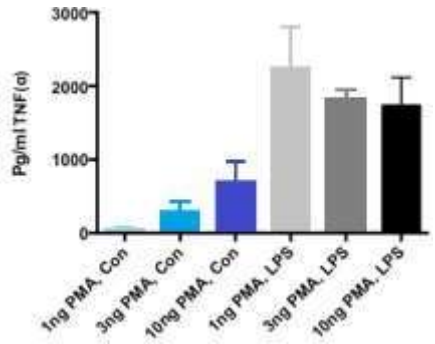
The two-way ANOVA shows that results are not statistically significant.4.5 Colony C; TNFα and IL-1β analysis

TNFα; 24 hour differentiation



(1; 3;10ng/ml); washed and treated for 4 hours with medium (Con) or LPS (100U/(Fig 5.10) TNFα assay on 1 day PMA Differentiated U937 (Colony C). U937 cells were differentiated for 24 hours with indicated concentrations of PMA ml).

Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = yes; 0.0004	1ng x 1ng = yes; 0.0100
1ng x 10ng= ns	1ng x 10ng= yes; 0.0107	3ng x 3ng= yes; 0.0012
3ng x 10ng= ns	3ng x 10ng= yes; 0.0074	10ng x 10ng= yes; 0.0033

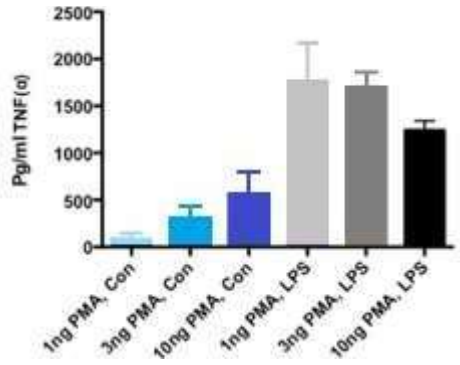


(Fig 5.11) TNFα assay on 3 days PMA Differentiated U937 (Colony C)

The readings for the 1ng and 3ng PMA controls were significant; with the highest TNFα production seen at the 3ng concentration. The readings for the LPS-treated cells are all statistically significant relative to the controls. TNFα levels were higher in the LPS-treated groups than the corresponding control group. In contrast to other figures; there is a dose-related reduction in TNFα production as the concentration of PMA increases.

Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0134	1ng x 3ng = ns	1ng x 1ng = yes; 0.0005
1ng x 10ng= yes; 0.0072	1ng x 10ng= ns	3ng x 3ng= yes; 0.0001
3ng x 10ng= ns	3ng x 10ng= ns	10ng x 10ng= yes; 0.0209

TNFα; 72 hour differentiation

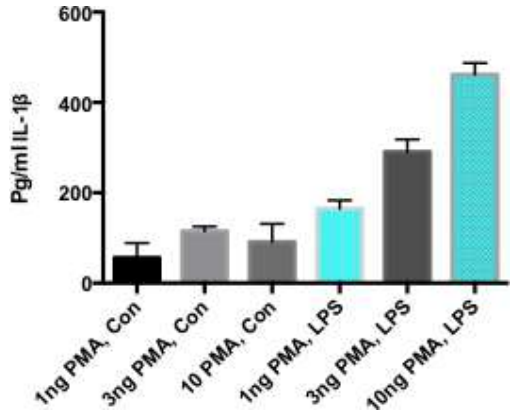


(Fig 5.12) TNFα assay on 3 days PMA Differentiated U937 (Colony C)

There is an apparent drop in TNFα production following LPS activation as the concentration of PMA rises. Comparing the levels produced by the 3ng PMA group treated with LPS and the control we can see that LPS stimulation induces higher TNFα levels than the controls. Compared to the 24 hour differentiation of the U937 cells; higher levels of TNFα were generated when left to differentiate for 72 hours.

Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = ns	1ng x 1ng = yes; 0.0002
1ng x 10ng= yes; 0.0123	1ng x 10ng= ns	3ng x 3ng= yes; 0.0003
3ng x 10ng= ns	3ng x 10ng= yes; 0.0134	10ng x 10ng= yes; 0.0105

IL-1β; 24 hours differentiation

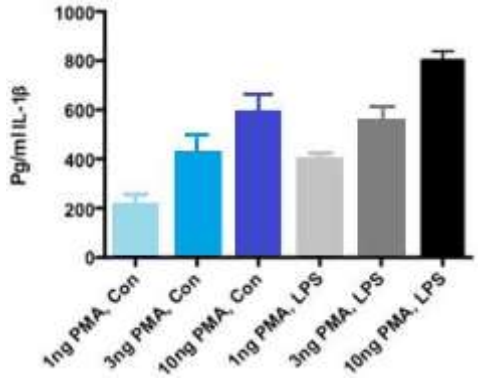


(Fig 5.13) IL-1β assay on 1 day PMA Differentiated U937 (Colony C)

The LPS-treated groups all provided significant results and from the graph we can see that as the PMA concentration increases the corresponding level of IL-1β also increases. The cells treated with 1ng PMA and LPS generated higher levels of IL-1β than the corresponding 1ng PMA control group.

Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0127	1ng x 3ng = yes; 0.0003	1ng x 1ng = yes; 0.0011
1ng x 10ng= ns	1ng x 10ng= yes; 0.0001	3ng x 3ng= yes; 0.0001
3ng x 10ng= ns	3ng x 10ng= yes; 0.0001	10ng x 10ng= yes; 0.0001

IL-1β; 72 hour differentiation

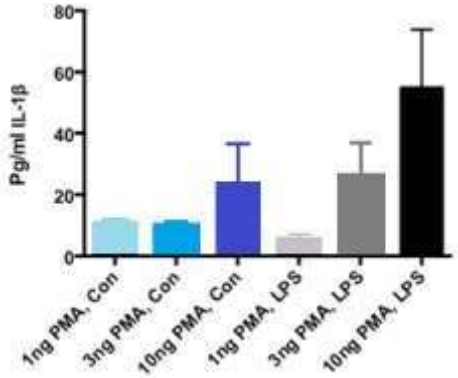


(Fig 5.15) IL-1β assay on 3 days PMA Differentiated U937 (Colony C)

All the readings are considered to be significant with reference to their corresponding p-values. At each concentration of PMA; we can see that a significantly higher level of IL-1β is generated by the LPS-treated groups compared to the controls. Compared to the 24 hour differentiation of the U937 cells; higher levels of IL-1β were generated when left to differentiate for 72 hours.

Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0027	1ng x 3ng = yes; 0.0023	1ng x 1ng = yes; 0.0004
1ng x 10ng= yes; 0.0001	1ng x 10ng= yes; 0.0001	3ng x 3ng= yes; 0.0313
3ng x 10ng= yes; 0.0184	3ng x 10ng= yes; 0.0004	10ng x 10ng= yes; 0.0021

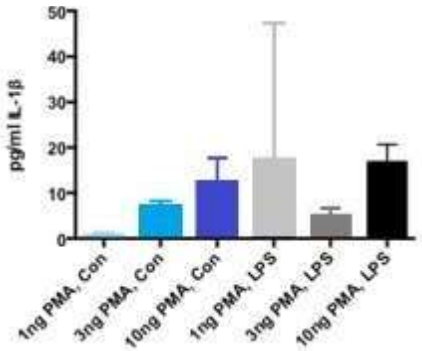
IL-β; 18 hours differentiation; no treatment with MSU



(Fig 5.17) IL-1β assay on 3 days PMA Differentiated U937 (Colony C)

P values indicate that the results obtained were not statistically significant and can therefore not be analysed.

Control	LPS	Con x LPS
1ng x 3ng = ns	1ng x 3ng = ns	1ng x 1ng = yes; 0.0095
1ng x 10ng= ns	1ng x 10ng= yes; 0.0112	3ng x 3ng= ns
3ng x 10ng= ns	3ng x 10ng= ns	10ng x 10ng= ns

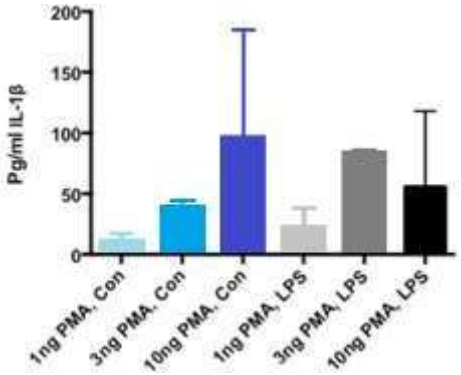


(Fig 5.18) IL-1β assay on 3 days PMA Differentiated U937 (Colony C)

The only significant readings that can be analyzed are those obtained for the 1ng and 3ng PMA control groups. We can see that higher IL-1β levels are generated at the 3ng concentration compared to the 1ng concentration.

Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0001	1ng x 3ng = ns	1ng x 1ng = ns
1ng x 10ng= yes; 0.0066	1ng x 10ng= ns	3ng x 3ng= ns
3ng x 10ng= ns	3ng x 10ng= yes; 0.0015	10ng x 10ng= ns

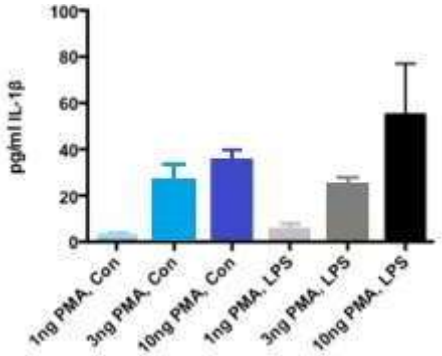
IL-β; 18 hour differentiation and 4 hours treatment with MSU



(Fig 5.19) IL-1β assay on 3 days PMA Differentiated U937 (Colony C)(MSU)

Interpretation of these results and comparison with the MSU-untreated group is difficult due to statistically insignificant results being obtained.

Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0064	1ng x 3ng = yes; 0.0029	1ng x 1ng =ns
1ng x 10ng= ns	1ng x 10ng= ns	3ng x 3ng= yes; 0.0002
3ng x 10ng= ns	3ng x 10ng= ns	10ng x 10ng= ns



(Fig 5.20) IL-1β assay on 3 days PMA Differentiated U937 (Colony C)(MSU)
Comparing the 1ng and 3ng PMA control groups (treated with MSU); we can see a sharp rise in IL-1β production when the concentration of PMA rises to 3ng. This also occurs with the 1ng and 3ng PMA LPS-treated cells.

Control	LPS	Con x LPS
1ng x 3ng = yes; 0.0005	1ng x 3ng = yes; 0.0001	1ng x 1ng = ns
1ng x 10ng= yes; 0.0001	1ng x 10ng= yes; 0.0043	3ng x 3ng= ns
3ng x 10ng= ns	3ng x 10ng= ns	10ng x 10ng= ns

4. Discussion

4.1. MTT

The MTT test is an indicator of cell viability in vitro; the more living cells available; the more enzymatic reactions and the more intense the colour result will be (with a corresponding increase in the spectrophotometric absorbance). Kim and Ha (2009) showed that LPS induces macrophage killing through the production of nitric oxide and the subsequent formation of the cytotoxic superoxide anions. Therefore; following LPS treatment we would expect the MTT level to drop due to the loss of cell viability. A steady decline in the AU values can be seen in the graphs of all the colonies in response to an increasing PMA concentration. This may signify that PMA reduces cell viability and becomes toxic to the cells when it is present in too high a concentration. This trend is also witnessed in cells that underwent a 24 hour and 72 hour differentiation.

4.2. TNFα

A dose-related increase in TNFα production was seen in accordance with the PMA concentration. It is possible that the presence of dead cells induce an inflammatory reaction in the remaining living macrophages. As the concentration of PMA increases; the number of dead cells increases thus a larger inflammatory result is seen. Higher TNFα levels were generated by the LPS-treated groups in comparison with the controls; confirming the inflammatory response elicited by LPS. Compared the 24 hour and 72 hour differentiations; the longer the cells were exposed to PMA; the more extreme the inflammatory response seen. This suggests that PMA has a toxic effect on the U937 cells; if used for too long a time or at too high a concentration.

A number of statistically insignificant results were generated in the TNFα analysis; leading to conflicting results. This highlights the importance of maintaining environmental parameters to generate reproducible results. A trend did happen to emerge from all the experiments- LPS-treatment led to a drop in viable macrophage numbers and a rise in TNFα release; due to the inflammatory response generated by the dead cells. This mirrors the findings from the MTT assay analysis and the work of Kim and Ha (2009)

4.3. IL-1β

Interleukin-1β (IL-1β); produced by activated macrophages; sets off an intracellular signalling cascade that subsequently causes the activation of transcription factors and expression of ROS-producing target genes. In monocytes; IL-1β interacts with NADPH oxidase in order to generate a pool of ROS (Bonizzi et al.; 1999). Graphs for each colony indicate that high concentrations of PMA induce high levels of IL-1β; confirming the previous idea that PMA exhibits a dose-related toxicity towards macrophages; and as the death toll rises the ensuing inflammation becomes larger. Elevated levels of IL-1β are generated in the LPS-treated groups in comparison to the controls; reaffirming LPS's ability to induce cell death in macrophages (Kim and Ha; 2009)

4.4. RNAi

The $gp91^{phox}$ protein is one of the primary elements of the NADPH-oxidase system in phagocytes (Segal et al.; 2012). A deficiency in the $gp91^{phox}$ subunit has been associated

with the development of CGD (e.g. Bjorgvinsdottir et al.; 1996; Rajakariar et al.; 2009; Sadat et al.; 2003; Zhen et al.;1993). It has been demonstrated in patients with a $gp91^{phox}$ deficiency that a defective NADPH is at play; resulting in a lower production of ROS (Hill et al.; 2010). It is predicted that mutations in CYBB; encoding $gp91^{phox}$ will lead to an insubstantial production of ROS via NADPH-oxidase; based upon findings of previous studies (for example; Bjorgvinsdottir et al.; 1996).
In studies with $p47^{phox}$ mutant strains; high concentrations of macrophages were found to be became activated; thus producing more ROS through a NADPH oxidase-independent mechanism; leading to an augmented production of cytokines (Yi et al; 2010).

Hill et al (2010) found that $gp91^{phox}$-deficient patients had an attenuated NADPH function resulting in a smaller release of ROS and a less impressive immune reaction. This highlights the importance of this subunit in cultivating an immune response. Cells treated with $gp91^{phox}$siRNA produced less TNFα than the cells treated with $p47^{phox}$siRNA; confirming Hill's findings (2010) that there is a reduced immune response when $gp91^{phox}$ is knocked out or present in a mutant form. It did; however; produce higher levels than the control which does not support the findings of Hill and colleagues (2010). Additionally; knock out of the gp91 subunit in the $gp91^{phox}$siRNA-treated cells resulted in a substantial increase in IL-1β release when compared to both the $p47^{phox}$siRNA-treated cells and the control which fails to correlate these findings as well.

5. Conclusion

These results supported our hypothesis that mutations in various subunits of NADPH oxidase cause an altered macrophage response to infection. A knockout or mutation of the p47 subunit in macrophages was shown to result in an amplified release of TNFα in response to LPS treatment when compared to control. This may suggest that it has a redundant role in the inflammatory response; however; additional research in $p47^{phox}$ mutant strains under a range of different experiment conditions will help to explain our findings. In the absence of the $p47^{phox}$ auto-inhibitory component (PBR); the macrophages produced only a modest amount of TNFα. This may infer that in a non-inhibited state; the p47 subunit may behave differently upon macrophage stimulation; leading to an abnormal form of NADPH being activated. This is difficult to postulate in the absence of other statistically significant results; and further research on this topic is recommended. Mutating or knocking out other components of the p47 subunit; for example; its tandem SH3 domain; may enable us to resolve our observed findings. Conflicting results were produced with reference to the inflammation generated by the mutant g91phox strains; prompting further analysis of this mutant and more diligence when performing the experiments. CGD is a genetic disorder caused by a dysfunctional NADPH oxidase; unable to surmount a sufficient immune response to invading pathogens. Our findings reveal that mutations in various subunits of NADPH lead to a spectrum of different inflammatory responses being produced by macrophages. The results of this study can be applied to better our understanding of the pathogenesis of CGD and aid in the development of more targeted and effective therapies against CGD.

References

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