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NAD$^+$ Levels Are Depleted in DMSO Differentiated Leukemia Cells.

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ABSTRACT

NAD$^+$ is an important cofactor and a main substrate for a signaling enzyme, CD38. CD38has dual functions as a cell surface receptor and as NAD-consumer enzyme. This enzyme produces second messengers and also controls cellular metabolism by regulating intracellular NAD$^+$ levels. The role of CD38,in negatively regulating NAD$^+$ levels and the consequences of this decline, has previously been investigated in HL60 cells, as a model of inducible CD38 expression, following treatment with ATRA up to 5 days of differentiation. In the present study, we are interesting in investigation functional properties of HL60 differentiation after treatment with DMSO, via evaluating NAD$^+$ levels and TNF-α secretion. Treatment with DMSO led to decrease NAD$^+$ levels alongside with granulocytes differentiation. This drop in NAD$^+$ levels was associated with reduction in Lactate levels indicating interesting change in glycolytic pathway during HL60 differentiation. We also measured TNF-α release to show the link between a pro-inflammatory response and NAD$^+$ levels in DMSO-differentiated HL-60 cells. These cells showed lower levels of TNF-α during differentiation. These findings suggest that low NAD$^+$ levels might be correlated with inflammatory response during differentiation. Collectively, using DMSO as inducing agents might be useful to generate a model for HL60 differentiation to granulocytes. Interestingly, this might provide an alternative model to study the regulation of NAD-signaling enzymes (CD38) and the consequence of lowering NAD$^+$ levels-mediated immune response manipulation. Finally, studying these changes in NAD$^+$ levels and the link to immune response, might provide a therapeutic strategy for treating immunity-associated conditions.

Keywords: NAD$^+$ levels, HL60 and DMSO, TNF-α, immune response

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INTRODUCTION

Nicotinamide adenine dinucleotide NAD(P)His an important substrate and a oxidative cofactor in eukaryotic cells. NAD participates in many redox reactions, for example, glycolysis and the citric acid cycle of cellular respiration[1]. As a substrate, NAD is required for activation of several NAD-consuming enzymes, such as poly-ADP-ribose-polymerases (PARPs) [2], mono-ADP-ribose transferases (ARTs; 3), sirtuins (NAD-dependent protein deacetylases) [4] and the CD38/CD157 system in cells. However,CD38, as a member of the cyclase family (except from been a receptor molecule) [5,6]appears to be a major NAD consuming enzyme in cells[7], and thus it limits the availability of extracellular and intracellular NAD^+ levels to all other consumers. On the other side, cellular NAD^+ is re-synthesized via three suggested recycling pathways, a *de novo* pathway(from tryptophan), salvage pathway: from nicotinic acid (NAc) or nicotinamide (NAM); vitamin PP, or niacin [8], and from nicotinamideriboside (NR) [9].

CD38-catalyzing NAD consumption participates in the synthesis of Ca^{2+} messengers; NAADP and cADPR [10-12], and it involves in the regulation of a wide variety of signaling pathways that is related to numerous metabolic diseases[13]. For instance, NAD depletion is associated with neoplasia and ischaemia[14], diabetes, cancers, Parkinson's disease [15-18], sickle cell disease [19] in addition to chronic lymphocytic leukemis (CLL)[20]. Leukaemia condition is one of the current major health problems. Elucidating the signalling mechanisms by which human leukaemia cell differentiation enhance genes expression or reduce a vital cofactors, such as intracellular NAD^+ levels, is critical for the understanding of this condition and for the development of therapeutic strategies for its treatment. Thus For the importance of NAD status, studies on NAD^+ levels and its related enzymes, mainly CD38, are in progress. In fact, it has previously been suggested that the change in NAD^+ levels might modulate protein activities, and have an effect on cellular functions [21,22].For instance, studies have showed significant high levels of NAD^+ in CD38 KO mouse compared to the wild-type[23]. However, recent study by Zainab and others [24] revealed an interesting link between CD38 expression and intracellular NAD^+ levels in ATRA-treated HL60 cells and RAJI cells. This study showed large changes in NAD^+ levels and a novel role of CD38 as a main NAD controlling enzyme rather than PARP or Sirtuins enzymes. A surprising significant drop in NAD^+ levels has been shown during HL60 differentiation with ATRA. This model has been used to study the status of intracellular NAD^+ levels upon the induction of CD38 expression in CLL cells during differentiation.

Similarly, it has been used HL-60 cells in the present study but was incubated with DMSO to induce HL60 differentiation to granulocyte cells. In fact, treating HL60 cells with different inducer (for 2 days) enhanced differential gene expression responses, mainly for CD38. CD38 is expressed on the cell surface by ATRA and isonicotinic acid. However CD38 expression did not occur when HL60 cells were treated with nicotinamide or nicotinamide N-oxide (niacin related compounds). On the other hand, CD38 expression was rarely observed in HL60 cells or cells treated with 1% DMSO. Interestingly, previous studies on differentiation of HL60 showed that treatment with ATRA caused to induce CD38 expression greater than treatment with DMSO [25].Here we have demonstrated a critical role for DMSO treatment in induction of HL60 cell differentiation and the consequences in decrease NAD^+ levels which might be through regulating NAD-catalyzing enzymes mechanism. The drop in intracellular NAD^+ levels was accompanied by a significant drop in glycolysis activity represented by low lactate levels. Moreover, we also observed low levels of TNF-α which might be correlated with the decline in NAD^+ levels. Indeed, it has recently been shown that NAD^+ levels are associated with TNF-α release in a model of differentiated human leukaemia cells [25].

The changes in NAD^+ levels, during cell differentiation by DMSO as granulocytes inducer, may provide some useful information to understand the mechanisms of HL60 cell differentiation. HL60-differentiation is mediated at least in part via activation ofCD38, a major NAD consumer enzyme, which is involved in the regulation of intracellular NAD^+ levels and the consequence on metabolic pathways [24].Additionally, these findings might also support the fascinating link between NAD^+ levels, immune response and glycolytic pathway but this time in neutrophil-like differentiated cells.

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MATERIALS AND METHODS

Cell culture

HL-60 and RAJI cells were maintained in suspension in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated FCS (Lonza), 100 U/ml penicillin, 100 g/ml streptomycin (Lonza), and 2 mM L-glutamine (Lonza). Cells were maintained in a humidified incubator supplied with 5% CO_2, at 37 ^oC. To induce HL-60 differentiation, 1.2% DMSO (Dimethyl sulphoxides) (DMSO; Sigma) was added to cells (0.2×10^6 ml^{-1}) and then incubated up to 5 days[26].

Intracellular NAD^+cycling assay

NAD^+ standards (5–60 µM) and/or 1.25–5 $\times 10^6$ ml^{-1} of indicated cells were extracted and prepared for NAD assay as previously reported protocol[27,24]. Briefly, extracted samples were placed in a 100 ^oC water bath for 10 min and then centrifuged at 5000g for 5 min. The supernatants were stored in -20 ^oC or used immediately. A prepared samples (49 µl) was added to 151 µl of a reaction mixture containing 98 mM Bicine (pH 8.0), 24 mM of NaOH, 1.62 mM PES, 0.41 mM MTT, 19.6 µl ethanol, 3.92 mMEDTA and 5 µl of yeast ADH (400 U ml^{-1}). After 30 min incubation in the dark, the absorbance at 565 nm was recorded by using a plate reader (VersaMax, Molecular Devices, Sunny vale, CA)

Lactate assay

50 µl of cell supernatant (Medium) or lactate standard solution (0.1–1 mM) was added to 96-well plate containing 250 µl of 315.8 mM glycine, 252.6 mM hydrazine, 4 mM NAD^+ and 16.6 U ml^{-1} lactate dehydrogenase (LDH). The solution was incubated at 37^oC for 30 min and measured at 340 nm in a plate reader.

Analysis of cytokine release by Elisa

To quantify extracellular TNF-α level, cell supernatants (samples) were analysed by sandwich ELISA according to the manufacturer's instructions. The samples were harvested from differentiated cells, HL60 and RAJI cells at the indicated time points, and protocols were followed and compared with standard curves (7-5000 pg/ml) available from NIBSC (Potter's Bar, UK).

Briefly, 96-well ELISA plates were coated with (4 μg/ml) of mouse anti-human TNF-α (BD Pharmingen), in PBS and then incubated overnight at 4^oC. Samples were added into the plate, and incubated overnight at 4^oC. For TNF-α detection, biotinylated mouse anti-TNF-α at 1 μg/ml, in 2% BSA buffer, was applied and then horseradish peroxidase streptavidin conjugate were added, followed by incubation with TMB reagent (Insight Biotechnology Ltd.). Absorbance was measured by an OPTI Max tune able micro plate reader set to 450 nm and analysed by Softmax Pro version 2.4.1.

NBT reduction assay

DMSO Differentiation of cells was performed in 96 well plates by using the Nitroblue tetrazolium (NBT) assay. In this assay 100 µl of 1×10^6 cells ml^{-1}(in RPMI) were incubated with 100 µl of 2 mg/ml NBT and 100 µl (200 ng/ml) of freshly prepared phorbolmyristate acetate (PMA). After one hour incubation time, in 5% CO_2 humidified incubator at 37 ^oC, cells were dissolved introduced to (DMSO,100 µl) and the absorbance was measured at 590 nm on a plate reader.

Statistical analysis

Statistical calculations were performed using Stat View statistical software (Abacus concepts, California, USA). One-way ANOVA or t-test was performed for all data. Data are reported as means±SEM for triplicate samples from three independent experiments. Differences were considered significant if $P<0.05$.

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RESULTS AND DISCUSSION

Studies on the relationship between intracellular NAD^+levels and cell metabolism during cell differentiation are in progress[28]. In fact, not all inducers of granulocytic and monocytic differentiation of HL-60 cells up-regulate CD38expression [29,30]. For instance, low CD38 expression was evaluated during cell differentiation by DMSO for 2 days [25] which suggest that these cells might generate high levels of NAD^+. However, a little is known about the status of intracellular NAD^+ levels in DMSO differentiated cells. Interestingly, we have shown in the current study that, the extracted intracellular NAD^+ levels, which have been measured in HL60 cells, proved to be declined with different time point during differentiation (Fig. 1A) when treated with 1.2% DMSO up to 5 days. The significant drop in NAD^+ levels, was mainly in the 3rdday of differentiation (74.88±2.34%)and 5th day of differentiation (63.08±1.69%)comparing to untreated control. This might be due to the regulatory role of NAD-consumer enzyme, CD38, even with its low expression levels comparing to HL60 cells treated with 1µMof ATRA. These results and our previous work is also support the hypothesis suggests CD38 as main regulator of intracellular NAD^+ levels [31].Thus the decrease in NAD^+ levels might be CD38-dependant and no other NAD^+-consuming enzymes.

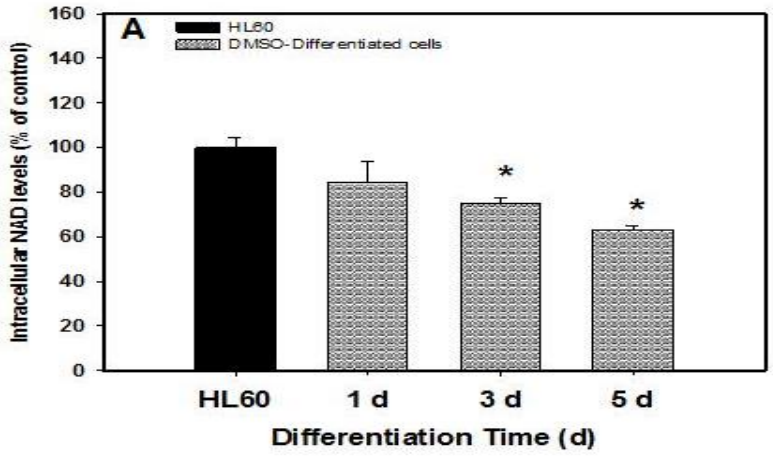

Fig 1 (A): the decrease of intracellular NAD^+ levels during HL-60 differentiation by DMSO, n = 3. Differences between groups were assessed by one-way ANOVA. *P < 0.05 versus control,

We have used HL60 cells as a reliable model that can terminally differentiate to granulocytic (neutrophil-like) cells after incubation with 1.2% DMSO for up to 5 days[26]. To confirm the characteristic of granulocyte differentiation, the NBT assay was used. By the end of the treatment, NBT levels in DMSO-differentiated cells were measured, and the results showed a significant increase ($p < 0.05$) in the NBT reduction capacity (Fig. 1B) after 5 days of differentiation(146.49± 9.42% of the control).

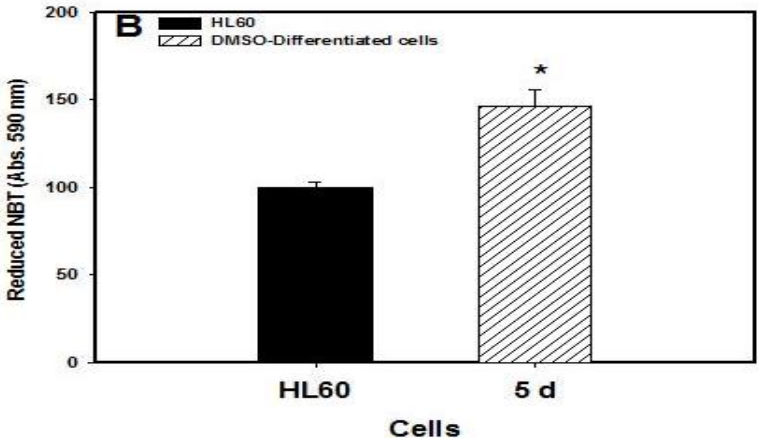

Fig 1 (B): The NBT reduction ability of DMSO treated HL60 cells for 5 days comparing to the control, n=3. Differences between groups were assessed by t-test. *P < 0.05 versus control. Data are expressed as mean ± SEM of three separate cultures.

Further studies might also be required to understand the full mechanism of cell differentiation and the status of NAD metabolites levels, i.e. cADPR. Notably, investigating CD157 (CD38 analogue), was not really important as this enzyme show one hundred fold lower catalytic activity (NADase or cyclase activity) than CD38 [32]. For this reason, low NAD^+ levels in DMSO treated cells may be influenced by CD38 activity rather than CD157. Moreover, evaluating NAD-recycling,(salvage and *de novo*) pathway enzymes (IDO, NAMPT and NMNAT),and NAD^+ consuming enzymes, (PARP, Sirtuins and ARTs),provide useful tool to fully understand the differentiation of human leukemia cells. This might offer an attractive therapeutic treatment for patients with human leukemia. Further investigating, on intracellular NAD^+ levels, using niacin-related compounds, the well-known NAD precursor, to induce granulocytic differentiation, might provide some useful information to clearly explain the mechanism underlying the differentiation of human leukemia cells.

In addition to evaluate intracellular NAD^+ levels, we have decided to investigate new functional properties of differentiated HL60 post treatment with DMSO. Therefore, we sought to determine the effect of NAD^+ levels depletion on cell physiology and glycolyticflux, represented by lactate production, during HL60 differentiation. The results show a significant decrease in lactate production during HL60 differentiation with DMSO on day 1 (75.68±4.97),day 3 (46.51±4.89) and 5 (38.40±0.77) of differentiation comparing to the control (Fig.2). The results might suggest that the reduction in lactate production as consequence of the decline in NAD^+ levels during differentiation, and that other NAD^+-dependent processes might be also affected by the lowered NAD^+ levels.

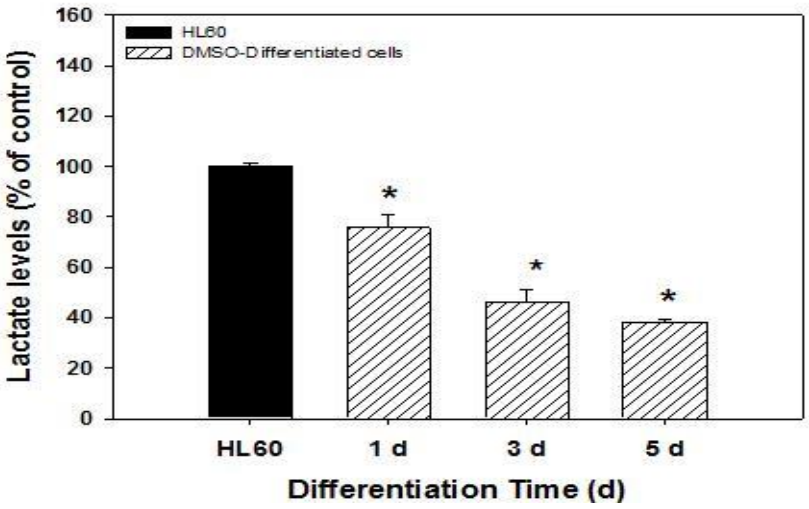

Fig 2: The Consequences of NAD^+ depletion on lactate production during HL-60 differentiation by DMSO. Data are expressed as mean ± SEM of three separate cultures. Differences between groups were assessed by one-way ANOVA. *P < 0.05 versus control.

Recently, researchers showed an important link between cellular Metabolism and immune responses. Indeed, it has recently been confirmed that intracellular NAD^+ was able to positively control TNF-*α* release as well as transcription of this pro-inflammatory cytokine in a model of differentiated-THP1 (macrophage-like) cells and primary macrophages [28, 33].Here, TNF-*α* levels were also assessed in HL60-differentiated cells following treatment with DMSO over 5 days (Fig. 3) comparing to the related control. The results showed low levels of TNF-*α* in DMSO-differentiated cells, particularly in day3 (66.03±6.93%) and day5 of differentiation(68.39±5.84%)compared to HL60 cells. These results further confirmed the link between a pro-inflammatory TNF-α and NAD^+ content but this time in DMSO-differentiated HL60 (neutrophil-like) cells. It is noteworthy that TNF-α secretion correlated with production of NAD^+ levels and it was suggested that NAD^+ regulate TNF-α secretion in NAD^+-dependent sirtuinde acetylase activity [33]. Therefore, it was mentioned that attenuating NAD^+ levels, SIRT6 knock out or inhibition block TNF-*α* translation and release. Thus, the drop in NAD^+ levels, upon differentiation with DMSO, might lead to similar drop in TNF-*α* level. Finally, more investigating on this link in DMSO- differentiated HL60 might provide insight on the impact of TNF-*α* on NAD^+ homoeostasis by regulating the expression of NAD^+ homoeostasis enzymes in neutrophil-like cells [34].

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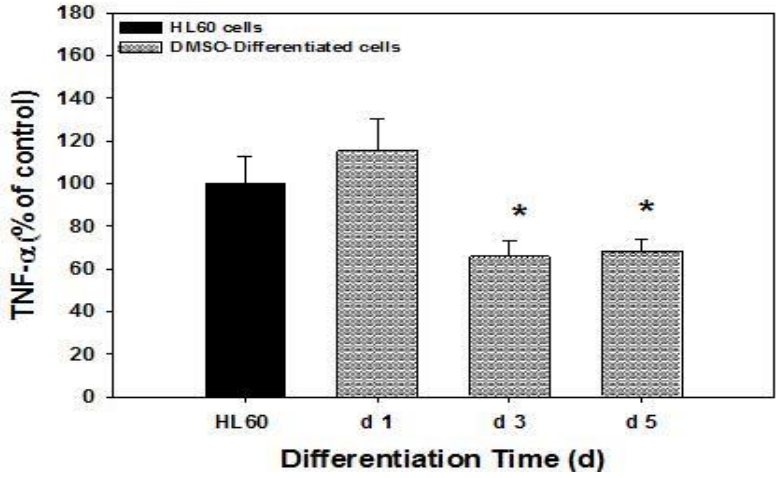

Fig 3: The decline in TNF-α levels during HL60 differentiation by DMSO up to 5 days, n = 3 Data are expressed as mean ± SEM of three separate cultures. Differences between groups were assessed by one-way ANOVA. *P < 0.05 versus control.

Moreover, Pharmacological modulation of TNF-α release was also evaluated in DMSO-differentiated cells, HL60 cells and RAJI cells following treatment with 100 µMNAD for 1day. A high TNF-α production has been observed in all cells (that has a different content of intracellular NAD^+ levels) following incubation with NAD^+ for a same period and compared to control (Fig. 4).Interestingly, Hl60 cells which display higher NAD^+ levels comparing to RAJI or differentiated cells [24]showed slight increase in TNF-α levels (162.55±6.49%) comparing to RAJI (122.40±2.82%) or differentiated cells (146.55±7.47%).

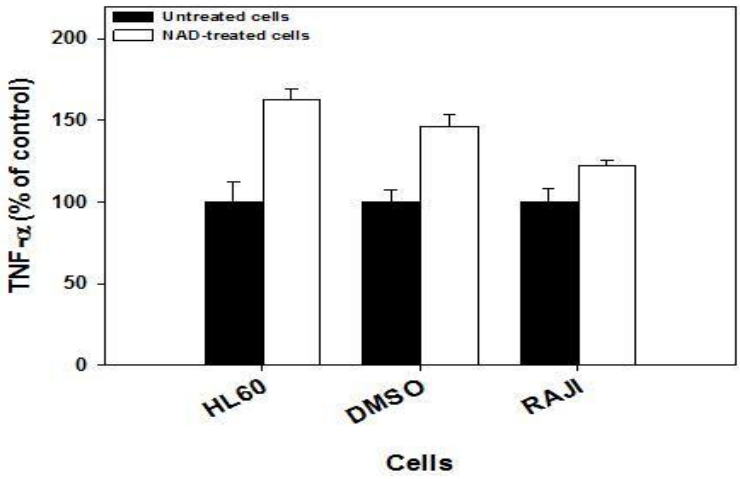

Fig 4: The effect of treatment with 100 µM NAD on TNF- α release in HL60, RAJI and DMSO-treated cells and for 1 day incubation, n = 3. Data are expressed as mean ± SEM of three separate cultures. Differences between groups were assessed by one-way ANOVA. *P < 0.05 versus control.

Our data suggest that NAD-supplementation increases the intracellular NAD content which might lead to induce TNF-α release in all cell lines. Indeed, our data are in agreement with previous work that observed an adequate intracellular NAD^+ levels are required for optimal TNF-α production and the decrease in intracellular NAD levels was mirrored by a reduced capacity to produce TNF-α[33]. Finally, Uncontrolled TNF-α synthesis is known to play an important role in numerous inflammatory disorders. Thus, manipulating NAD^+ levels might provide promising therapy to treat various inflammation associated-disorders via controlling TNF-α production. Altogether these results might provide a reliable model mimicking the pathway of neutrophil-like differentiation to study the link might exist between intracellular NAD^+ levels and immune response as well as the link to glycolytic metabolism. Additionally, lowered NAD^+ levels, as a consequence of CD38 up-regulation which has been shown in recent observations (24), could be a predictor marker for chronic lymphocytic

leukemia (CLL) patients. Indeed, in chronic lymphocytic leukemia, CD38 expression is very important predictor marker for this disease [35].

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Conflict of interest: None

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