Scientific Journal for the Faculty of Science-Sirte University Vol. 5, No. 1 (2025) 46-52

Scientific Journal for the Faculty of Science-Sirte University

Journal home page: http://journal.su.edu.ly/index.php/JSFSU/index

DOI: 10.37375/issn.2789-858X

Effect of hypoxia on release TNF alpha by dendritic cells and macrophage derived from myeloid cells of mice

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DOI: https://doi.org/10.37375/sjfssu.v5i1.3206

ARTICLE INFO:

Received 16 February 2025

Accepted 03 April 2025.

Published 17 April 2025

Keywords:

Hypoxia, Dendritic cells, Macrophage, Mouse myeloid cells.TNF-alpha.

A B S T R A C T

Hypoxia is low levels of oxygen and is a common feature during inflammation associated with numerous inflammatory diseases and infections. Dendritic cells and macrophages are significant immune defensive cells with a range of functions that include phagocytosis, secretion and release of an extensive kind of proteins including cytokines and chemokines, and are typically found in areas where oxygen availability is low, especially during inflammation. Tumor necrosis factor type alpha (TNF α) is a cytokine that is expressed by various cell types including macrophages, dendritic cells, and other non-immune cells against infections. It is an important pro-inflammatory cytokine that regulates inflammatory cells and responses.

This study focused on comparing the effects of hypoxia on the release of TNF alpha levels by dendritic cells and macrophages derived from myeloid cells of C57BL/6J mice in vitro using the ELISA test. Hypoxia is used because of the associated changes in the living body of the mouse.

Non-stimulated macrophages and dendritic cells derived from myeloid incubated in parallel in hypoxia and normoxia showed the production of TNF-α in both conditions; however, both dendritic cells and macrophages released more TNF-α in normoxia than in hypoxia. The production of TNF-alpha was higher in dendritic cells compared to macrophages. In brief, the comparisons between normoxia and hypoxia on the release of TNF alpha by dendritic cells and macrophages derived from myeloid cells of non-stimulated mice were shown.

1 Introduction

Hypoxia (low oxygen in intracellular cells or in the tissues) is a feature of several inflammatory *disorders*, and found in pathological tissues an, has been defined at nearly every inflammation site. It is mediated by some cellular adaptations to maintain balanced oxygen levels in the tissues of the human body. The mechanism by which progenitor cells respond to hypoxia-induced changes to mediate subsequent signalling responses remains a fundamental question underlying oxygen delivery (Luo *et al*., 2022).

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Immune cell components arise and distinguish into myeloid cells such as macrophages, dendritic cells, as well as B and T lymphocytes cells, and NK cells. These cells process the cellular mechanisms of innate and acquired immunity. Myeloid-obtained dendritic cells are the best-known antigen presenting cells that found in most of the tissues. The cells are activators of resting T lymphocytes and thus bridge the innate and adaptive immune systems. Differentiation of dendritic cells can be done *in vitro* by using several cytokines for instance IL-4 and GM-CSF, particularly from blood and myeloid cells. Cultured mouse myeloid dendritic cells express an extensive kind of receptors, complement proteins, and regulators and when they are stimulated with or lacking LPS (Peng *et al*., 2008). It plays an important part in connecting the innate and adaptive immune systems. Dendritic cells function normally under conditions of inflammatory hypoxia, affecting their cellular functions.

Dendritic cells are significant defensive cells of the immune system and are typically found in areas where oxygen availability is low especially during inflammation. Macrophages are active immune cells that protect the body from infection and are present in early inflammation, appear in various diseases, and have a variety of purposes that contain phagocytosis and the relief of an extensive kind of cytokines and chemokines.

The effect of hypoxia on altering dendritic cell purpose has been studied. Dendritic cell function in innate immunity is favored by reducing their maturation and decreasing the expression of their surface molecules (Rius *et al*., 2008). The oxygen tension in normal tissues ranges from 2.5-9%, but it has been found that deficient perfusion of diseased tissues may lead to temporary or chronic hypoxia, where the oxygen tension drops to less than 1% oxygen.

HIFs (Hypoxia inducible factors) are genes that respond to low oxygen concentrations or hypoxia and are involved in proliferation and stem cell function under low oxygen. In low oxygen, the myeloid cells offer many essential roles in environments generated by infection, inflammation, damage of tissue, and solid cancers. HIF factors in myeloid macrophages regulate proliferation, enhance innate immunity, can control gene expression proinflammatory, help in mediating the killing of bacteria, and affect the survival of the cell (Imtiya and Simon 2010).

Infection can lead to the formation of areas in tissues with low oxygen tension. The role of cellular response to hypoxia has been studied and focused on their interest as a particular metabolic stimulus or signal. (Yun *et al*., 1997; Neubauer 2001).

Tumour Necrosis Factor-alpha (TNF–α) show essential roles in local inflammation by up-regulating the cell surface proteins in hypoxic conditions. Hypoxia results in inhibition of dendritic cell migration and increased cytokine production. The lack of oxygen that occurs in tumour areas due to the spread of cancer cells is considered one of the obstacles that limit the success of therapeutic effectiveness in treating solid tumours.

Under hypoxic conditions in myeloid cells, the hypoxia-inducible factor (HIF-1 alpha) plays an essential role in amplifying the inflammatory response due to the activation of HIF-1 alpha. Macrophages are important effectors of the innate immune response that can be categorized into two phenotypes: M1 (classic activation) and M2 (regulatory activation). Typically activated M1 phenotype macrophages have an essential part in defense against bacterial infections; also, they create amounts of nitric oxide (NO) (Hu *et al.*, 2003). Phagocytosis by macrophages in killing bacteria shows better in hypoxic conditions compared to normoxic conditions (Zinkernagel *et al.*, 2007). Hypoxia is occurs in nasty tumors and inflamed tissues, macrophages are present at tumor sites and express many hypoxia-induced factors.

Lymphocyte cells are rapidly assembled in tissues that are damaged and are hence affected by deficiency of oxygen. It has been observed that different cell behaviors in lower hypoxia conditions, for instance, T lymphocyte cells in hypoxia, may inhibit several effector functions, such as cytokine production (Sitkovsky and Lukashev 2005). HIF1α is an essential regulator in responses to hypoxia and is expressed in nearly all mammalian cells, being necessary in the effectiveness and survival of immune cells.

TNF-α is a causative factor of tumour necrosis and has recently been identified as a pathogen and causative agent of autoimmune diseases. TNF-alpha has been shown to bind to two diverse receptors, which form

signal transduction pathways that cause different cellular responses, counting cell differentiation, proliferation, and survival. It was shown that TNF-alpha is associated with chronic inflammation due to inappropriate or excessive activation of its signals, which can cause the development of pathological complications for instance autoimmune diseases (Jang *et al* 2021).

Inflammatory low level of oxygen is progressively known as the most important determinant of the immune response. TNF-α showed an essential part in inflammation by up-regulating adhesion molecules under hypoxic.

The purpose of this study was to investigate the effect of hypoxia on release TNF alpha levels by dendritic cells and macrophage-derived from myeloid cells of mice *in vitro* using ELISA test. Hypoxia is used because of the associated changes *in vivo* body of the mouse model.

2 Materials and Methods

2.1 Mice macrophages and dendritic cells

2.1.1 Preparation and differential of the cells

In this study, non-stimulated mouse macrophages and dendritic cells were used after myeloid cells culture in Leicester University lab, and was prepared according to the methods in a paper by Westcott *et al*., 2007, and was performed in accordance with Home Office regulations and institutional guidelines. *In vitro* myeloid dendritic cells were produced as shown in a study by Inaba *et al*., 1992; and a study by Lutz *et al*., 1999. Myeloid macrophages were produced as described in a study by Zhang *et al*., 2008, but with some modifications. The growth medium in cell culture used the cytokines GM-CSF and cytokines and IL-4 (Inaba *et al*., 1992; Sallusto *et al*., 1994).

The stimulation protocol with the IL- 4 and GM-CSF was used with some modifications as described in the papers of Xu *et al*, 2007 and in study by Westcott *et al*, 2007. 10 ml culture media of RPMI 1640 were used, from Gibco, with 10% (v/v) fetal calf serum (FCS), the mouse cells were suspended to neutralise the lysis, and centrifuged for 5 minutes at 433g to achieve the cells from myeloid. marrow was prepared in a sterilized laminar flow cabinet, and both IL-4 and GM-CSF were used together (Menges *et al*., 2005). Both mouse myeloid dendritic cells and macrophages are widely used because *in vitro* differentiation allows for the generation of relatively homogeneous cells. After myeloid cells preparation and by using the Hemocytometer slide, the lymphocyte cells of 2 x 10^6 cells /ml were counted in RPMI 1640 culture media (20 ml) with added penicillin at a concentration of 100 U/ml and streptomycin at a concentration of 100 µg/ml, also L-glutamine at the concentration of 2 mM, in addition to FCS of 10%, after that all of these cells were put in a tissue culture flask (75 cm^2). Next, cytokines were added to the flask: 10 ng/ml of the cytokines rm GMCSF and cytokines mouse Interleukin 4 (IL-4) 10 ng/ml (by PEPROTECH) as designated by Xu *et al*., 2007, with a change to a different concentration in IL-4.

Afterward, the flasks were incubated to differentiate the cells for 6 days at 37°C with 5% CO_2, both cytokines rm GM-CSF and IL-4 were used to distinguish between macrophages and dendritic cells. After that on the third day of differentiation, cells in old media were changed by centrifugation and a new fresh media containing cytokines were added to the cell pellets. The cells were separated into two different type of cells adherent (attached) and nonadherent cells (floating in the media) on day six of differentiation. Nonadherent cells on culture supernatants were centrifuged to obtain dendritic cells, and then adherent cells were collected using scarpering the cells to obtain macrophages. Dendritic cells were obtained by centrifuging the culture supernatants. Adherent cells were collected to obtain macrophages using scarpering the cells. The cells were adjusted and counted, re-fed with new media and cytokines then the cells were incubated at 37°C overnight to allow the cells to adhere to obtained dendritic cells.

Both dendritic cells and macrophages in 75 cm^2 tissue culture flasks were incubated under normoxic and hypoxic conditions at 37˚C for 24 h. For hypoxic conditions, the cells were incubated at 0.2% O_2 with 5 % CO_2 and 94.8% N_2 at 37 °C using a low level oxygen incubator chamber (The RS Biotech Galaxy CO_2 Incubator). Then inside the hypoxic incubator, the Analox miniature O_2 oxygen analyzer was placed and was used to ensure that the oxygen levels in 0.2 %. For

normoxia conditions, the cells were cultured in similar standard incubator conditions in 37˚C for 24 h, and the supernatants were used to evaluate the levels of cytokines TNF-alpha.

2.1.2 Measurement of tumor necrosis factor-alpha level

Cytokines of TNF-alpha released from non-stimulated mouse dendritic cells and macrophages were measured in normoxia and hypoxia. The supernatants of the cell culture were saved in minus 80 ˚C and were used to evaluate the mouse TNF-alpha cytokine levels. The development kit of the mouse TNF-alpha ELISA test (900-M54) (PeproTech) was used, and using ELISA microplates (Nunc Maxisorp), and following the ELISA protocol as said by the manufacturer's instructions, and a dilution of murine TNF-alpha standard was used, starting at 2 ng /ml and then lower to zero.

2.2 Statistical Analysis

Paired samples t-tests were used to parallel mean TNF-alpha activity from un-stimulated dendritic cells and macrophages of mice in hypoxia and normal oxygen levels. p-values <0.05 for the results were considered statistically significant. Experiments were repeated twice

3 Results

Mice dendritic cells and macrophages were differentiated and incubated in normoxia and hypoxia conditions to measure the level of TNF-alpha. It is believed that lack of oxygen affects the removal of pathogens. TNF-alpha is a cytokine created by macrophages, dendritic cells, and other non-immune cells against infections. It is an important pro-inflammatory cytokine that regulates inflammatory cells and responses.

The supernatants from cultures of dendritic cells and myeloid-obtained macrophages of mice were used to measure the TNF-alpha level by using ELISA test in normal level of oxygen and low level of oxygen for 24 hours.

In non-stimulated macrophage and dendritic cell the concentrations of TNF-α measured were low but it showed different concentrations between hypoxia and normixia.

Dendritic cells secreted significantly higher TNF-α levels in normoxia paralleled to hypoxia *(P=0.0071)* (Figure 1). A comparable pattern appears when measuring the TNF-alpha of macrophages derived from myeloid cells, macrophages secreted significantly higher levels of TNF-α in normoxia paralleled to hypoxia condition (*P= 0.0234*) (Figure 1).

Under hypoxia, macrophages express higher levels of TNF-alpha. Nevertheless, it has been revealed that the decreased production of TNF-α after using supernatants from murine macrophage-like cells RAW 264.7 (RAW) under low level of oxygen (2% O2) was used for 24 h paralleled to normal level of oxygen, and a TNF-α ELISA kit was used to measure the protein produce (Yun *et al.*, 1997).

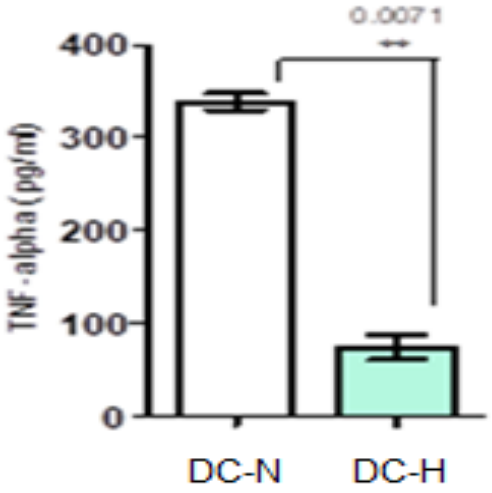

Figure 1: TNF-alpha level in supernatants of dendritic cells (DC) from the mouse in both condition Hypoxia (H) and Normoxia (N). Determinations three times were represented in each bar. This experiment was done two times. The values were given as mean and SD error bars. The collection levels TNF alpha were detected by using ELISA which the format was 16 - 2000 pg/ m.

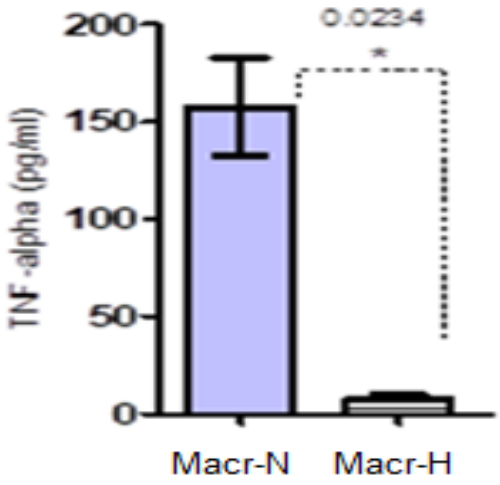

Figure 2: TNF-alpha levels in supernatants of macrophage cells (Macr) from the mouse in both condition Hypoxia (H) and Normoxia (N). determinations three times were represented in each bar. This experiment was done two times. The values were given as mean and SD error bars. The collection levels TNF alpha were detected by using ELISA which the format was 16 - 2000 pg/ m.

4 Discussion

Hypoxia or low levels of oxygen tension in the tissues, is a common *feature* of several inflammatory diseases and is found in pathological tissues, and it has been defined at nearly every inflammation site. Tumour necrosis factor-alpha is a cytokine that has diverse effects on many cell kinds and is a key regulation of inflammatory responses (Bradley 2008).

The current study was showed to study the effect of hypoxia on release TNF alpha by dendritic cells and macrophage-obtained from myeloid cells of mice.

For dendritic cells and macrophages, it has been shown that can be generated *in vitro* by culturing them in growth medium supplemented with GM-CSF and IL-4. Dendritic cells could be obtained from myeloid cells, splenocytes, or blood- obtained monocytes (Liu *et al*., 2002). In current research paper, the generated of myeloid- obtained dendritic cells were as described. Furthermore, the generated myeloid-obtained macrophages were as described in study by Zhang *et al*., 2008 but with certain modifications.

In this study, the level of TNF-alpha produce from both dendritic cells and macrophages was measured under both conditions normoxic and hypoxic. Secrete TNF-α from macrophages and activate further cells for example natural killer cells to secrete IFN-gamma to stimulate macrophages in order to raise their levels during the making of nitric oxide (Edelson and Unanue 2000).

The levels of TNF-α in both dendritic cells and macrophages was reduced in low levels of oxygen in parallel with normal levels of oxygen . Dendritic cells secreted an important higher levels of TNF-α in normoxia paralleled to hypoxia condition (*P= 0.0071*). Also, macrophages secreted significantly higher levels of TNF-α in normoxia paralleled to hypoxia condition *(P= 0.0234*). This result is similar to a study by Kohler T et al. in 2012 that showed that bone marrow-derived dendritic cells cultured under hypoxic conditions for six days had decreased TNF-α production (*Köhler T et al., 2012)*, although the time and duration of exposure to hypoxia varied.

Dendritic cells have been shown to respond to hypoxia by prompted expression gene of cytokines which are identified to prompt survival of epithelium, adhesion and recruitment of mono-nuclear phagocytes, also activate and recruitment of Th-1/Th-17 cells (Blengio *et al*., 2013).

Moreover, a study confirmed that macrophages respond to hypoxia by inducing the expression of TNF-alpha and its soluble cytokine receptors by the cell line of a human macrophage (Scannell *et al*.,1993). The reduction of TNF-alpha was showed in supernatant cell lines of peritoneal macrophage from mice (RAW 264.7) incubated for 24 hours in hypoxia condition (oxygen 2%) paralleled to normal levels of oxygen (Yun *et al.,* 1997). The difference in incubation in hypoxia shows that the macrophages are multi-functional cells and capable adjust their reaction to the condition of hypoxic.

Hypoxia causes many changes, such as vascular, metabolic, and the immunity works, to equilibrium damage and protection, and suppressive immune cells can protect compared to extreme tissue injury (Xu *et al*., 2024). Such studies may help uncover the mechanisms that attract macrophages or dendritic cells

to hypoxic tumor sites and lead to new strategies for combating cancer.

It was found that incubation of primary peritoneal macrophages (which contribute to several parts of innate and acquired immunity in the peritoneal cavity) in normal levels of oxygen or in hypoxia conduction for 24 and 48 h was not enough to secret TNF-alpha, suggesting that LPS was necessary for stimulation. Furthermore, the use of ELISA and biological assays to measure TNF-alpha concentrations can be different and these differences may reflect the vital biological differences between the techniques (Lahat *et al*., 2008).

Conclusions

Hypoxia can occur in various pathological conditions changed in cells of C57BL/6J mice obtained from myeloid mouse cells *in vitro*. Incubation of non-stimulated macrophages and dendritic cells obtained from myeloid mouse cells generated *in vitro* in hypoxia showed that the concentrations of TNF-α measured were low, but it showed different concentrations between hypoxia and normoxia, and hypoxia showed an effect on releasing TNF-α compared to normoxia.

Acknowledgements

I thank Dr. Cordula M. Stover for her support.

Conflict of interest:

I declare that there are no conflicts of interest.

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