Nicolaus Copernicus University in Toruń

Faculty of Biological and Veterinary Sciences

Mgr Konkonika Roy

The dark side of endotoxin tolerance: increased susceptibility to cancer

Dissertation for a doctoral degree

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Supervisor: dr hab. Sylwia Wrotek, prof. NCU

Assistant supervisor: Dr Tomasz Jędrzejewski

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I wish to express my profound gratitude to my supervisor, Dr. hab. Sylwia Wrotek, prof. NCU, for her mentorship, unwavering guidance, and support throughout my academic journey. Her passion, expertise, wisdom, and openness for always being willing to listen and provide thoughtful feedback has been helpful in shaping both my research and my personal growth. I am deeply grateful for her encouragement during challenging times and for celebrating the small milestones along the way. Working under her supervision has been an invaluable and transformative experience for which I will always be thankful.

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1. Abbreviations

APCs	Antigen-presenting cells
AP-1	Activator protein-1
Arg-1	Arginase-1
BC	Breast cancer-bearing mice
CM	Conditioned medium
CM_{4T1}	Conditioned medium obtained from 4T1 cells
CM_{ET}	Conditioned medium obtained from endotoxin-tolerant macrophages
CM_{NT}	Conditioned medium obtained from non-treated macrophages
COX-2	Cyclooxygenase-2
CSF-1	Colony-stimulating factor 1(known as M-CSF)
ERKs	Extracellular signal-regulated kinases
ET	Endotoxin tolerance
ETBC	Breast cancer-bearing mice with endotoxin tolerance
GSDM	Gasdermin D
IFN-γ	Interferon γ
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IKKs	Inhibitor of NF-κB kinases
IRAK	IL-1 receptor-associated kinase
IRF3	INF regulatory factor 3
LBP	LPS-binding protein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MD2	Myeloid differentiation protein 2
MHC	Major histocompatibility complex
MMPs	Matrix metalloproteinases
Mo_{ET}	Endotoxin-tolerant macrophages
Mo_{LPS}	Macrophages stimulated once with LPS
Mo_{NT}	Non-treated macrophages
Myd88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa B
NLRP3	NOD-like receptor pyrin domain containing 3
NO	Nitric oxide
NOS2	Nitric oxide synthase 2
PAMP	Pathogen-associated molecular pattern
PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin H_2
$PI(4,5)P_2$	Phosphatidylinositol (4,5)-bisphosphate
$PI(3,4,5)P_3$	Phosphatidylinositol 3,4,5-trisphosphate

RHIM	Receptor interacting protein (RIP) homotypic interaction motif
RIPK1 and 3	Receptor-interacting serine/threonine-protein kinase 1 and 3
RIP1 and 3	RHIM-containing proteins
ROS	Reactive oxygen species
STAT6	Signal transducer and activator of transcription 6
TAMs	Tumor-associated macrophages
TAK1	Transforming growth factor-β-activated kinase 1
TAB2 and 3	TAK1-binding proteins 2 and 3
TBK1	Tank-binding kinase 1
TGF-β	Transforming growth factor-β
TIR	Toll-interleukin-1 receptor
TIRAP	TIR domains-containing adapter protein
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor α
TRIF	Toll/IL-1 receptor domain-containing adaptor inducing INF-β
TRAM	TRIF-related adaptor molecule
VEGF	Vascular endothelial growth factor

2. Publication list

This dissertation contains selected results detailed described in the following articles #1, #2, and #3:

Article #1	**Roy, K.**; Kozłowski, H.M.; Jędrzejewski, T.; Sobocińska, J.; Maciejewski, B.; Dzialuk, A.; Wrotek, S. Endotoxin Tolerance Creates Favourable Conditions for Cancer Development. *Cancers* 15 (20), 5113, **2023**.	Impact Factor$_{2023}$: 4.5 5-year Impact Factor: 4.9 Ministry points$_{2023}$: 200
Article #2	**Roy, K.**; Jędrzejewski, T.; Sobocińska, J.; Spisz, P.; Maciejewski, B.; Hövelmeyer, N.; Passeri, B.; Wrotek, S. Divergent impact of endotoxin priming and endotoxin tolerance on macrophage responses to cancer cells. *Cellular Immunology* 411-412, 104934, **2025**.	Impact Factor$_{2024}$: 3.7 5-year Impact Factor: 4.1 Ministry points$_{2024}$: 100
Article #3	**Roy, K.**; Maciejewski, B.; Jędrzejewski, T.; Spisz, P.; Sobocińska, J.; Di Pentima, M; Passeri, B.; Wrotek, S. Endotoxin tolerance enhances breast cancer aggressiveness and alters inflammatory marker expression in tumor and spleen of mice. *International Immunology*. (Manuscript ID: INTIMM-25-0140)	Impact Factor$_{2023}$: 5.0 5-year Impact Factor: 5.0 Ministry points$_{2024}$: 140 (Submitted)

3. Contribution to the development of the publications

Article #1: *Endotoxin Tolerance Creates Favourable Conditions for Cancer Development*

As part of my thesis investigation, and with the guidance of my supervisor, I took the lead in designing and conducting key experimental procedures central to this study. I performed the cell survival assays, investigated the migratory capacity, and assessed the clonogenic potential of the cancer cells. Additionally, I carried out ELISA assays to quantify the production of pro-inflammatory cytokines, such as TNF-α and IL-6. To further explore immune cell characteristics, I conducted flow cytometry analysis to determine the phenotypic profiles of macrophages under different conditions. I was also responsible for data curation, interpretation, and analysis. I also participated in writing the entire manuscript, prepared the figures, and proofread the manuscript after the review process.

Article #2: *Divergent impact of endotoxin priming and endotoxin tolerance on macrophage responses to cancer cells*

With the guidance of my supervisor, I conducted an *in vitro* study, including analyzing the gene expression of pro-inflammatory mediators (TNF-α, IL-6, and iNOS) in macrophages and evaluating the protein expression levels of CD14 and COX-2 using the Western blot method. I performed nitric oxide production assays and ROS analysis to investigate inflammatory responses further. I designed and executed co-culture experiments with 4T1 cancer cells to assess macrophage phenotypes within a cancer-associated environment, utilizing flow cytometry. Additionally, I performed metabolic profiling of the macrophages using the SCENITH assay, which was also analyzed by flow cytometry. I was responsible for data curation, interpretation, and statistical analysis. I also participated in writing the entire manuscript, prepared the figures, and proofread the manuscript after the review process.

Article #3: *Endotoxin Tolerance Enhances Breast Cancer Aggressiveness and Alters Inflammatory Marker Expression in Tumor and Spleen of Mice*

Guided by my supervisor, I was responsible for planning and executing the *in vivo* experiments described in the manuscript. I was responsible for developing the endotoxin tolerance model in mice through the administration of LPS, followed by the injection of cancer cells to study tumor development in this context. I closely monitored the mice throughout the experimental period, documenting tumor progression and overall health status. At the endpoint, I collected relevant tissues for downstream analysis, including blood samples for blood

morphology, spleens for assessing size and evaluating the gene expression of immune mediators, and tumor tissues for measuring the expression of key inflammatory genes. I was also responsible for data curation and analysis, and contributed to manuscript writing and figure preparation.

4. Abstract in English

Endotoxin tolerance (ET) is a mechanism that develops in organism due to repeated or prolong endotoxin exposure, for example during infections caused by Gram-negative bacteria. A hallmark of ET, among others is, a significant suppression of pro-inflammatory cytokine synthesis and a weakened febrile response. Many years ago, it was observed that a persistently weakened febrile response during infection could predispose individuals to the development of cancer. However, the causes of this medically important phenomenon remained unknown. Considering that one of the characteristic features of ET is the absence of a febrile response, a hypothesis was proposed that ET may be responsible for the reduced fever-related immune response observed in cancer patients. Therefore, the aim of this study was to investigate whether ET promotes cancer progression.

I began my research by developing ET models in two systems: cellular and organismal. Under *in vitro* conditions, I analyzed macrophages exhibiting endotoxin tolerance (Mo_{ET}) by assessing the levels of various markers associated with the inflammatory response, such as CD14, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nitric oxide (NO), and reactive oxygen species (ROS). Compared cells exposed to LPS only one (Mo_{LPS}), Mo_{ET} showed significantly reduced expression of iNOS and COX-2. Moreover, despite decreased NO production in the tumor microenvironment, ROS levels in these cells remained significantly elevated. Then, I focused on analyzing the interactions between tumor cells and Mo_{ET}. My most important finding was that Mo_{ET}, unlike non-treated with LPS cells (Mo_{NT}), significantly supported tumor cell survival, increased their migratory capabilities, clonogenic potential, and spheroid-forming ability, all of which suggest pro-tumorigenic potential of ET. Furthermore, I demonstrated that in the tumor microenvironment, Mo_{ET} adopt a phenotype similar to M2-type macrophages, which are considered immunosuppressive and pro-tumorigenic. These cells also displayed an altered metabolic profile, shifting away from classical glycolysis and oxidative phosphorylation pathways towards alternative metabolic routes.

In the next stages of the research, I validated the *in vitro* results using an animal model. The results of *in vivo* studies confirmed that ET significantly influences the cancer progression, leading to a reduction in survival and accelerated tumor growth in endotoxin-tolerant mice with breast cancer (ETBC group) compared to non-endotoxin-tolerized cancer-bearing mice (BC group). Assessment of immune mediators in spleens collected from ETBC mice revealed decreased expression of interleukin (IL) 6 and interferon (IFN) γ, alongside increased

expression of iNOS (NOS2), IL-1β, COX-2, STAT6, and colony-stimulating factor 1 (CSF-1) compared to BC mice. Similarly, analysis of tumors from ETBC mice showed elevated expression of IL-10, NOS2, IL-1β, vascular endothelial growth factor (VEGF), COX-2 compared to BC mice.

The obtained results provide evidence that ET locally reprograms macrophages towards a pro-tumoral phenotype and simultaneously modifies their response to tumor cells. Consequently, at the organismal level, ET shapes a systemic environment conducive to tumor development.

Key words: endotoxin tolerance; pro-inflammatory factors; M1/M2 macrophage phenotype; cancer; immunosuppression; tumor microenvironment

5. Abstract in Polish

Do wytworzenia tolerancji endotoksynowe (ET) dochodzi w organizmach, które są poddawane powtarzającej się lub długotrwałej ekspozycji na endotoksynę, np. podczas infekcji bakteriami Gram-ujemnymi. Charakterystyczną cechą ET jest min. zahamowanie syntezy cytokin prozapalnych i osłabiona reakcja gorączkowa. Już wiele lat temu zaobserwowano, że długotrwale utrzymująca się osłabiona reakcja gorączkowa w przebiegu infekcji może stanowić czynnik predysponujący do rozwoju chorób nowotworowych. Przyczyny tego istotnego z medycznego punktu widzenia zjawiska nie były znane. Mając na uwadze, że jednym z charakterystycznych objawów ET jest brak reakcji gorączkowej, wysunięto hipotezę, iż to właśnie ET może odpowiadać za osłabienie gorączkowej odpowiedzi immunologicznej u pacjentów onkologicznych. Celem pracy było zbadanie, czy ET sprzyja progresji nowotworowej.

Badania rozpoczęłam od opracowania modeli ET w dwóch systemach tj. komórkowym i organizmalnym. W warunkach *in vitro* wykonałam analizę makrofagów wykazujących tolerancję endotoksynową (Mo_{ET}), poprzez badanie poziomu różnych markerów związanych z odpowiedzią zapalną, takich jak CD14, indukowana syntaza tlenku azotu (iNOS), cyklooksygenaza 2 (COX-2), tlenek azotu (NO) oraz reaktywne formy tlenu (ROS). W porównaniu z komórkami jednorazowo eksponowanymi na LPS (Mo_{LPS}), Mo_{ET} charakteryzowały się zmniejszoną ekspresją iNOS i COX-2. Ponadto, mimo obniżonej produkcji NO w warunkach mikrośrodowiska nowotworowego, poziom ROS w tych komórkach pozostawał znacząco podwyższony.

Następnie, skupiłam się na analizie interakcji między komórkami nowotworowymi a Mo_{ET}. Moim najważniejszym odkryciem było stwierdzenie, że Mo_{ET} silniej niż kontrolne komórki nietraktowane LPS (Mo_{NT}) wspierają przeżywalność komórek nowotworowych, zwiększają ich zdolności migracyjne, potencjał klonogenny oraz zdolność do tworzenia sferoidów, co łącznie wskazuje na pronowotworowy potencjał ET. Wykazałam także, że Mo_{ET} w warunkach mikrośrodowiska nowotworowego przyjmują fenotyp zbliżony do makrofagów typu M2, który jest uznawany za immunosupresyjny i pro-nowotworowy. Ponadto, komórki te charakteryzowały się zmienionym profilem metabolicznym, przejawiającym się odejściem od klasycznych szlaków glikolizy i fosforylacji oksydacyjnej na rzecz alternatywnych dróg metabolicznych.

W kolejnych etapach badań zweryfikowałam wyniki uzyskane *in vitro*, wykorzystując model zwierzęcy. Wyniki badań *in vivo* potwierdziły, że ET wpływa na przebieg choroby

nowotworowej, prowadząc do wyraźnego zmniejszenia przeżywalności oraz znacząco przyspieszonego wzrostu guza u stolerowanych na endotoksynę myszy z rakiem piersi (grupa ETBC) w porównaniu do myszy z rakiem piersi, które nie były stolerowane na endotoksynę (grupa BC). Ocena mediatorów immunologicznych w śledzionach pobranych od myszy ETBC wykazała obniżoną ekspresję interleukiny (IL) 6 i interferonu (IFN) γ oraz podwyższoną ekspresję iNOS (NOS2), IL-1β, COX-2, STAT6 i czynnika stymulującego tworzenie kolonii (CSF-1) w porównaniu do myszy BC. Podobnie, analiza guzów myszy ETBC, pod kątem istotnych czynników związanych z ze stanem zapalnym, wykazała podwyższoną ekspresję IL-10, NOS2, IL-1β, czynnika wzrostu śródbłonka naczyniowego (VEGF), COX-2 w porównaniu do myszy BC.

Uzyskane wyniki dostarczają dowodów na to, że ET lokalnie przeprogramowuje makrofagi w kierunku fenotypu pro-nowotworowego i jednocześnie modyfikuje ich odpowiedź na komórki nowotworowe. W konsekwencji można zaobserwować na poziomie całego organizmu, że ET kształtuje warunki sprzyjające rozwojowi nowotworów.

Słowa kluczowe: tolerancja endotoksynowa; czynniki prozapalne; fenotyp makrofagów M1/M2; nowotwór; immunosupresja; mikrośrodowisko guza

6. Introduction

Organisms are exposed to various endotoxins throughout their lifetime, with lipopolysaccharide (LPS) being a prime example. LPS is a well-characterized pathogen-associated molecular pattern (PAMP) derived from the outer cell wall of Gram-negative bacteria (1). The Gram-negative bacteria, such as *Escherichia coli,* have been associated with various human health conditions, including urinary tract infections, gastrointestinal illnesses, and respiratory tract infections (2-7).

LPS comprises of three parts: O-antigen, core and lipid A. When released into the bloodstream, lipid A triggers a cascade of toxic effects, prompting a powerful immune response to counter invading pathogens. This involves the engagement of a variety of immune cells, ultimately leading to the production of pro-inflammatory cytokines and fever (8,9). Short-term endotoxin stimulation induces a transient inflammatory response, which is a beneficial mechanism designed to protect the body from pathogens (10). However, with repeated or prolonged endotoxin exposure, an adaptive immune response known as endotoxin tolerance (ET) develops, which helps dampen excessive inflammation and prevent tissue damage.

The first documentation of ET dates back to Paul Beeson (1946), who observed a diminished febrile response in rabbits following multiple injections of typhoid vaccine (11). This phenomenon was later confirmed by Greisman and Hornick (1975), who reported a reduction in fever with continuous endotoxin administration in healthy individuals (12). Subsequent studies further reinforced these findings, consistently demonstrating the absence of fever during ET (13-18). This phenomenon was also observed in a clinical trial involving 14 healthy individuals subjected to 5 consecutive doses of LPS, leading to an attenuated pro-inflammatory response (19). These findings have been in parallel to the observations of Kiani et al. (1997), who suggested that inducing ET in at-risk patients may help prevent complications during sepsis, and with Astiz et al. (1995), who reported that the pretreatment of human volunteers with monophosphoryl lipid A, a hydrolyzed derivative of endotoxin from *Salmonella minnesota*, attenuated the inflammatory response during the endotoxin challenge (20,21). Further research on ET highlighted Toll-like receptor (TLR) 4 as a key factor in the inflammatory loop due to its involvement in the production of the pro-inflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin (IL) 6, and IL-1β (22,23). Significantly, the suppression of the inflammatory response during ET can be associated with the overexpression of anti-inflammatory cytokines, such as IL-10 and transforming growth factor β (TGF-β) (24). Yin et al. (2023) observed that TGF-β neutralizing antibody and TGF-β

receptor inhibitors, i.e., metformin and resveratrol, induced an upregulation of TNF-α and IL-6 via LPS restimulation in tolerized macrophages (25). Consequently, the downregulation of pro-inflammatory factors and the absence of fever following LPS administration are considered signs of ET (26,27).

ET is regarded as an adaptive mechanism that is a protective response in disease conditions, such as sepsis (28-30). During sepsis, an uncontrolled production of pro-inflammatory cytokines, known as a cytokine storm, may occur (31,32). If ET develops, it helps mitigate this by reducing excessive inflammation and limiting tissue damage. Reportedly, the ET can occur as early as 24 to 48 hours after sepsis induction (33). However, according to the concepts outlined in the research grant under which this study was conducted, ET may be a double-edged sword. While ET protects against excessive inflammation, it is important to emphasize that it also functions as an immunosuppressive mechanism. Consequently, ET may impair immune surveillance, potentially allowing malignant conditions, like cancer, to evade detection.

These findings emphasize ET's complexity and dynamic nature, which is tightly regulated by an intricate network of molecular mediators. To fully understand the mechanism of ET, it is essential to explore the key signaling pathways involved in its regulation. Since my thesis focuses on the ET model induced by LPS from *Escherichia coli*, I provide below a brief overview of the TLR4 signaling pathway and its crucial role in the development of ET.

6.1. Molecular mechanism involved in the immune response to endotoxin

6.1.1. Recognition of LPS in TLR4 signaling cascade

LPS, as stated above, consists of three parts, i.e., lipid A, a core oligosaccharide, and an O side chain. Lipid A has been identified as the primary modulator of the immunogenic response in the context of TLR4 activation. The O-specific chain, which can vary in length, determines the distinction between smooth (long O-specific chain) and rough (short or absent O-specific chain) forms of the LPS (34).

When present inside the outer bacterial membrane, lipid A cannot be recognized by the host TLR4 receptors. For LPS sensing to occur, its release from the bacterial cell wall is necessary, typically through bacterial lysis or growth (35). Once released, lipid A part of LPS binds to the LPS-binding protein (LBP), an acute phase protein, known for its opsonic activity. The importance of this protein in the context of LPS signaling is its capacity to bind to the lipid A (part of the LPS) and then catalyze its transfer to CD14 (36,37). Structurally, LBP comprises

of a N-terminal, which enables the binding of the LPS to LBP, and a C-terminal domain, which is important for the transfer of the LPS to CD14 (38). It has been shown that LBP binds to the aggregate (micelles) form of LPS, and then transfers monomeric LPS to either membrane bound or soluble CD14 (39).

6.1.2. Interaction LPS with TLR4

CD14 is a glycoprotein present predominantly on the surface of myeloid-lineage cells, such as monocytes, macrophages, neutrophils and dendritic cells (40-42). This molecule exists in either glycosylphosphatidyl-inositol (GPI)-anchored membrane form or a soluble form and is required for the response to low concentrations of LPS (43). Soluble CD14 is required for the LPS response in cells that do not ordinarily express CD14 (44).

CD14 is localized within the cholesterol and sphingolipids enriched nanodomains of the plasma membrane, known as lipid rafts, which are considered sites of TLR4 activation. During the LPS signaling, the lipid A binds to the N-terminal hydrophobic pocket of the CD14, followed by the association of the CD14-LPS complex with myeloid-differentiation protein 2 (MD-2), a protein with a cleavable signal sequence (45). MD-2 is bound to TLR4 in the Golgi apparatus and is secreted as a soluble molecule from MD-2-expressing cells. Secreted MD-2 has been observed to bind TLR4 at higher affinity, thereby enhancing the response of TLR4 receptor cells to LPS (46). The crystallographic analysis of TLR4-MD-2 revealed the presence of five out of six acyl groups of the lipid A of LPS in the hydrophobic pocket of MD-2 while the remaining one interacted with the other TLR4-MD-2 complex, promoting dimerization (47), and activates downstream signaling pathways, including the myeloid differentiation primary response 88 (MyD88)-dependent and MyD88-independent pathways (48).

The intracellular fragments of the TLR4 contain Toll/IL-1R homology (TIR) domain that is responsible for the interaction of the TLR4 with pairs of adaptor proteins: Toll-interleukin-1 receptor (TIR) domains containing adapter protein (TIRAP)/MyD88 in case of the presence of the TLR4 in the plasma membrane, and Toll/IL-1 receptor domain-containing adaptor inducing INF-β (TRIF)/TRIF-related adaptor molecule (TRAM) (49). The MyD88-dependent signaling activation occurs at the plasma membrane, while the TRIF-dependent signaling is activated after the endocytosis of the TLR4 (50). Studies have shown that the MyD88-dependent pathway is responsible for the production of pro-inflammatory cytokines (51), while the TRIF-dependent pathway is responsible for the production of type I interferons (IFNs) and chemokines (52).

6.1.3. MyD88 dependent signaling

Following the dimerization of the TLR-MD-2 complex, the TIR domains of the TLR4 interact with TIRAP, which also contains its own TIR domain and a domain enriched in basic and aromatic residues that interacts with phosphatidylinositols (PIs) and phosphatidylserine (PS) (53). The binding of TIRAP to phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) associates the protein with the plasma membrane. This interaction is essential for the interaction of TIRAP with TLR4 (54). The role of $PI(4,5)P_2$ is also critical for the later steps of the signaling cascade.

The TLR4-bound TIRAP recruits MyD88, another adaptor protein. Upon docking, the MyD88 death domain (DD) is exposed, which then recruits the Interleukin-1 Receptor-Associated Kinases 4 (IRAK4) (55). Subsequent, phosphorylation and activation of IRAK1 and IRAK2 (56) lead to the formation of the myddosome complex (57), which then recruits the E3 ubiquitin ligase TRAF6 (58) and activates the TAK1, a heterotrimeric kinase complex consisting of transforming growth factor-β-activated kinase 1 (TAK1) subunit and TAK1-binding proteins 2 and 3 (TAB2 and TAB3) structural subunits (60,61).

The activation of TAK1 leads to the activation of IκB kinases (IKK) and mitogen-activated protein kinase (MAPK) pathways. IKK is a complex composed of IκB kinases α/β (IKKα/β) subunits and IKKγ structural subunit. The phosphorylation of IKKα/β triggers the degradation of IκB proteins and subsequent nuclear translocation of transcription factors NF-κB (60,61). On the other hand, activation of MAPK pathways, such as extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK pathways, leads to the induction of the transcription factor activator protein-1 (AP-1) and cyclic AMP response element-binding protein (CREB) (62,63). As mentioned previously, $PI(4,5)P_2$ also plays crucial role in the NF-κB, as the activation of TIRAP and MyD88 also activates type I PI3-kinase which phosphorylates $PI(4,5)P_2$ to phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$), triggering the activation of protein kinase B (Akt) (64). Collectively, the activated NF-κB and MAPK pathways induced the expression of genes encoding the pro-inflammatory mediators, such as TNF-α, IL-6, IL-1β, and pro-IL-1β that drive the inflammatory response (65,66). It is important to note that the MyD88-dependent pathway also produces anti-inflammatory cytokines, such as IL-10, essential in terminating the MyD88-dependent signaling to prevent an exaggerated pro-inflammatory response (67,68).

6.1.4. TRIF-dependent signaling

For TRIF-dependent signaling to occur, TLR4 internalization is essential, and CD14 plays a critical role in facilitating the endocytosis of TLR4. In cells with lower CD14 expression, such as murine splenic B lymphocytes, TLR4 endocytosis was not observed (69).

Following the MyD88-dependent signaling the TLR4 is internalized and the dissociation of the TIRAP and MyD88 occurs, allowing the interaction of the TLR4 with the other two adaptor proteins: TRAM and TRIF. TRAM, similar to TIRAP, is the bridging adaptor between the TIR domain of TLR4 and TRIF (70). TLR4 and TRAM have been reported to localize in the same regions, such as the plasma membrane, endosomes, endocytic recycling compartment, and the Golgi apparatus (73-75). Moreover, TRAM has been observed to be present in CD14-enriched regions of the plasma membrane (74).

During the TRIF-mediated TLR4 signaling, two major processes are initiated, i.e., the activation of interferon regulatory factor 3/7 (IRF3/7), which leads to the synthesis of type I IFNs and chemokines CCL5/RANTES, and the late activation of NF-κB pathway (75,76). The activation of IRF3/7 has been observed to be mediated by TNF receptor-associated factor 3 (TRAF3), a ubiquitin ligase. TRAF3 activation is followed by non-canonical IKK kinases: TANK binding kinase 1 (TBK1) and IKKε. TBK1 phosphorylates the consensus motif of TRIF, which is essential for IRF3 recruitment. Initially, IRF3 and IRF7 are present in the cytoplasm in their inactive form and upon phosphorylation by TBK1, they dissociate from TRIF, dimerize and translocate to the nucleus (77,78). Finally, IRF3 and IRF7, to a lesser extent, IRF7 induce the expression of genes encoding type I IFN, the chemokine CCL5/RANTES, and interferon-regulated genes, such as those encoding the chemokine CXCL10/IP-10 (74,79). This pathway also leads to the production of IL-10 (80).

TRIF pathway, as stated previously, also initiates a late activation of NF-κB signaling, through the recruitment and activation of TRAF6 or via receptor-interacting serine/threonine-protein kinase 1 (RIPK1) (81). TRIF was observed to possess a receptor interacting protein (RIP) homotypic interaction motif (RHIM). Upon its interaction with RHIM-containing proteins RIP1 and RIP3, TRIF was found to be crucial in apoptosis and contributed to NF-κB activation (82). RIPK1 and RIPK3 have also been associated with LPS-induced ERK1/2 activation and cytokine production (83). The representative of the TLR4 signaling pathways has been depicted in Figure 1.

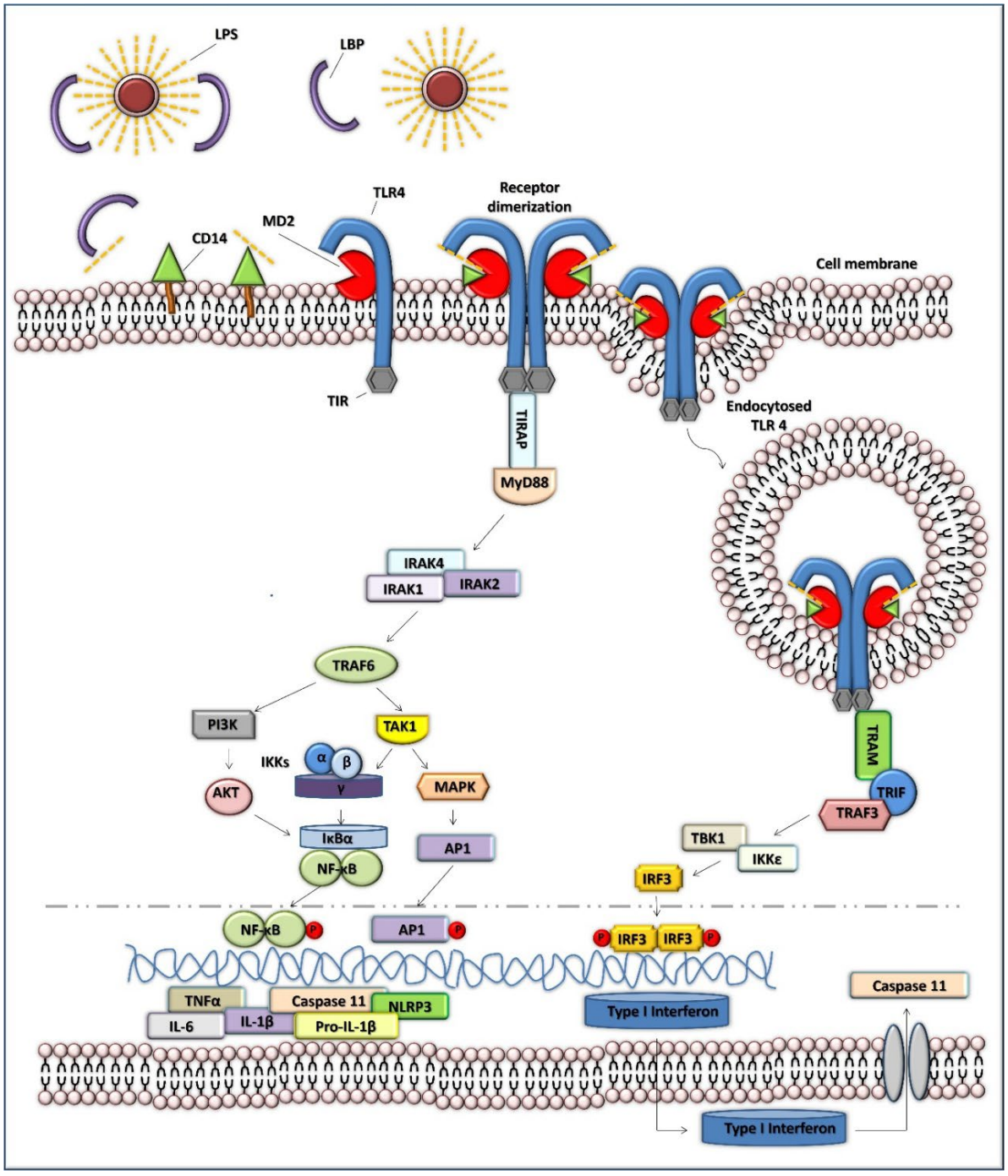

Fig. 1. TLR4 signaling pathway in response to LPS. The figure illustrates the activation of the MyD88-dependent and MyD88-independent signaling initiated by the binding of the LPS to TLR4-MD2 through the CD14 and LBP. These pathways mediate the production of the pro-inflammatory mediator and type I interferons. Figure scheme was prepared by Dr. Paulina Spisz from Department of Immunology, NCU.

6.1.5. Canonical inflammasome signaling

Caspases are a family of conserved endoproteases that play a crucial role in apoptosis and inflammation. It has been recognized that TLR4 also plays an important role in the so-called canonical activation of the NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome. The NLRP3 inflammasome is a multi-protein complex that forms in the cytoplasm in response to PAMPs or damage-associated molecular patterns (DAMPs). It is critical for the host's immune defenses against bacterial, fungal, and viral infections (84,85).

For the activation of the NLRP3 inflammasome two signals are essential. The first signal promotes the upregulation of NLRP3 and the production of the precursors of IL-1β and IL-18. And the second signal is from the extracellular ATP or pore-forming toxins, which activate the NLRP3 inflammasome (85). Stimuli, such as ligands for TLRs, NLRs (e.g., NOD1 and NOD2), or cytokine receptors, which activate NF-κB, are necessary for priming. The MyD88 and TRIF pathways have been observed to regulate the induction of NLRP3 and pro-IL-1β in response to TLR ligand (86,87).

During the second signal, the activation of the NLRP3 inflammasome complex occurs. NLRP3 inflammasome complex consists of: NLRP3, a protein comprising three distinct subunits, i.e., an amino-terminal pyrin domain (PYD), a central nucleotide-binding and oligomerization domain (NOD; a.k.a the NACHT domain), and a C-terminal leucine-rich repeat (LRR) domain (88); apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD) (ASC) and pro-caspase-1. During its activation, the ASC and the NLRP3 interact with the pro-caspase 1 through their CARD domain, which leads to the autoproteolysis and activation of caspase-1 (89). Active caspase-1 then cleaves the pro-IL-1β and pro-IL-18 into their active forms. It also cleaves gasdermin D (GSDMD), a pore-forming protein that binds to the $PI(4,5)P_2$ and releases the IL-1β and IL-18 from the cells (90,91). Knowledge regarding the exact mechanism leading to the inflammasome assembly is yet lacking. NLRP3 has been observed to be activated by a variety of stimuli, such as ATP, K^+ ionophores, heme, particulate matter, pathogen-associated RNA, and bacterial and fungal toxins and components. However, NLRP3 has not been observed to interact with these stimuli directly. Therefore, leading researchers to believe that all these agonists induce common cellular signaling, such as ionic flux, mitochondrial dysfunction, production of ROS, and lysosomal damage, which has been observed to activate the NLRP3 inflammasome (85,92–95).

6.1.6. Non-canonical inflammasome signaling

The non-canonical pathway acts as a protective mechanism towards the pathogens that have evolved to bypass the TLR4 (96). This pathway involves human caspase-4 and -5 and mouse caspase-11. Activation of the non-canonical inflammasome occurs by the direct binding of the LPS to human caspase-4 and -5 (similar to mouse caspase-11) (97,98). The component of the LPS that is sensed by these caspases is pent-acylated and hexa-acylated lipid A. Unlike the canonical inflammasome, this pathway does not require priming. TLR4-dependent and TRIF-dependent IFN-α/β production are partially involved in the pro-caspase-11 expression. However, these pathways are necessary for the caspase-11 activation in macrophages (99,100).

Caspases-4/5/11 induce apoptosis through GSDMD and pannexin-1, a protein channel that releases ATP from the cell (101,102). The release of ATP leads to the activation of P2X7 receptor (P2X7R), an ATP-gated cation selective channel, which opens a pore leading to the K^+ efflux. The inhibition of the pyroptosis then occurs through the oxidized phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC), which directly binds to the caspase-4 or caspase-11 in macrophages and results in the inactivation of non-canonical inflammasome (103). The representative scheme of the inflammasome signaling is present in Figure 2.

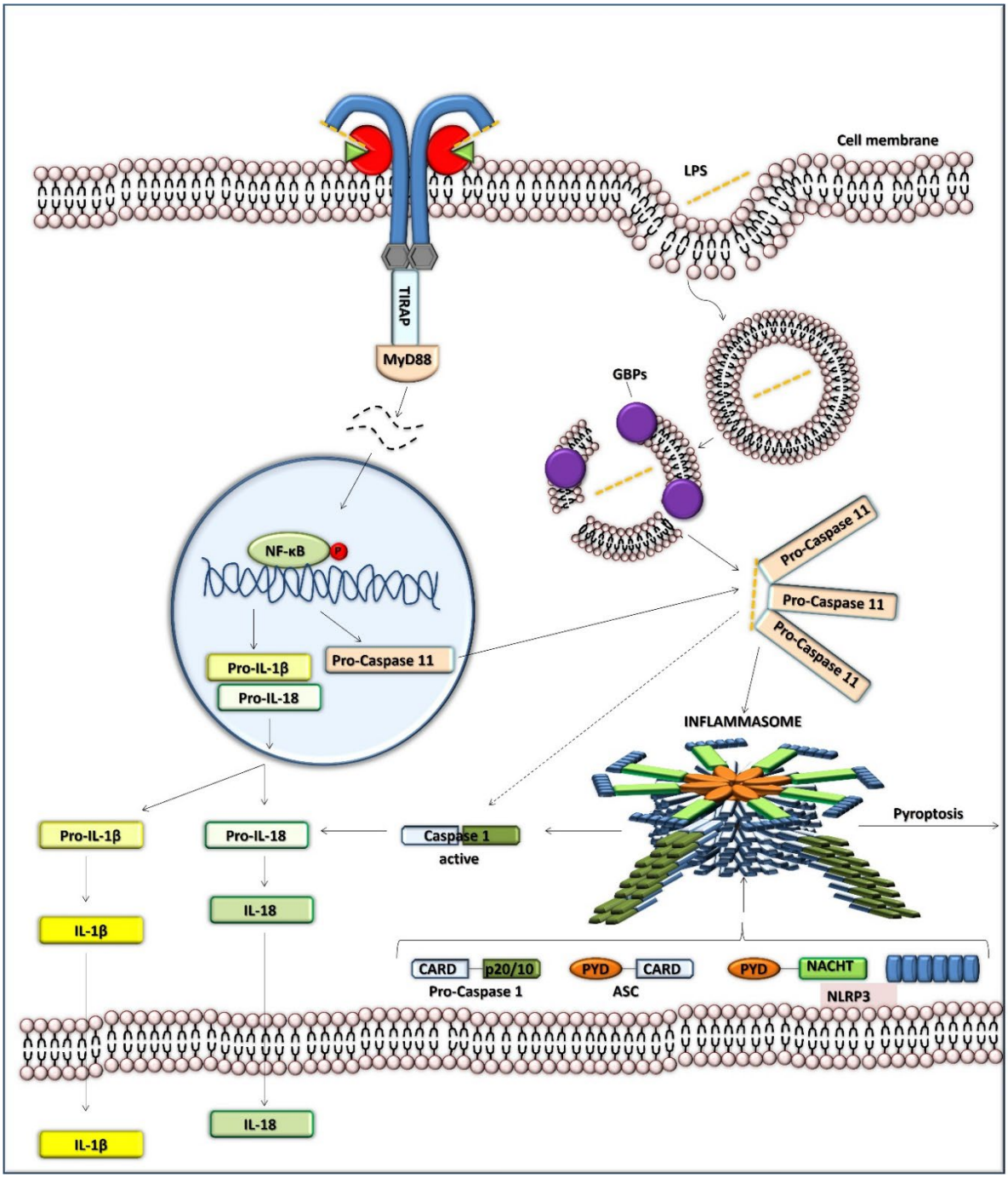

Fig. 2. Inflammasome signaling in response to LPS. The figure illustrates the activation of the inflammasome pathway in response to LPS stimulation. It highlights the interplay between TLR4 signaling and inflammasome activation, leading to the maturation and release of the pro-inflammatory cytokines (IL-1β and IL-18). The NLRP3 inflammasome complex assembles upon stimulation, comprising NLRP3, CARD, and pro-caspase 1. This assembly facilitates the autocatalytic activation of caspase 1, which subsequently processes pro-IL-1β and pro-IL-18 into their active forms. Figure scheme was prepared by Dr. Paulina Spisz from Department of Immunology, NCU.

6.1.7. Significance and mechanism of fever

In response to LPS exposure and as a result of the TLR4 and inflammasome mechanism described above, organisms develop fever. Its significance extends beyond infection control, with historical observations suggesting a potential therapeutic role in disease management. Aulus Cornelius Celsus (circa 25 BC - 50 AD) identified fever alongside the classic signs of inflammation, famously describing them as '*Rubor et tumor cum calore et dolore*' – redness, swelling, heat, and pain. The significance of fever continues to be a subject of ongoing research, with its foundations traced back to the observations of German physician Wilhelm Busch, who, in 1868, recognized that infectious fever might contribute to cancer remission, thus paving the way for further investigations into its therapeutic potential (104). These findings were further solidified by William Coley, a pioneer of immunotherapy, who treated numerous cancer patients with fever-inducing bacterial extracts known as Coley's toxin, leading to remission in many of them (105). In 1882, German physician Wilhelm Fehleisen achieved remissions in cancer patients by injecting cultured *Streptococcus pyogenes* (106). Although these observations suggested a beneficial effect of infectious fever in cancer treatment, the use of bacterial extracts was eventually discontinued due to their potential adverse effects.

To understand the role of fever in immune regulation, it is essential to explore the underlying mechanisms that trigger its induction and progression. Fever is a physiological response triggered by the release of pro-inflammatory mediators known as endogenous pyrogens. Exposure to inflammatory stimuli, such as LPS, leads to the production of pro-inflammatory cytokines. These factors induce the liberation of arachidonic acid from the phospholipids of the cell membrane and the activation of COX-2. Phospholipase A_2 (PLA_2) then catalyzes the release of arachidonic acid, which is subsequently metabolized by cyclooxygenase-2 (COX-2) into prostaglandin H_2 (PGH_2). Next, the conversion of PGH_2 to PGE_2 is catalyzed by prostaglandin E synthase (PGE synthase), with the microsomal isoform mPGES-1 playing a predominant role under inflammatory conditions. This enzymatic reaction is glutathione-dependent, as mPGES-1 requires reduced glutathione as a cofactor to facilitate the isomerization of PGH_2 into PGE_2 (107,108). PGE_2 crosses the blood-brain barrier via the *organum vasculosum laminae terminalis* (OVLT), and binds to prostaglandin E receptor 3 (EP3 receptor) in the preoptic area of the hypothalamus (109,110). This interaction signals the hypothalamus to raise the body’s thermal “set point”, thereby initiating the fever response as part of the immune regulation. The scheme for the mechanism of fever induction is presented in the Figure 3.

While patients often dislike fever, it offers numerous advantages in the body's defense against infection. The elevated body temperature enhances the activity of immune cells, such as T cells, neutrophils, and macrophages, making them more effective in combating pathogens (111). Additionally, it inhibits pathogen growth by creating an unfavorable environment for many microorganisms. Fever also accelerates the production of antibodies by B cells (9) and boosts the activity of enzymes (e.g., peroxidases, lysozyme, nitric oxide synthase) involved in immune functions, such as pathogen destruction and tissue repair (112,113).

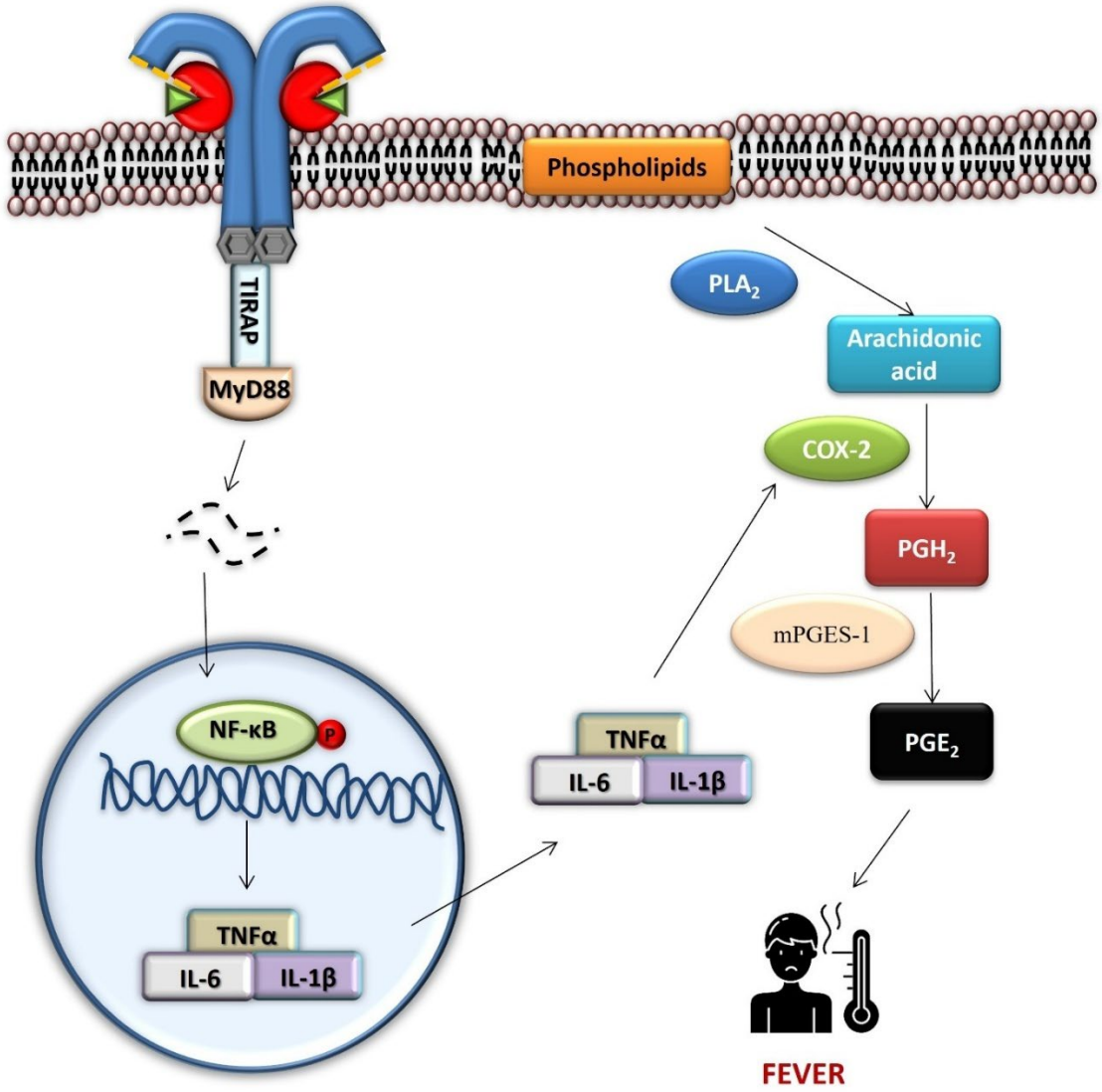

Fig. 3. Mechanism of fever induction. The figure illustrates how fever is triggered by the production of pro-inflammatory cytokines called endogenous pyrogens (TNF-α, IL-6, and IL-1β). These cytokines stimulate the upregulation of cyclooxygenase-2 (COX-2), which is involved in the conversion of arachidonic acid into prostaglandin E_2 (PGE_2). The increase in PGE_2 levels ultimately leads to the onset of fever. Figure created by the author of this dissertation.

6.1.8. Molecular mechanism involved in endotoxin tolerance

Considering the TLR4 and inflammasome signaling in LPS sensing, the precise mechanism underlying ET is not yet fully understood. However, studies report that IRAK-M, a negative regulator of TLR4 signaling, may be a key modulator of ET (114-116). Reportedly, IRAK-M inhibits the TLR4 downstream signaling by blocking the dissociation of the IRAK1 and IRAK4 from MyD88, thereby preventing the formation of the IRAK-TRAF6 complex. Upregulation of the IRAK-M expression was also observed in the monocytes after the second LPS challenge (117). Another important factor, which is believed to be a regulator of ET, is hypoxia-inducible factor 1α (HIF-1α) (118). Importantly, its elevation has been observed to positively correlate with IRAK-M levels, a relationship that can be associated with an immunosuppressive characteristic of ET (119).

Another pathway that can be modified during ET development is the TRIF signaling cascade, a MyD88-independent phenomenon. The involvement of TRIF in ET has been reported by Biswas et al. (2007), who observed ET-related downregulation of Myd88-dependent pro-inflammatory cytokines (TNF-α and CCL3), and a simultaneous upregulation of TRIF-dependent cytokine INF-β (120). Another study reported inhibition of TLR4-TRIF and TRIF-TBK1 complex formation in endotoxin-tolerant monocytes, which leads to the suppressed expression of IRF3, subsequently leading to decreased expression of IFN-β (121,122).

In the context of ET, a shift in NF-κB composition has been observed, resulting in the upregulation of genes encoding TGF-β, IL-10, and COX-2 (123). Moreover, the elevated expression of IL-10 in endotoxin-tolerant macrophages is associated with the activation of signal transducer and activator of transcription 6 (STAT6), a transcription factor known to drive M2-like macrophage polarization (124). Although numerous studies aimed at understanding the mechanism of ET have significantly advanced our knowledge of this complex phenomenon, substantial gaps remain, particularly concerning the regulatory pathways and cellular mechanisms that sustain the tolerant state.

6.2. The role of macrophages in infections and tumorigenesis

As integral components of the mononuclear phagocyte system, macrophages are crucial sentinel cells within tissues, playing a central role in immune responses and tissue repair. They originate from circulating monocytes, which differentiate into macrophages upon migrating into inflamed tissues and perform a broad range of functions in response to tissue-specific

signals (125,126). In inflammation, macrophages play three distinct roles: antigen presentation, phagocytosis, and immunomodulation by producing various cytokines and growth factors (127). The major role of macrophages is to defend the host against pathogens by producing ROS (128) and NO (129). Notably, previous studies conducted by members of my team have shown that cells of the innate immune system (such as macrophages), rather than those of the adaptive immune system (T and B lymphocytes), are essential for initiating the fever response (130).

Macrophages are broadly classified into two distinct phenotypes, i.e., classically activated M1 cells and alternatively activated M2 cells. M1 phenotype has been majorly associated with the increased expression of CD80 and CD86 (131,132), and induction of inflammatory response through the increased expression of pro-inflammatory factors, such as TNF-α, IL-6, IL-1β, ROS, iNOS and COX-2 (133,134). In contrast, M2 macrophages are characterized by elevated levels of arginase-1 (Arg-1), mannose receptor (CD206) and anti-inflammatory cytokines, such as IL-10, TGF-β, as well as chemokines, including CCL17 and CCL22. They are activated by exposure to specific cytokines and factors, including IL-4, IL-13, IL-10, immune complexes, hormones, or adenosine A2A receptors (A2AR) agonists. M2 macrophages are essential in tissue repair, angiogenesis, and metabolic processes (135).

Although macrophages have long been classified based on traditional criteria, recent studies have shown that their phenotypic classification is more flexible and dynamic than previously thought. Notably, the M2 phenotype has been subdivided into distinct subsets: M2a, M2b, M2c and M2d, which do not exhibit an anti-inflammatory profile uniformly. As reported, the M2b subtype also produces pro-inflammatory cytokines similar to M1 macrophages (136).

Macrophages have also been identified within tumor tissues, where they are commonly referred to as tumor-associated macrophages (TAMs). In the tumor microenvironment, macrophages have been known to mainly originate from bone marrow-derived monocytes, which are recruited from the tumor or stroma by released chemokines, such as colony-stimulating factor 1 (CSF-1), also known as macrophage colony-stimulating factor (M-CSF), and CCL2 (137,138). Recent studies have reported TAMs to be in a constant transition state between M1 and M2. However, these cells exhibit an immunosuppressive environment, critically impacting cancer progression. Reportedly, M1-like TAMs promote tumor suppression through two different mechanisms: macrophage-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC). In contrast, M2 macrophages are known to facilitate tumor metastasis by degrading the endothelial cell matrix membrane. They promote this process through the secretion of matrix metalloproteinases (MMPs), serine

proteases, and cathepsins, which degrade various extracellular matrix components, including collagen. This degradation process enables the migration of tumor cells and tumor-associated stromal cells, thereby promoting the tumor metastasis (139). Additionally, M2-like TAMs contribute to cancer progression and invasion by promoting cell proliferation and tumorigenesis through the secretion of mediators, such as CCL2, IL-6, IL-10 and vascular endothelial growth factor (VEGF) (140). Due to their predominant role in promoting tumor progression within the tumor microenvironment, TAMs have become a key focus of cancer research.

7. Aim of the study

Endotoxin tolerance (ET) is characterized by immune reprogramming, where the immune system becomes less responsive to subsequent endotoxin exposure. ET has garnered considerable attention recently due to its potential role in modulating immune responses, particularly in chronic infectious conditions. However, after reviewing the existing literature, it is clear that there is a significant gap in our understanding of ET within the context of cancer. This study seeks to fill this gap. Therefore, the overarching goal of my research was to explore the impact of ET on cancer, with specific objectives aimed at examining:

a. The impact of endotoxin tolerance on macrophages and cancer cells (Article #1)

To achieve this goal, I have developed an *in vitro* model of ET in macrophages (RAW 264.7 cell line) and evaluated the induction of ET through the expression analysis of the pro-inflammatory factors, such as TNF-α and IL-6. Then, the effect of ET on the survival capacity, clonogenic potential, cancer cell motility and spheroidal development was analyzed in the 4T1 breast cancer and CT26 colon cancer cells by stimulating them with conditioned media (CM) obtained from non-tolerant and endotoxin-tolerant macrophages. The effect of ET on the production of the pro-inflammatory cytokines was also evaluated in a co-culture model of macrophages and cancer cells. Finally, I assessed the M1/M2 phenotype of the endotoxin-tolerant macrophages through the expression of the CD80 (M1) and CD163 (M2) surface markers.

b. The effect of the tumor microenvironment on endotoxin-tolerant macrophages (Article #2)

To advance this goal, in collaboration with Professor Benedetta Passeri from the University of Parma (Italy), I analyzed the infiltration of the macrophages into the mouse tumor tissues *in vivo* using immunohistochemical analysis of the MAC387 antibody expression. Next, I continued my research using the *in vitro* ET model by examining the gene expression of pro-inflammatory mediators, such as TNF-α, IL-6 and iNOS in the non-tolerant macrophages (Mo_{NT}), tolerant macrophages (Mo_{ET}) or macrophages treated only once with LPS for 24 hours (Mo_{LPS}). Additionally, I analyzed the protein expression of CD14 and COX-2 in these cell conditions. Given the significant influence of cancer on inflammation, I investigated the effect of ET in the context of cancer by measuring NO and ROS production, which are key mediators of the primary inflammatory response. This analysis was performed in the Mo_{NT}, Mo_{ET}, and

Mo_{LPS} cell models following stimulation with CM derived from 4T1 breast cancer cells (CM_{4T1}). Since it is also well-established that the cancer microenvironment can influence macrophages, promoting pro-tumorigenic conditions that support tumor growth and progression, I also examined the M1/M2 phenotype of Mo_{NT}, Mo_{ET} and Mo_{LPS} in a co-culture system with 4T1 cells to evaluate this phenomenon. Subsequently, I assessed the survival capacity of these macrophages in the presence of CM_{4T1}. Finally, in collaboration with Dr. Nadine Hövelmeyer the Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg University (Mainz, Germany), I analyzed their metabolic profiles within the cancer-associated environment.

c. The effect of endotoxin tolerance on tumor development in mice (Article #3)

I established an *in vivo* ET model in mice to achieve this goal. Since the absence of fever is a hallmark of ET, I monitored the mice's body temperature (T_b) and their motor activity. Subsequently, the mice were inoculated with 4T1 cells to induce cancer, and tumor progression and survival rates were evaluated in cancer-bearing mice (BC) and cancer-bearing mice with ET (ETBC). I performed histological analysis of the tumor tissues obtained from both groups. Since ET is thought to impair immunocompetence, I analyzed blood morphology to assess potential changes in white blood cell populations. Additionally, I assessed spleen size and analyzed the expression of key immune-related genes in this organ, including IL-6, IL-1β, COX-2, VEGF, IFN-γ, NOS2, IL-10, STAT6, and CSF-1, due to the spleen's central role in immune regulation. Finally, tumor tissues were also analyzed for the expression of IL-10, NOS2, IL-1β, VEGF, COX-2, CSF1, CD206, and STAT6 to further investigate the immunological landscape within the tumor microenvironment.

8. Materials and methods

The research was carried out with the support of the National Science Centre (Poland) under the Preludium Bis 2 grant (Grant no. 2020/39/O/NZ5/00915).

I conducted my research using two models of ET that I developed: *in vitro* and *in vivo*. *In vitro* studies were carried out using cancer cells and macrophages, and conditioned media (CM_{NT} from non-treated macrophages; CM_{ET} from endotoxin-tolerant cells). *In vivo* studies were conducted using mice, in which I induced breast cancer in addition to endotoxin tolerance. Detailed descriptions of the procedures can be found in the attached publications as follows:

8.1. *In vitro* methodology

Cell culture: Murine macrophage cell line RAW 264.7 was obtained from the European Collection of Authenticated Cell Cultures, whereas the breast cancer cell line 4T1 and colon cancer cell line CT26 were purchased from the American Type Culture Collection. These cell lines were cultured according to the protocol in our research articles #1, # 2 and #3.

Preparation of LPS solution: LPS derived from *Escherichia coli* (strain 0111:B4) was used at a concentration of 100 ng/mL (articles #1 and #2).

Model of endotoxin tolerance in vitro: The ET model *in vitro* was established according to the protocol mentioned in articles #1 and #2.

Preparation of conditioned media: Conditioned media (CM) were obtained from non-tolerant macrophages (CM_{NT}) and tolerant macrophages (CM_{ET}) (article #1) and 4T1 cancer cells (CM_{4T1}) (article #2).

Cell viability assay: To assess the cell survival capacity of 4T1 and CT26 cancer cells (article #1) as well as macrophages (article #2), the MTT assays were performed.

Analysis of inflammatory mediators: Inflammatory responses were evaluated by analyzing the expression levels of key cytokines and mediators using multiple techniques. TNF-α and IL-6 expressions were quantified using enzyme-linked immunosorbent (ELISA) assays and real-time polymerase chain reactions (RT-PCR) (Articles #1 and #2). The expressions of iNOS and COX-2 were assessed using RT-PCR and Western blot analysis, respectively (Article #2). CD14 expression was examined using the Western blotting technique.

Analysis of NO and ROS production: NO production was measured using the modified Griess reagent assay, while ROS levels were determined through H_2DCFDA staining and using flow cytometry (article #2).

Colony formation assay: The evaluation of colony formation capacity of the 4T1 and CT26 cancer cells treated with CM derived from macrophages was presented in article #1. This method allows for the evaluation of the long-term survival of cancer cells.

Scratch assay: This method was used to assess how CM derived from macrophages affects the motility of 4T1 and CT26 cancer cells (article #1).

3D spheroidal assay: Three-dimensional spheroids derived from 4T1 and CT26 cancer cells were generated using the hanging drop method, which allows for the formation of uniform, compact cellular aggregates that better mimic the *in vivo* tumor microenvironment. The effect of macrophage-conditioned media on these spheroids' morphology and growth dynamics was subsequently evaluated (article #1).

Polarization of macrophages: The M1/M2 polarization of non-treated macrophages (Mo_{NT}), tolerant macrophages (Mo_{ET}), and macrophages stimulated only once with LPS for 24 hours (Mo_{LPS}) was assessed in monoculture and co-culture systems by evaluating the CD80 and CD163 expression through flow cytometry (articles #1 and #2).

Single Cell Energetic Metabolism by Profiling Translation Inhibition (SCENITH): The metabolic profiles of Mo_{NT}, Mo_{ET}, and Mo_{LPS} macrophages, both alone and following stimulation with CM_{4T1}, were assessed using the SCENITH assay (Article #2). The cells were treated with metabolic inhibitors: 2-Deoxy-D-glucose to inhibit glycolysis, oligomycin to block oxidative phosphorylation (OXPHOS), or a combination of both. Flow cytometry was used to analyze the resulting data and determine the metabolic activity of the cells. This part of the study was conducted during my three-month internship at the Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg University Mainz, Germany, funded by the Polish National Agency for Academic Exchange (NAWA).

8.2. *In vivo* methodology

Experimental animals: Female BALB/c mice were housed in a controlled environment and were subjected to the procedures approved by the Local Bioethical Committee for Animal Care in Bydgoszcz (Poland; permission no. LKE 50/2022) (articles #2 and #3).

Temperature and motor activity measurement: Body temperature (T_b) and the motor activity of the mice were monitored using a temperature-sensitive miniature biotelemetry system (PhysioTels model TA10TA-F40; Data Sciences International, USA) with intra-abdominally implanted transmitters (articles #2 and #3).

Preparation of LPS solution for ET induction in mice: LPS derived from *Escherichia coli* (strain 0111:B4) was administered at a dose of 50 µg/kg in the *in vivo* experiments (article #3).

Model of endotoxin tolerance in mice: The ET model was established according to the protocol described in article #3.

Survival studies and tumor growth rate analysis: Tumor growth dynamics and survival rates in two experimental groups: cancer-bearing mice (BC) and cancer-bearing mice with ET (ETBC), were monitored over time to evaluate the impact of ET treatment on disease progression (article #3).

Tumor induction and tissue collection: Mice were inoculated with 4T1 breast cancer cells to induce tumor growth and were monitored regularly throughout the course of the study. After approximately 3 weeks, the mice were euthanized. Tissues were then collected for further analysis, including blood morphology, spleen size measurement, and RT-qPCR analysis of immune related genes (IL-6, IL-1β, COX-2, VEGF, INF-γ, NOS2, IL-10, STAT6, and CSF-1) in both tumor and spleen samples (articles #2 and #3).

Immunohistochemical analysis: The infiltration of the macrophages in the tumor tissues from mice with and without ET was analyzed through Mayer's hematoxylin and eosin (HE) staining, along with using the antibody MAC387 (articles #2). The immunohistochemical tissue analysis was done in collaboration with Professor Benedetta Passeri at the University of Parma, Italy.

Histological assessment: Mice mammary tumor tissues were assessed by pathologists. The tumor tissues were fixed in 10% neutral buffered formalin for 24 hours. The tissue samples were then routinely processed for histopathology and embedded in paraffin wax (FFPE). Paraffin sections of 5 µm thickness, were stained for histology with Mayer's hematoxylin and eosin (H&E). All images were captured using a Nikon Eclipse E800 microscope.

Tumor growth rate analysis: Tumor samples obtained from the group of mice with BC and ETBC were measured using Vernier calipers. The tumor volume was calculated using the formula

$$V = \frac{\pi}{6} * L * W$$

In this equation, L represents the average length of the tumors and W represents the average width of the tumors. The comparison analysis between the BC and ETBC mice groups was conducted using non-linear regression analysis.

Statistical analysis: Statistical comparisons were conducted using the GraphPad Prism 7.0 software (articles #1, #2 and #3). All of the data in articles #1 and #2 are expressed as the mean ± standard error of the mean (SEM) of three independent experiments and analyzed using one-

way ANOVA followed by Bonferroni's multiple comparisons test. For the data in article #3, relative gene expression levels were evaluated and compared using the $2^{-\Delta\Delta CT}$ method. Multiple group comparisons were performed using a one-way analysis of variance (ANOVA) followed by the Sidak test. An unpaired t-test was used to evaluate the gene expression between two individual groups. Non-linear regression was used to assess the tumor growth rate. In all papers, the statistical standard of significance was set at $p < 0.05$.

9. Articles

9.1. Article #1

Roy, K.; Kozłowski, H.M.; Jędrzejewski, T.; Sobocińska, J.; Maciejewski, B.; Dzialuk, A.; Wrotek, S. Endotoxin Tolerance Creates Favourable Conditions for Cancer Development. . *Cancers*, 15, 5113, **2023**. https://doi.org/10.3390/cancers15205113.

cancers

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Article

Endotoxin Tolerance Creates Favourable Conditions for Cancer Development

Konkonika Roy [1,*], Henryk Mikołaj Kozłowski [1,2], Tomasz Jędrzejewski [1], Justyna Sobocińska [1], Bartosz Maciejewski [1], Artur Dzialuk [2] and Sylwia Wrotek [1,*]

[1] Department of Immunology, Faculty of Biology and Veterinary Sciences, Nicolaus Copernicus University, 1 Lwowska Street, 87-100 Torun, Poland
[2] Department of Genetics, Faculty of Biological Sciences, Kazimierz Wielki University, 10 Powstańców Wielkopolskich Ave., 85-090 Bydgoszcz, Poland
* Correspondence: konkonika.roy@doktorant.umk.pl (K.R.); wrotek@umk.pl (S.W.)

Simple Summary: Macrophages, depending on their phenotype, can either destroy or stimulate cancer cells. Therefore, it is very important to identify the conditions under which they adopt a dangerous phenotype. Our study investigated the impact of endotoxin tolerance (ET) on macrophage behaviour and its role in cancer development. By utilizing in vitro models and diverse research methods, including examining conditioned medium effects on 3D cancer cell cultures and studying macrophage-cancer cell crosstalk, we discovered that ET-induced macrophage reprogramming leads to the release of factors that promote a cancer-favourable environment. Our findings highlight the dual nature of ET as a mechanism, potentially contributing to cancer progression. This work suggests that targeting ET could offer novel avenues for cancer prevention and treatment. To the best of our knowledge, our research group is the first to uncover this adaptive mechanism's potential role in cancer development.

Abstract: Endotoxin tolerance (ET) is an adaptive phenomenon of the immune system that protects the host from clinical complications due to repeated exposure of the body to endotoxins such as lipopolysaccharide (LPS). Since ET is an immunosuppressive mechanism in which a significant reprogramming of macrophages is observed, we hypothesized that it could influence cancer development by modifying the tumour environment. This study aimed to explore whether ET influences cancer progression by altering the tumour microenvironment. Endotoxin-tolerant macrophages (Mo_{ET}) were examined for their impact on breast and colon cancer cells via direct interaction and conditioned media exposure. We characterized cancer cell behaviour by viability, clonogenic potential, motility, scratch assays, and 3D spheroidal assays. Mo_{ET}-derived factors increased cancer cell viability, motility, and clonogenicity, suggesting a conducive environment for cancer development. Remarkably, despite reduced TNFα and IL-6 levels, Mo_{ET} exhibited M1 polarization. These findings uncover an ET-associated macrophage reprogramming that fosters a favourable context for cancer progression across diverse tumours. Targeting ET could emerge as a promising avenue for cancer therapy and prevention.

Keywords: cancer; endotoxin tolerance; macrophage polarization M1/M2; tumour microenvironment; immunosuppression; pro-cancerogenic conditions; cytokines

Citation: Roy, K.; Kozłowski, H.M.; Jędrzejewski, T.; Sobocińska, J.; Maciejewski, B.; Dzialuk, A.; Wrotek, S. Endotoxin Tolerance Creates Favourable Conditions for Cancer Development. *Cancers* **2023**, *15*, 5113. https://doi.org/10.3390/cancers15205113

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1. Introduction

Cancer is one of the leading causes of death for individuals under the age of 70 years in 112 out of 183 countries, according to the World Health Organization (WHO). The most commonly diagnosed cancers are female breast, lung, and colorectal cancers [1]. There have been many advancements in cancer therapy and treatment, such as targeted therapy and immunotherapy. However, even with such advancements in early screening and treatment,

the American Cancer Society has estimated that there will be approximately 300,590 new cases of breast cancer and 153,020 new cases of colorectal cancer in the United States in 2023 [2]. While the mortality rate of cancer patients still remains high, it is important to note that the survival rate has improved over the years. For instance, a study in Italy indicates the 5-year survival rate for breast cancer has increased from 75% in the mid-1970s to 91% today. Similarly, the 5-year survival rate for colorectal cancer has increased from 51% in the mid-1970s to 66% [3,4]. To further improve the outcomes of cancer patients, novel, effective therapeutic strategies are in high demand.

Cancer development is influenced by factors that are released from both the cancer cells and the cells in the surrounding environment. In research on cancer, macrophages are the subject of many studies [5–7]. It has been found that not only tumour-associated macrophages (TAM) but also peripheral macrophages affect cancer growth [8]. Furthermore, it is commonly accepted that they may exhibit anti-cancer and pro-cancer properties, depending on their M1 and M2 phenotypes. Briefly, the M1 phenotype is responsible for anti-cancer effects through the production of pro-inflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin (IL-6). In contrast, M2 phenotype macrophages are reported to produce anti-inflammatory factors such as tumour growth factor (TGF) β and IL-10 and are considered a pro-cancer pool [9,10].

The main role of macrophages is to protect organisms from bacterial and viral infections by secreting antimicrobial mediators and pro-inflammatory cytokines [11]. These cells are very sensitive to lipopolysaccharide (LPS), an endotoxin derived from the outer membrane of Gram-negative bacteria that is one of the most common factors that humans and animals are exposed to throughout their lives [12]. In response to LPS, macrophages shift towards the M1 phenotype and produce pro-inflammatory factors [13,14]. However, if the macrophages are exposed to LPS for a prolonged period, a state of endotoxin tolerance (ET) develops [15]. As a result, the pro-inflammatory response of macrophages is attenuated, and fever is abolished [16]. This is believed to be a protective mechanism against the harmful effects of endotoxin-induced acute inflammation [17]. On the other hand, ET turned out to be a "double-edged sword" because it is involved in promoting secondary infections, sepsis, and eventually death [18–20].

Since reprogramming of endotoxin-tolerant macrophages adversely affects the course of infection, we wondered whether ET may also affect cancer development. Thus, in the current study, we hypothesized that ET-related immune paralysis can create conditions favourable to cancer. To test this, we first established an in vitro model of ET, and then we studied the effect of endotoxin-tolerant macrophages on cancer cell behaviour.

Our data clearly shows that endotoxin-tolerant macrophages release factors that support tumour development and enhance cancer aggressiveness.

2. Materials and Methods

2.1. Cell Culture

The murine macrophage cell line RAW 264.7 was obtained from the European Collection of Authenticated Cell Cultures (Salisbury, UK), while the breast cancer cell line 4T1 and the colon cancer cell line CT26 were purchased from the American Type Culture Collection (Manassas, VA, USA). All the cell lines were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 IU/mL penicillin (all compounds from Sigma-Aldrich, Darmstadt, Germany) and were incubated at 37 °C in a humidified atmosphere with 5% CO_2. The cells were sub-cultured every 2–3 days. To detach the adherent 4T1 and CT26 cells, 0.25% trypsin-EDTA solution (Sigma-Aldrich) was used after the cells reached 70–80% confluency, and RAW 264.7 cells were easily detached by the light scraping.

2.2. Preparation of Lipopolysaccharide (LPS) Solution

Lipopolysaccharide (LPS) derived from *Escherichia coli* (0111: B4, Sigma Aldrich) was dissolved in a sterile phosphate-buffered saline (PBS). LPS was used in the experiments at a working concentration of 100 ng/mL.

2.3. Induction of Endotoxin Tolerance (ET) in RAW 264.7 Macrophage Cells

RAW 264.7 macrophages were seeded in a 24-well plate at a concentration of 2×10^5 cells/well in 2 mL of DMEM medium supplemented with 10% FBS and pre-incubated for 24 h. The cells were then maintained in the following three conditions: non-tolerant macrophages (Mo_{NT}), tolerant macrophages (Mo_{ET}), or macrophages treated only once with LPS for 24 h (Mo_{LPS}). Mo_{LPS} were used as a positive control. To obtain Mo_{ET} cells, RAW 264.7 cells were stimulated for 24 h with 100 ng/mL of LPS, followed by a wash with PBS and further culturing in a similar dose of LPS-containing media for another 24 h. Finally, the post-culture supernatants were collected and stored at −80 °C until the ELISA assays were performed to determine cytokine levels.

2.4. Preparation of Conditioned Media from RAW 264.7 Macrophages

The RAW 264.7 cells were seeded in Petri dishes at a density of 3×10^6 cells/plate in 8 mL of DMEM medium supplemented with 10% FBS and then pre-incubated for 24 h. For the induction of ET, these RAW 264.7 cells were cultured in a media containing 100 ng/mL of LPS and 1% FBS for 24 h, followed by a wash with PBS and stimulation with 100 ng/mL of LPS for another 24 h. Non-tolerant macrophages were cultured in the same condition in the absence of LPS. Finally, the cell culture supernatants were collected and centrifuged (2000× g, 5 min.) to remove the cell debris. The conditioned media (CM) from non-tolerant macrophages (CM_{NT}) and tolerant macrophages (CM_{ET}) were stored at −80 °C until needed in further experiments.

2.5. Cell Viability Assay

For the evaluation of 4T1 and CT26 cancer cell viability after stimulation with different concentrations of CM_{NT} and CM_{ET}, a 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma Aldrich) assay was performed. First, the cells were seeded into 96-well plates at a density of 2×10^3 cells/well and pre-incubated for 24 h in the DMEM containing 10% FBS. The cells were then stimulated with CM^{NT} or CM^{ET} in 1% FBS/DMEM media at 10, 25, 50, and 75% concentration for 24 h and 48 h. After the treatment, the cells were washed with PBS and incubated in a red phenol-free culture medium containing 0.5 mg/mL MTT solution for 3 h at 37 °C. Then, to dissolve the formazan crystals, the media was aspirated, 100 µL of DMSO was added, and the plate was placed on an orbital shaker and mixed horizontally for 15 min. Lastly, the optical density was measured at the wavelength of 570 nm (with a reference wavelength of 630 nm) using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). The viability of conditioned media-treated cells is shown as the percentage of cells incubated in a complete DMEM medium containing 10, 25, 50, or 75% of culture medium supplemented with 1% FBS.

2.6. Colony Formation Assay

The effect of the CM_{NT} and CM_{ET} on the colony formation capacity of both the 4T1 and CT26 cells was evaluated by seeding 1×10^5 cells/well in a 12-well plate and incubating overnight. The cells were then treated with different concentrations of CM_{NT} and CM_{ET} (10–50%) for 48 h. After the stimulation, the media were removed, and the cells were washed with PBS and trypsinized. These cells were then seeded at a density of 200 (4T1 cells) or 400 (CT26 cells) cells/well and maintained in the normal culture media supplemented with 10% FBS for 5 to 7 days, respectively. The fresh media were added every 2–3 days. At the end of the time point, the colonies were fixed with 100% v/v methanol for 20 min, washed once with distilled water, and then stained with 0.5% crystal violet

(the solution prepared in 25% v/v methanol) solution for 20 min. After the staining, the images of the colonies from each cell line were obtained and analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA) using the colony counter plugin.

2.7. Scratch Assay

To evaluate the influence of the CM_{NT} and CM_{ET} on 4T1 and CT 26 cancer cell motility, the cells were seeded at a concentration of 3×10^4 cells/well in a 24-well plate and then cultured in DMEM containing 10% FBS until 90% confluency was obtained. Scratch was made mechanically using a 10 µL pipette tip, followed by the removal of the media, and immediately washed with PBS to remove any unbound cells. The cells were stimulated with different concentrations (10–50%) of the conditioned media CM_{NT} and CM_{ET} for 20 to 24 h. Images of the scratch closure were obtained at 0 h and 20 h for 4T1 cells and at 0 h and 24 h for CT26 cells with an inverted Leica Dmi1 microscope with a digital camera (Wetzlar, Germany). These images were analyzed with the ImageJ software (National Institutes of Health, Bethesda, MD, USA) by calculating the distance between the edges of the scratch. The percentage of scratch closure was calculated by the following formula:

$$Scratch\ closure\ (\%) = (D0 - D[20\ or\ 24])/D0 \times 100$$

In this equation, $D0$ represents the distance between the edges of the wound at 0 h, and D [20 or 24] is the distance at 20 and 24 h, respectively.

2.8. 3D Spheroidal Assay

To examine the effect of the CM_{NT} and CM_{ET} on the process of spheroid formation of 4T1 and CT26 cancer cells, the spheroids were generated using the hanging drop method. The 4T1 and CT26 cells at a density of 3×10^4 were cultivated on the upper lips of Petri plates in a volume of 30 µL of the different concentrations of the CM (10–50%), and cancer cell spheroids cultured in the different concentrations of the non-treated culture media (10–50%) was used as a positive control. The spheroids were maintained in the conditioned media for 48 h, and then their images were obtained using a simple microscope using 100 times magnification. These images were analyzed with the ImageJ software (National Institutes of Health, Bethesda, MD, USA) by calculating the area observed of each spheroid.

2.9. Co-Cultures of Cancer Cells and RAW 264.7 Macrophages

To elucidate the role of the endotoxin-tolerant microenvironment in tumourigenesis, the 4T1 and CT26 cancer cells were cultured either independently or co-cultured with Mo_{NT} and Mo_{ET} cells. Five experimental conditions were evaluated, i.e., Mo_{NT}, Mo_{ET}, or 4T1/CT26 cancer cells cultured independently or 4T1/CT26 cells co-cultured with Mo_{NT} or Mo_{ET}. Co-culture of 4T1/CT26 cells and Mo_{NT} treated with LPS for 24 h was used as a positive control. In a common 6-well plate, the Mo_{NT} or Mo_{ET} cells were seeded at a concentration of 1×10^5 cells/well together with the 4T1 or CT26 cells, which were seeded at a concentration of 3×10^5 cells/well. The cells were then maintained in DMEM media supplemented with 1% FBS for 24 h. After the incubation, the cell culture supernatants were collected and stored at -80 °C until the evaluation of cytokine levels (TNFα and IL-6) by ELISA technique.

2.10. Analysis of Cytokine Production

The protein level of pro-inflammatory cytokines (TNFα and IL-6) in the Mo_{NT}, Mo_{ET}, and Mo_{LPS} post-culture and the co-culture experiment supernatants were analyzed with ELISA kits from R & D Systems (Minneapolis, MN, USA) according to the manufacturer's protocols. Colourimetric changes in the assays were detected with Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). The same method was also used to assess the level of cytokines produced by the cancer cells.

2.11. Flow Cytometry Analysis

To identify the M1/M2 polarization phenotype of the Mo_{ET}, flow cytometry was performed by staining these macrophages with fluorescein isothiocyanate (FITC)-labelled anti-CD80 MoAb and allophycocyanin (APC)-conjugated anti-CD163 MoAb (Sony Biotechnology Inc., San Jose, CA, USA). In this analysis, two experimental conditions were maintained, i.e., Mo_{NT} (control) and Mo_{ET}. Cells were seeded at a density of 1×10^6 cells in small flasks and pre-incubated in DMEM containing 10% FBS overnight. The culture media was removed the next day, and the cells were gently washed with PBS, followed by the first stimulation with LPS 100 ng/mL for 24 h. After 24 h, the cells were again washed with PBS and stimulated for another 24 h. At the end of the stimulation, the media was removed, the monolayer of the cells was washed once with ice-cold PBS, then again by adding another 5 mL of cold PBS, and cells were detached by gentle scraping. These cells were collected and washed with PBS three times (1300 rpm, 5 min) and incubated with Mouse Seroblock FcR (Bio-Rad, Hercules, CA, USA) for 10 min. After the initial incubation, staining with anti-CD80 and anti-CD163 antibodies was done in the dark for 30 min. To remove unbounded antibodies, three washes with PBS were performed, followed by a final resuspension in 500 µL of PBS. A BriCyte E6 flow cytometer (Mindray, Shenzhen, China) was used to perform the analysis.

2.12. Statistical Analysis

Statistical comparisons were conducted with the GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA). All of the data are expressed as the mean ± standard error of the mean (SEM) of three independent experiments and analyzed using one-way ANOVA followed by Bonferroni's multiple comparisons test with the level of significance set at $p < 0.05$.

3. Results

3.1. Endotoxin-Tolerant Macrophages Displayed Decreased Expression of TNFα and IL-6

An indicator of endotoxin tolerance (ET) is the decreased production of pro-inflammatory cytokines such as TNFα and IL-6 from macrophages during continued endotoxin exposure. Therefore, to determine the induction of ET, in this study, we evaluated the level of protein expression of TNFα and IL-6 in macrophages treated with a dose of 100 ng/mL of LPS based on the previous literature and preliminary data produced in our laboratory [21].

It is well-established that macrophages stimulated with a single dose of LPS exhibit an increase in pro-inflammatory cytokines production, which was confirmed in our results showing the upregulation of TNFα and IL-6 in the LPS-stimulated cells in comparison with non-treated cells $p < 0.001$ (Figure 1A,B, respectively). However, the continued stimulation of the cells with LPS attenuated this effect since the reduced production of both cytokines was observed in the LPS-tolerated macrophages compared with the cells stimulated with LPS only once ($p < 0.001$).

3.2. Conditioned Media Derived from Endotoxin-Tolerant Macrophages Increases the Survival Capacity of Cancer Cells

To study the effects of macrophage-released mediators on 4T1 and CT26 cell viability, the cancer cells were cultured in different concentrations of RAW 264.7 cell-conditioned media (CM) derived from non-treated (CM_{NT}) and LPS-tolerated cells (CM_{ET}) (10%, 25%, 50% and 75%). The dose-dependent toxicity of both CM_{ET} and CM_{NT} was observed for 4T1 and CT26 cell lines (Figure 2). However, this cytotoxic effect was stronger for the cells cultivated in CM_{NT} than in CM_{ET}, which was observed for CM at a concentration ranging from 25–75% after 24 (Figure 2A,C) and 48 h (Figure 2B,D) of incubation.

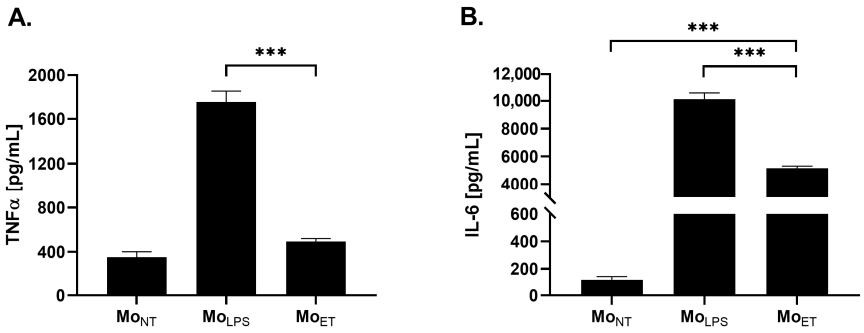

Figure 1. The concentration of TNFα and IL-6 produced by the following groups of RAW 264.7 cells: non-treated macrophages (Mo_{NT}), LPS-tolerated macrophages (Mo_{ET}), and macrophages treated with LPS only once (Mo_{LPS}). Cells were stimulated either once for 24 h, Mo_{LPS} (24 h) or twice for 24 h, Mo_{ET} (48 h) with LPS at a concentration of 100 ng/mL. The amount of TNFα (**A**) and IL-6 (**B**) was assessed by ELISA. The data are shown as the means ± SEM of three independent experiments with three wells for each condition. Asterisks denote a significant difference between Mo_{NT} and Mo_{LPS} (*** $p < 0.001$).

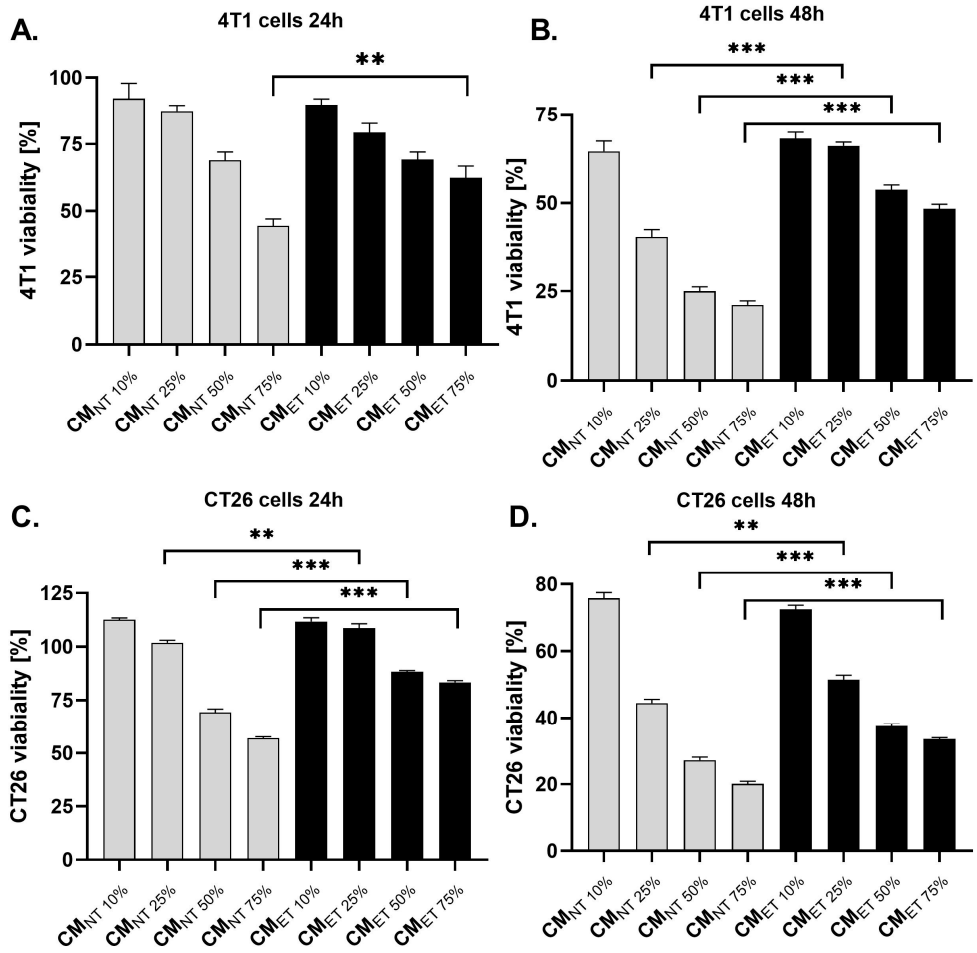

Figure 2. Cell viability of breast cancer 4T1 cells (**A**,**B**) and colon cancer CT26 (**C**,**D**) cells stimulated with different concentrations (10–75%) of conditioned media (CM) derived from endotoxin-tolerant macrophages (CM_{ET}) and non-treated macrophages (CM_{NT}) for 24 and 48 h. Cell viability was assessed by the MTT colourimetric method. The results are expressed as a percentage of control non-stimulated cells (which is represented as 100%). The data are shown as the mean ± SEM of three independent experiments with six wells for each condition. Asterisks denote significant differences between the respective concentrations of CM_{ET} and CM_{NT} (*** $p < 0.001$, ** $p < 0.01$).

3.3. Exposure of the Cancer Cells to the Conditioned Media Derived from the Tolerant Macrophages Influences Their Clonogenic Potential

To evaluate the effect of CM_{NT} and CM_{ET} on the colony-forming capacity (the capability of a single cancer cell to grow into a large colony through clonal expansion), we performed colony-formation assays in 4T1 breast cancer and the CT26 colon cancer cell lines. Both the cancer cell lines were treated with different concentrations of CM (10–50%) for 48 h before the seeding and culturing them in DMEM supplemented with 10% FBS.

After 7 days, the 4T1 cells treated with the $CM_{ET\ 50\%}$ showed a significant increase in the number of colonies when compared to the cells treated with $CM_{NT\ 50\%}$ ($p < 0.001$) (Figure 3A,B). However, in the case of other concentrations of the CM_{NT} and CM_{ET}, more colonies were observed in $CM_{NT\ 10\%}$ when compared to $CM_{ET\ 10\%}$ ($p < 0.05$), and no significant difference was observed in the case of CM at a concentration of 25%.

Comparatively, the CT26 cells, after 5 days of culture, showed an increased number of colonies in the case of $CM_{ET\ 10\%}$ and $CM_{ET\ 25\%}$ when compared to the $CM_{NT\ 10\%}$ and $CM_{NT\ 25\%}$ ($p < 0.01$) (Figure 3C,D). Moreover, all the concentrations of $CM_{ET\ (10–50\%)}$ also showed a significant increase in the number of colonies when compared to the control cells, which were treated with DMEM containing 1% FBS for 48 h ($p < 0.01$).

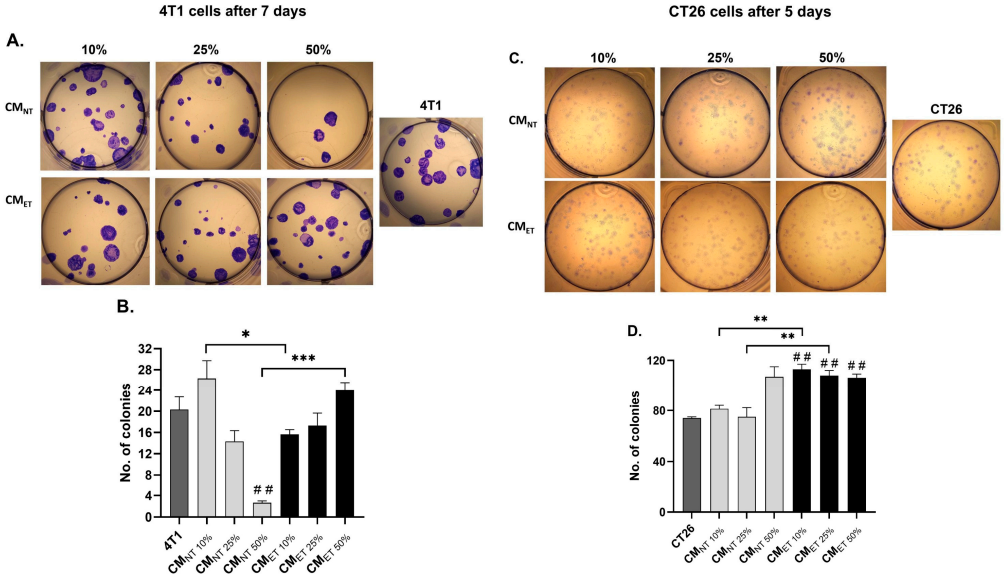

Figure 3. The number of colonies of 4T1 (**A**,**B**) and CT26 (**C**,**D**) cancer cells counted after 7 and 5 days of culture, respectively. Before seeding, the cells were cultivated in the conditioned media (CM) derived from endotoxin-tolerant macrophages $CM_{ET\ 10–50\%}$ and non-treated macrophages $CM_{NT\ 10–50\%}$ for 48 h. The data are shown as the mean ± SEM of three independent experiments with 3 wells for each condition. Asterisks denote a significant difference between the cells cultured in CM derived from non-treated cells (Mo_{NT}) and LPS-tolerated cells (Mo_{ET}) (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$). Hashes denote the significant difference between CMET and the control cells (## $p < 0.01$).

3.4. Conditioned Media Derived from Endotoxin-Tolerant Macrophages Increase Cancer Cell Motility

To further study the effect of the RAW 264.7 cell-conditioned media on the migration capacity of the 4T1 and CT26 cell lines, scratch assays were performed. The 4T1 and CT26 cells were cultured in CM_{ET} and CM_{NT} at different concentrations (10, 25, and 50%) for 20 and 24 h, respectively. The results showed that both 4T1 (Figure 4A,B) and CT26 cells (Figure 4C,D) cultured in $CM_{ET\ 10–50\%}$ demonstrated a significantly higher rate of scratch closure when compared to $CM_{NT\ 10\ to\ 50\%}$ ($p < 0.001$). Interestingly, all tested concentrations of CM_{ET} also increased the cell motility when compared with the 4T1 and CT26 control cells. In contrast, the motility of 4T1 cancer cells cultivated in all tested concentrations of

CM_{NT} was at the same level as the control cells, and in the case of CT26 cells, the CM_{NT} 10% showed increased cell motility when compared to the control ($p < 0.001$).

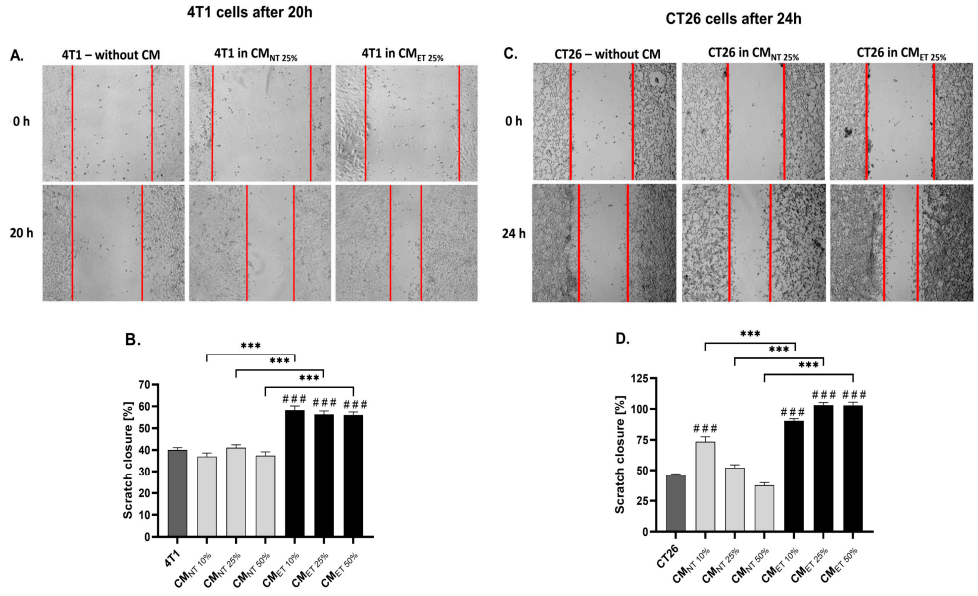

Figure 4. The motility of 4T1 (**A**,**B**) and CT26 (**C**,**D**) cancer cells cultured in the conditioned media (CM) derived from endotoxin-tolerant macrophages (CM_{ET}) and non-treated macrophages (CM_{NT}) at a concentration of 10–50% for 20 h and 24 h, respectively. Cell migration was assessed with a scratch assay. (**A**,**C**) present the representative images of the cells treated with CM at a concentration of 25%. (**B**,**D**) show the quantitative scratch closure measured between 0 h and 20 or 24 h (%) using ImageJ software. (*** $p < 0.001$). The data are shown as the mean ± SEM of three independent experiments with 3 wells for each condition. Asterisks denote a significant difference between the cells cultured in CM_{NT} and CM_{ET} (*** $p < 0.001$). Hashes denote the significant difference between MoET and the control cells (### $p < 0.001$).

3.5. Conditioned Media from the Tolerant Macrophages Can Affect the Survivability of the Cancer Cells at the 3D Level

After the evaluation of the effects of the CM_{ET} on the cancer cell monolayers, we wanted to understand whether the CM can also affect the growth of the cancer cell spheroids.

We observed that breast cancer cell spheroids treated with $CM_{ET\ 10–50\%}$ showed an increased area when compared to the $CM_{NT\ 10–50\%}$ ($p < 0.001$) and $4T1_{10–50\%}$ ($p < 0.001$). Among tested concentrations of CM, the $CM_{ET\ 50\%}$ showed a significant increase in the spheroidal area after 48 h when compared to the CMNT 50%. Importantly, the cancer cell spheroids treated with $CM_{ET\ 10–50\%}$ showed an evidently significant increase in the area when compared to the unstimulated 4T1 cells at all concentrations (10–50%). However, this effect was reversed in the case of colon cancer cells, as the cancer cell spheroids stimulated with $CM_{NT\ 25–50\%}$ showed an increased growth when compared to $CM_{ET\ 25–50\%}$. Also, the untreated CT26 control cancer cell spheroids appeared to have significantly larger spheroidal area compared to spheroids stimulated with $CM_{NT\ 10–50\%}$ and $CM_{ET\ 10–50\%}$ conditions ($p < 0.001$) (Figure 5).

3.6. Endotoxin Tolerance Affects Crosstalk between Macrophages and Cancer Cells by Downregulating Expression of IL-6 and TNFα

In separate experiments, we co-cultured 4T1 and CT26 cells with Mo_{ET}, Mo_{NT} or Mo_{LPS} cells to determine if direct contact with cancer cells affects endotoxin-related macrophage reprogramming of pro-inflammatory cytokines expression. The 24 h lasting co-culture of 4T1 and CT26 cells with macrophages (Mo_{ET} or Mo_{NT} or Mo_{LPS}) resulted in strong stimulation of IL-6 production when compared with 4T1 and CT26 cultured alone (for both, $p < 0.001$). Additionally, we showed that ET conditions attenuated the

IL-6 expression in co-cultured cells (4T1 or CT26 and Mo_{ET}) compared to the IL-6 level measured in the co-culture of cancer cells with macrophages exposed previously to LPS once (i.e., for 4T1 and CT26, $p < 0.001$) (Figure 6A,C).

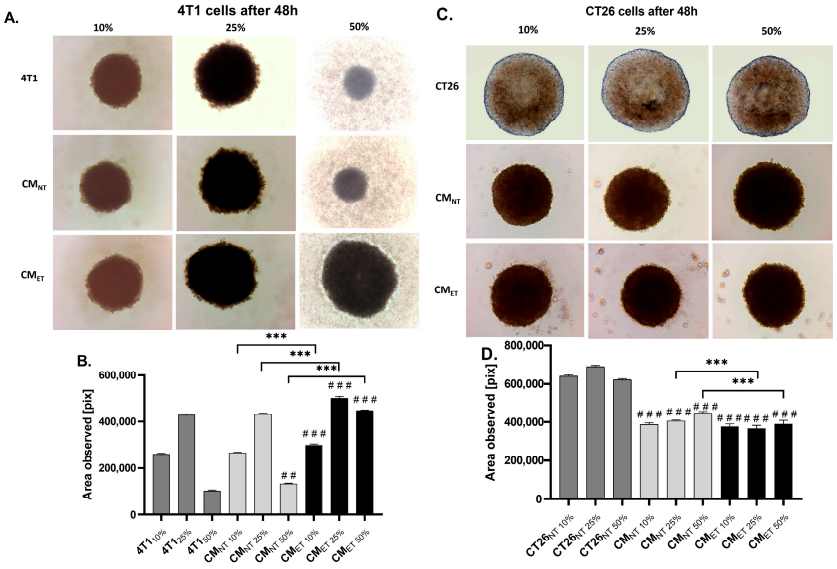

Figure 5. The area of 3D spheroids formed by the breast cancer 4T1 cells (**A**,**B**) and colon cancer CT26 cells (**C**,**D**) in the conditioned media (CM) derived from endotoxin-tolerant macrophages (CM_{ET}), non-treated macrophages (CM_{NT}), and normal media at a concentration of 10–50% after 48 h. Asterisks show a significant difference between the cells cultured in CM_{ET} and CM_{NT} (*** $p < 0.001$). Hashes denote the significant difference between Mo_{ET} and the control cells (### $p < 0.001$; ## $p < 0.01$).

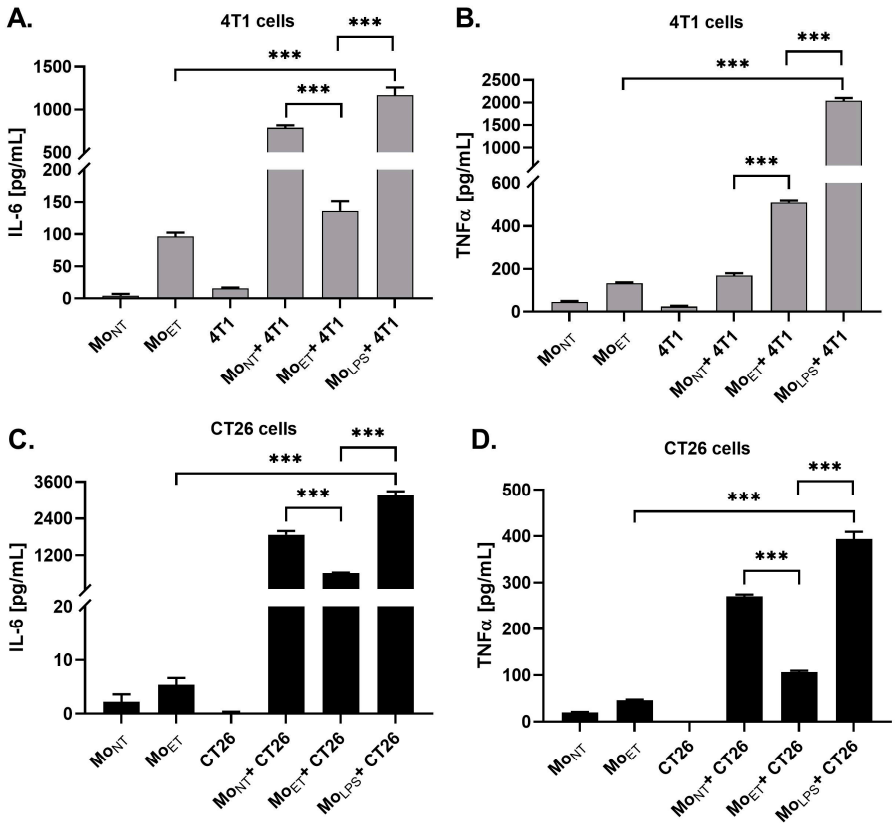

Figure 6. Protein concentration of IL-6 (**A**,**C**) and TNFα (**B**,**D**) produced in a monoculture of Mo_{NT}, Mo_{ET}, 4T1 or CT26, and co-culture of 4T1 or CT26 cells with Mo_{ET}, Mo_{NT}, and Mo_{LPS} that was determined by

ELISA assays. Asterisks denote significant differences between the co-culture conditions of 4T1 or CT26 cells with Mo_{ET} and Mo_{NT} (*** $p < 0.001$). The data are shown as the mean ± SEM of three independent experiments with three wells for each condition.

We also observed a significant increase in TNFα production in both the 4T1 and CT26 cells co-cultured with Mo_{LPS}, whereas prolonged stimulation with LPS that induced ET attenuated this effect. Moreover, the expression level of TNFα in the co-culture of 4T1 and Mo_{NT} was similar to that of the monoculture of Mo_{ET} and was significantly lower when compared with 4T1 cells co-cultured with Mo_{ET} ($p < 0.001$). In the case of CT26 cells, a similar pattern of expression was observed in almost all the conditions of co-culture when compared to 4T1, except in the case of CT26 cells co-cultured with Mo_{ET}, which showed a significantly low expression of TNFα when compared to Mo_{NT}+ 4T1 ($p < 0.001$) (Figure 6B,D).

3.7. Macrophages Maintain the M1 Phenotype Even after Prolonged Exposure to LPS

To determine whether endotoxin tolerance (ET) influences the polarization of macrophages, we assessed the expression of M1 (CD80) and M2 (CD163) polarization markers. We observed that the Mo_{LPS} and Mo_{ET} showed a statistically significant increase in the number of cells with an M1 phenotype compared with Mo_{NT} ($p < 0.001$); however, endotoxin tolerance reduced the number of M1 cells compared with Mo_{LPS} ($p < 0.01$). Additionally, there was a small but statistically significant increase in the number of cells with the M2 phenotype ($p < 0.01$ for Mo_{LPS} and $p < 0.05$ for Mo_{ET}, respectively) (Figure 7).

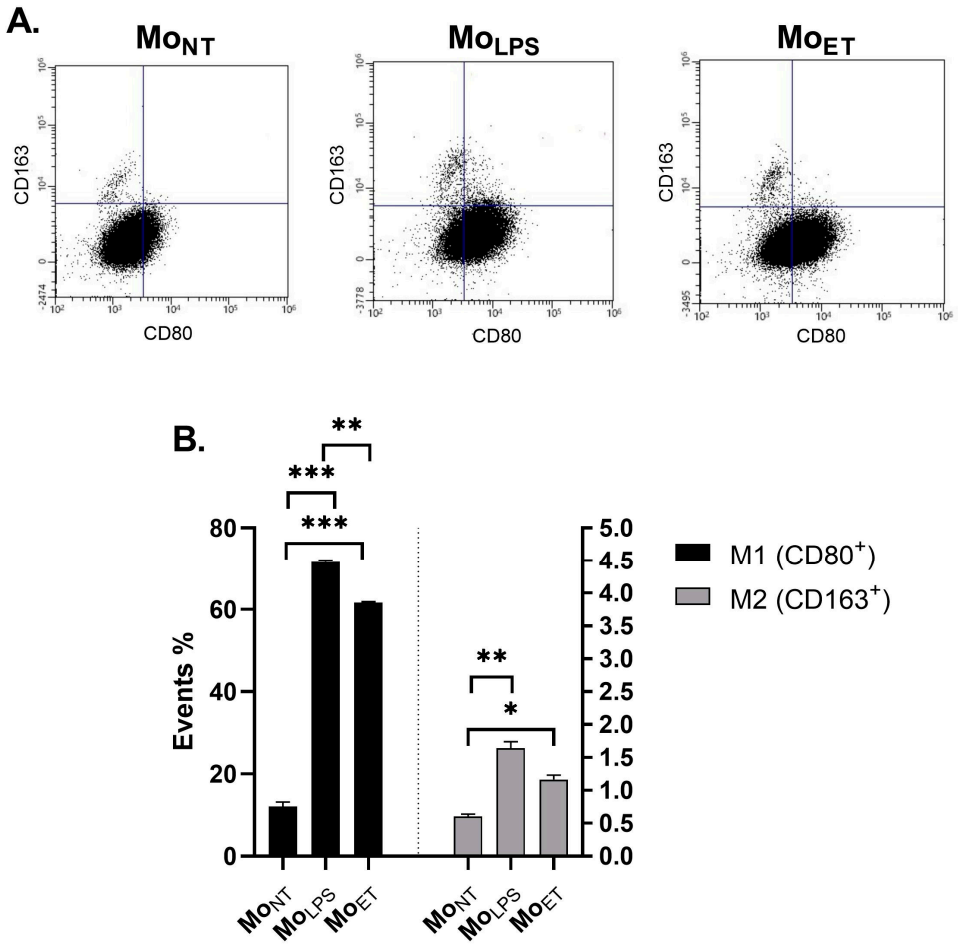

Figure 7. Characterization of the macrophage phenotype in LPS-tolerated RAW264.7 cell. The percentage of M1 cells is plotted on the left *Y*-axis and M2 cells on the right *Y*-axis, respectively. To induce endotoxin

tolerance, the cells were stimulated twice with 100 ng/mL of LPS. The data are shown as the mean ± SEM of three independent experiments. Asterisks denote a significant difference in individual groups of cells (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

4. Discussion

In the current study, we revealed that endotoxin tolerance (ET) is a novel factor that may contribute to cancer progression. ET is an adaptive phenomenon of the immune system that can occur from repeated exposure to endotoxins like LPS. Interestingly, though this immunosuppressive characteristic is considered to be a protective mechanism against endotoxin, it is identified in severe septic patients [22]. Furthermore, it has been reported that LPS-tolerant human monocytes are also hyporeactive to heat-killed *Streptococcus pyogenes*, *Staphylococcus aureus*, and *zymosan* [23]. Similarly, pre-treatment of macrophages and monocytes with other Toll-like receptors (TLRs) ligands, such as cholera toxin B chain [24] mycobacterial components such as arabinose-capped lipoarabinomannan and soluble tuberculosis factor, can lead to cross-tolerance of LPS [25]. Since experimental evidence from both animal models and clinical trials about the endotoxin utility in anti-cancer treatments has not been consistent [26–28], we suppose that it may have been associated with the development of endotoxin tolerance in some experimental settings. Thus, it seems plausible that ET may increase the probability of occurrence of unfavourable outcomes not only in infectious diseases but also in cancer.

ET is characterized by a decrease in the production of pro-inflammatory cytokines such as TNFα [29,30] and IL-6 [29]. We assessed two models of tolerance in macrophages, i.e., first stimulation with LPS (in a dose of 100 ng/mL) for 24 h, followed by a second stimulation for either 6 h or 24 h. Our data proved that the second scheme (24 h + 24 h) of cell stimulation is optimal since we observed significant changes in the expression of pro-inflammatory factors (e.g., TNF-α, IL-6). This model is similar to the model used in previous studies by Nomura et al. (2000) and Xiang et al. (2009) [31,32]. Additionally, we assessed the expression of IL-1β, which in some research is also considered as the marker of ET condition [33]; however, similarly to Erroi et al. (1993) who observed only a moderate decrease in IL-1β expression in spleen homogenates of LPS tolerant mice [34], we did not notice significant changes in the expression of this cytokine between macrophages treated only once with LPS and endotoxin-tolerant macrophages.

In our previous studies, we observed many times that macrophages are key cells involved in response to LPS [21,35]. It is commonly accepted that they may exhibit anti-cancer or pro-cancer properties, depending on their M1 and M2 phenotypes that are associated with the production of different cytokines. Although we observed a decreased expression of pro-inflammatory cytokines in Mo_{ET}, these cells were polarized toward the M1 phenotype. Several studies reported that the M2 differentiation state resembles ET macrophages phenotypically [36,37] as it showed that induction of tolerance by LPS exposure of murine and human macrophages induced gene expression profiles that were consistent with M2 polarization. Nonetheless, the authors also reported that LPS re-stimulation of LPS-pre-treated macrophages resulted in sustained, rather than limited, expression of the assessed chemokines. Our results are in part parallel with Pena et al. (2011) [37], who reported that the macrophages pre-treated with LPS, which induced endotoxin tolerance, exhibited no significant change in the expression of the M2 marker CD206 despite developing tolerance with respect to pro-inflammatory cytokines. Moreover, these data are also in agreement with the findings of Rajaiah et al. (2013), who noted that the polarization of macrophages to the M2 phenotype is not clearly associated with ET [38]. Therefore, our data seem to confirm the hypothesis that the signalling pathways leading to endotoxin tolerance and differentiation of M2 are dissociable.

Although ET has been thought of as a protective mechanism against septic shock [39], the relationships between endotoxin sensitivity and carcinogenesis and protection against cancer are poorly understood. Therefore, using an experimental approach helpful in mim-

icking the microenvironmental conditions of the tumour, in this work, we studied the effect of endotoxin-tolerant macrophages on two different cancers, i.e., breast cancer cells and colon cancer cells.

Our results of the viability test demonstrated that conditioned media obtained from endotoxin-tolerant macrophages exhibit a diminished dose-dependent cytotoxic effect on breast cancer cells and colon cancer cells as compared to conditioned media obtained from non-tolerant macrophages. Furthermore, these data are in accordance with our observation of colony-forming capacity. This test showed that exposure to the conditioned media obtained from the tolerant macrophages triggers an increase in the number of colonies when compared to the controls at the highest concentration in the case of both cell lines. It means that factors released by endotoxin-tolerant macrophages stimulate each single cancer cell to grow into a large colony through clonal expansion.

Apart from characteristics proving the increased potential of cancer cells to proliferate in the environment created by Mo_{ET}, we analyzed the capacity of these cells to move. The migration of the cancer cells is a pivotal step of metastasis, which is the primary cause of death for patients with solid tumours [40,41]. Similar to previous tests, we observed an enhanced migration of breast cancer and colon cancer cells when stimulated by a conditioned medium from endotoxin-tolerant macrophages in comparison to a conditioned medium from control, non-tolerant macrophages. To the best of our knowledge, the effect of conditioned media derived from endotoxin-tolerant macrophages on cancer cell motility has not been investigated so far.

To evaluate the effect of the conditioned media from endotoxin-tolerant macrophages on solid tumours, we used a 3D spheroidal cancer cell model. It is a critical issue since tumours exhibit greater resistance to treatment compared to cancer cells grown as a single layer. This occurs due to a phenomenon called multicellular resistance, which is caused by factors such as cell–cell interactions, cell–matrix interactions, and the three-dimensional structure of tissues [42,43]. After 48 h, we observed a major increase in the spheroidal area in the case of breast cells at the highest concentration of the conditioned media from tolerant macrophages, while the inverse effect was observed in the case of colon cancer. It is indicative of the variance of the effect towards types of cancer that differ with LPS exposure. As Zhu et al., 2016 reported that the LPS was responsible for promoting migration and invasion of colon cancer through VEGF-C activation but not proliferation [44]. A reason behind this can be the continuous exposure of the colon cancer cells to LPS from the intestinal bacteria, unlike breast cancer cells. These data correlate with the various literature [6,45,46], which states that the higher infiltration of the macrophages can improve the survival capacity in the case of colorectal cancer patients. Thus, our results suggest that the factors released by endotoxin-tolerant macrophages may increase at least breast cancer cell growth and survival, while colon cancer reacts differently.

Since we observed that factors released by endotoxin-tolerant macrophages may affect cancer cells, we were further interested in whether contact with cancer cells affects endotoxin-related macrophage reprogramming of pro-inflammatory cytokines expression. Therefore, we studied the direct cell-to-cell crosstalk between endotoxin-tolerant macrophages and breast cancer cells or colon cancer cells. We found that in the co-culture of endotoxin-tolerant macrophages and cancer cells, the lower level of both IL-6 and TNFα cytokines was still observed. It proves that direct crosstalk between macrophages and cancer cells does not eliminate this effect in macrophages. In particular, in the co-culture of endotoxin-tolerant macrophages and breast cancer cells, the IL-6 expression appeared to be at a level comparable to that of the monoculture of endotoxin-tolerant macrophages.

Our research is grounded in the hypothesis that the immunosuppression triggered by endotoxin tolerance (ET) in macrophages, a crucial component of an organism's immune system, as well as within the tumour microenvironment, may foster conditions that do not inherently hinder the progression of cancer. Our approach involves investigating the impact of these tolerant macrophages on various aspects of cancer cells, such as viability, motility, clonogenic potential, and spheroid formation. Through this examination, we aim

to substantiate the influence of ET on cancer development. By analyzing these findings, we propose a potential correlation between immune system impairment caused by ET and the onset of cancer (Figure 8). However, due to the intricate nature of ET as a multifaceted mechanism, more comprehensive investigations are needed. Consequently, our future objective is to conduct further in-depth studies to unravel the intricate relationship between cancer and endotoxin tolerance by studying the effect of ET on cancer in in vivo and also by examining the factors that are being secreted by these endotoxin-tolerant macrophages. Crucially, it is essential to examine how these factors impact various types of tumour models. Additionally, it is valuable to explore other aspects of cancer cell migration when influenced by ET macrophages in depth.

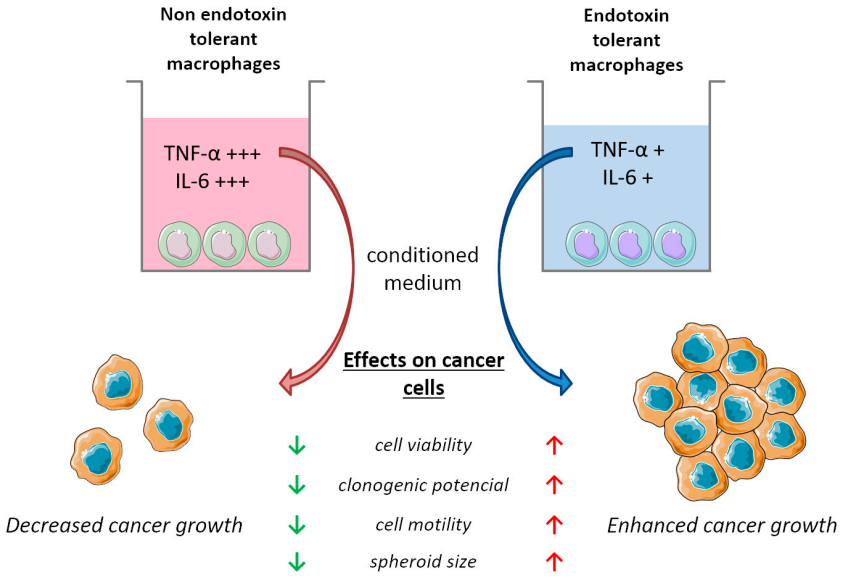

Figure 8. Effect of endotoxin tolerance on cancer cell growth in vitro. Endotoxin-tolerant macrophages release low levels of TNF-α and IL-6 into the medium. Conditioned medium collected from these macrophages enhances cancer aggressiveness measured by cell viability, clonogenic potential, cell motility, and spheroid size. Up and down arrows indicate the direction of effect in response to each conditioned medium. (Partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.)

5. Conclusions

In this study, we confirmed macrophages' susceptibility to endotoxin that changes over time, and finally, a state of endotoxin tolerance develops. Importantly, we proved for the first time that endotoxin-tolerant macrophages are reprogrammed and release factors that can affect cancer development and behaviour. Although there are many studies showing that macrophages may stimulate cancer development [47], our experiments proved that endotoxin-tolerant macrophages stimulate even more cancer-friendly conditions. Thus, in experiments on cancer cells and in clinical trials with endotoxin, it is important to monitor whether a state of endotoxin tolerance has developed. If so, it should be reversed to prevent a cancer-conductive environment. Furthermore, it is likely that many attempts to use endotoxin to cure cancer were inconclusive due to not considering the possibility of developing a state of endotoxin tolerance.

We believe that exploring the reprogramming mechanism of endotoxin tolerance may be an important factor to consider in achieving better outcomes in cancer patients. Therefore, further experiments are needed to fully understand the molecular mechanisms underlying the pro-cancer properties of ET.

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resources, S.W. and A.D.; data curation, K.R.; writing—original draft preparation, K.R.; writing—review and editing, K.R., H.M.K., T.J., S.W., J.S., B.M. and A.D.; visualization, K.R., T.J., J.S. and B.M.; supervision, S.W.; project administration, S.W.; funding acquisition, S.W. and A.D. All authors have read and agreed to the published version of the manuscript.

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9.2. Article #2

Roy, K.; Jędrzejewski, T.; Sobocińska, J.; Spisz, P.; Maciejewski, B.; Hövelmeyer, N.; Passeri, B.; Wrotek, S. Divergent impact of endotoxin priming and endotoxin tolerance on macrophage responses to cancer cells. *Cellular Immunology* 411-412, 104934, **2025**. https://doi.org/10.1016/j.cellimm.2025.104934.

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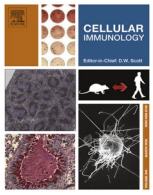

Divergent impact of endotoxin priming and endotoxin tolerance on macrophage responses to cancer cells

Konkonika Roy [a,*], Tomasz Jędrzejewski [a], Justyna Sobocińska [a], Paulina Spisz [a], Bartosz Maciejewski [a], Nadine Hövelmeyer [b,c], Benedetta Passeri [d], Sylwia Wrotek [a]

[a] Department of Immunology, Faculty of Biology and Veterinary Sciences, Nicolaus Copernicus University, 1 Lwowska Street, Torun, 87-100, Poland
[b] Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany
[c] Research Centre for Immunotherapy (FZI), University Medical Center of the Johannes-Gutenberg University Mainz, Mainz, Germany
[d] Pathology Unit, Department of Veterinary Science, University of Parma, Strada del Taglio 10, 43126 Parma, Italy

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ABSTRACT

Endotoxin tolerance (ET) is an adaptive phenomenon that arises from the repeated exposure of immune cells, such as macrophages, to endotoxins like lipopolysaccharide (LPS). Initially, when macrophages are activated by LPS, they produce inflammatory mediators that drive the primary immune response. However, this response is significantly diminished during the establishment of ET, creating an immunosuppressive environment. This environment can facilitate the development and progression of malignant conditions, including cancer.

Our research focused on the interactions between immune cells and the tumor microenvironment under ET conditions. Through comprehensive *in vivo* and *in vitro* studies employing various research techniques, we have demonstrated that interactions between endotoxin-tolerant macrophages (Mo_{ET}) and cancer cells contribute to a pro-tumorigenic condition. Notably, we observed that Mo_{ET} adapt a pro-tumorigenic, immunosuppressive M2 phenotype (CD163 expression). These macrophages involves distinct metabolic pathways, not depending solely on glycolysis and oxidative phosphorylation. Furthermore, our *in vivo* findings revealed macrophage infiltration within tumors under both ET and non-ET conditions, highlighting the suppressed immune landscape in the presence of ET. These findings suggest that ET plays a pivotal role in shaping tumor-associated immune responses and that targeting ET pathways could offer a novel and promising therapeutic approach for cancer treatment.

1. Introduction

It is known that when primed with endotoxin, macrophages are more reactive and produce higher levels of inflammatory mediators upon subsequent stimulation, whereas endotoxin-tolerant macrophages have a blunted response and lower production of these mediators [1]. The effect of endotoxin-treated macrophages on cancer development has been explored [2,3], with interesting findings showing that endotoxin-tolerant macrophages create favourable conditions for tumor progression. It is much less known however, how such endotoxin-tolerant macrophages respond to contact with cancer cells.

One of the key effects of endotoxin tolerance is a shift in macrophage polarization. This process involves macrophages adapting to different functional states in response to environmental signals, including pathogen-associated molecular patterns (PAMPs) like bacterial endotoxins [2]. These states are broadly categorized into two main phenotypes: M1 (classically activated) [3,4] and M2 (alternatively activated) macrophages [5,6]. Each phenotype plays a distinct role in immune responses, inflammation and tissue homeostasis. Recognizing macrophage polarization is essential because it provides insight into the immune system's balance between pro-inflammatory and anti-inflammatory responses. This understanding is critical for developing targeted therapies for various conditions, including infections, chronic inflammatory diseases, and cancer.

To identify macrophage polarization, various markers and factors indicative of different macrophage phenotypes can be assessed. These include cytokines, enzymes, surface markers, and the production of reactive oxygen species (ROS). It is well known that interleukin (IL) 6, tumor necrosis factor (TNF) α and IL-1β are produced in high levels by M1 macrophages and are key indicators of the pro-inflammatory state [7]. They drive inflammation and help recruit other immune cells to sites of infection [8] or injury [9]. Enzymes such as inducible nitric oxide

* Corresponding author.
E-mail addresses: konkonika.roy@doktorant.umk.pl (K. Roy), wrotek@umk.pl (S. Wrotek).

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synthase (iNOS) [10,11] and cyclooxygenase-2 (COX-2) [12] are directly involved in the metabolic activities and responses of macrophages. For instance, iNOS catalyzes nitric oxide production, which plays a role in pathogen killing and inflammation [13], but can also contribute to tissue damage if overproduced, while COX-2 generates pro-inflammatory prostaglandins [14,15]. ROS, generated by various sources including NADPH oxidase, play a role in pathogen destruction and tissue damage, further amplifying the inflammatory response [16,17]. These enzymatic activities provide insights into the functional roles of macrophages in inflammation and tissue repair that surface markers alone cannot reveal. In contrast to the M1, M2 macrophages are characterized by elevated expression of arginase-1 (Arg-1), mannose receptor (CD206), and the anti-inflammatory cytokine IL-10. These macrophages are primarily activated by cytokines and factors such as IL-4, IL-13, IL-10, immune complexes, hormones, or agonists of adenosine A2A receptors (A2AR). M2 macrophages play a crucial role in tissue repair, angiogenesis, and various metabolic processes, contributing to the resolution of inflammation and promoting healing [18–22].

Finally, surface markers, such as CD80 and CD163 complement cytokine and enzymatic markers by providing additional specificity for identifying M1 and M2 macrophages. CD80 marks the pro-inflammatory M1 state [23], while CD163 is a hallmark of the anti-inflammatory M2 state [24,25].

This study aims to determine whether endotoxin tolerance triggers a shift in macrophage polarization and metabolism, focusing on evaluating the response of endotoxin-tolerant macrophages upon contact with cancer cells *in vitro*.

2. Materials and methods

2.1. Experimental animals

Female BALB/c mice of 6–8 weeks old were purchased from the Mossakowski Medical Research Centre of the Polish Academy of Sciences (Warsaw, Poland) and allowed to acclimatize for 14 days before experimentation. The animals were housed individually in polycarbonate cages within a controlled environment. The room was maintained at a consistent relative humidity of 50 ± 10 % and a temperature of 24 ± 1 °C, with a 12-h light-dark cycle, where lights were turned on at 7:00 a.m. Food and water were provided *ad libitum*. All procedures were approved by the Local Bioethical Committee for Animal Care in Bydgoszcz (Poland; permission no. LKE 50/2022).

2.2. Preparation of lipopolysaccharide (LPS) solution

Lipopolysaccharide (LPS) from *Escherichia coli* (strain O111:B4, Sigma-Aldrich) was dissolved in sterile phosphate-buffered saline (PBS) and was applied at a final concentration of 100 ng/mL for the experiments.

2.3. Induction of endotoxin tolerance and breast cancer in mice

Mice were injected with LPS 50 μg/kg intraperitoneally (i.p.) for three consecutive doses to induce endotoxin tolerance, and on the day 4 along with the LPS injection the mice were inoculated with 2.5×10^4 4T1 breast cancer cells subcutaneously (s.c.) on the right mammary gland. The mice were then monitored daily to document the tumor growth. After approximately 3 weeks the mice were sacrificed by overdosing them with ketamine and the tumor tissues were obtained for further analysis.

2.4. Immunohistochemical analysis

The breast tumor tissues were obtained and fixed with 10 % formalin for 24 h. Samples were routinely processed, and 5 μm thick sections were stained using Mayer's hematoxylin and eosin (HE) and prepared for immunohistochemistry. The primary antibody MAC387 (sc-66,204, monoclonal, host: mouse, Santa Cruz Biotechnology, Heidelberg, Germany), was titrated according to the manufacturer's recommendations: 1:200 for MAC387. Briefly, after dewaxing–rehydration, tissue sections were exposed to antigen retrieval; then, sections were cooled at room temperature for 20 min before being soaked into 3 % H_2O_2 for 12 min. Slides were rinsed twice in PBS, pH 7.4, followed by serum blocking with normal goat serum. Incubation with primary antibody was carried out overnight at 4 °C. After being washed twice in PBS, pH 7.4, the slides were incubated for 30 min with a biotinylated goat anti-rabbit, IgG antibody. Afterwards, a avidin–biotin complex (ABC) peroxidase kit (Vectastain, Elite, ABC-Kit PK-6100, Vector Labs, Burlingame, CA, USA) and 3′3′-diaminobenzidine (DAB) system (DAB-Kit-SK4100, Vector Labs) were used for the detection of antigen – antibody reactions. Nuclei were counterstained with Mayer's hematoxylin. For negative controls, the primary antibodies were replaced by rabbit or goat serum, or Balb/c ascitic fluid at corresponding concentrations. All the images were captured using Nikon Eclipse E800.

2.5. Cell culture

The murine macrophage cell line RAW 264.7 was sourced from the European Collection of Authenticated Cell Cultures (Salisbury, UK), while the breast cancer cell line 4T1 was obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10 % fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 IU/mL penicillin (all from Merck, Darmstadt, Germany). Cell cultures were maintained at 37 °C in a humidified atmosphere with 5 % CO_2 and sub-cultured every 2–3 days. Adherent 4T1 cells were detached using 0.25 % trypsin-EDTA solution (Merck) upon reaching 70–80 % confluency, while RAW 264.7 cells were detached by gentle scraping.

2.6. Induction of endotoxin tolerance in RAW 264.7 macrophages

RAW 264.7 macrophages were seeded in a 24-well plate at a concentration of 2×10^5 cells/well in 2 mL of DMEM medium supplemented with 10 % FBS and pre-incubated for 24 h. The cells were then maintained in the following three conditions: non-tolerant macrophages (Mo_{NT}), tolerant macrophages (Mo_{ET}), or macrophages treated only once with LPS for 24 h (Mo_{LPS}), which were used as a positive control. To obtain Mo_{ET} cells, RAW 264.7 cells were stimulated for 24 h with 100 ng/mL of LPS, followed by a wash with PBS and further culturing in a similar dose of LPS-containing media for another 24 h. Finally, the media was removed and the cells were directly lysed with PureZOL™ RNA Isolation Reagent (Bio-Rad, Hercules, CA, USA). The samples were then collected and stored at −80 °C for future gene expression analysis.

2.7. Analysis of cytokine expression by quantitative real-time PCR

Total RNA was isolated from the samples by PureZOL™ RNA Isolation Reagent following the manufacturer's protocol and the reverse transcription was performed using 1 μg of total RNA and iScript™ cDNA Synthesis Kit following manufacturer's protocol. Real-Time PCR was performed in a final volume of 10 μL, with each reaction mixture consisting of cDNA, SsoAdvanced Universal SYBR Green Supermix and the PrimePCR™SYBR® Green Assay designed for IL-10 (Unique Assay ID: qMmuCED0044967), TNF-α (Unique Assay ID: qMmuCED0004141) and iNOS amplification. Amplification was carried out using the CFX Connect Real-Time PCR Detection System. For data normalization, the housekeeping gene GAPDH (Unique Assay ID: qMmuCED0027497) was used to ensure accuracy. The double delta Ct method ($2 - \Delta\Delta Ct$) was employed for data analysis. To check for non-specific primer binding, a melt curve analysis was performed during each qPCR run. All reagents used in the analysis of cytokine expression were purchased from Bio-Rad

(Hercules, CA, USA).

2.8. Western blot analysis

To analyse the expression of COX-2 and CD14, RAW 264.7 cells were seeded at a density of 1×10^5 cells/well in 12-well plate and pre-incubated for 24 h in 2 mL of DMEM medium supplemented with 10 % FBS. The three conditions of Mo_{NT}, Mo_{LPS}, and Mo_{ET} were maintained as described previously. Finally, the cells were washed with ice-cold PBS and lysed using

100 µL of a 1 × RIPA buffer supplemented with 1 % sodium dodecyl sulphate (SDS) and 0.5 % protease inhibitor cocktail (all the reagents were procured from Merck). After mechanical homogenization, the lysates were centrifuged to remove cellular debris. The samples were then heated at 95 °C for 5 min. Protein concentrations in the lysates were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. Lysates were diluted with sample buffer to a final concentration of 30 µg/mL, and 20 µL of each sample was subjected to SDS-PAGE on 4–20 % precast polyacrylamide gels (Bio-Rad). Proteins were transferred to nitrocellulose membranes and immunoblotted with specific primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase (HRP). Immunoreactive bands were detected using the SuperSignal West Pico substrate (Thermo Fisher Scientific), and densitometric analysis was performed using Image Lab Software 5.2.1 (Bio-Rad). The details about the antibodies used in this research are provided in the Table 1.

2.9. Preparation of conditioned media derived from 4T1 cancer cells

To prepare the conditioned media, 4T1 cells were seeded at a density of 1×10^6 cells in a

75 cm^2 cell culture flask and were maintained in DMEM high glucose supplemented with 10 % FBS till 70–80 % confluency was reached. The cells were then washed with PBS and maintained in DMEM high glucose supplemented with 1 % FBS for 24 h. Finally, the supernatant was collected, centrifuged and filtered to remove any cell debris. The conditioned media was then aliquoted and stored at −80 °C for further use.

2.10. Nitric oxide production analysis

The Griess reagent (modified) (Sigma-Aldrich) was used according to the manufacturer's protocol to evaluate nitric oxide production. The assay solution was prepared with ultrapure distilled water, with the analysis being conducted in the presence of standards ranging from

0.5–65 µM of NO_2. The RAW 264.7 cells were seeded at a density of 5×10^5 cells/well in

24-well plate and were maintained in the condition of Mo_{NT}, Mo_{LPS} and Mo_{ET} as we described above. Then, the cells were treated with the conditioned media at concentrations of 10, 25 and 50 % obtained from 4T1 cells ($CM_{4T1\ 10–50\%}$) for 16 h. The NO levels were also measured in unstimulated control cells, as well as in cells stimulated once or twice with LPS for 24 h, to serve as controls for the assay. After the treatment, the supernatants were collected, centrifuged to remove the cell debris and then mixed with an equal volume of Griess reagent. After 15 min, the absorbance was read using Synergy HT Multi-Mode microplate reader (BioTek Instruments, Winooski, VT, USA) at 540 nm.

Table 1
List of the antibodies used for western blot analysis.

Primary Antibodies				
Protein Name	Protein Symbol	Cat. No.	Source/ Isotope	Company
Cyclooxygenase 2	COX-2	#12282	Rabbit IgG	Cell Signaling Technology (Danvers, MA, USA)
CD14	CD14	#93882	Rabbit IgG	Cell Signaling Technology
Actin	Actb	612657	Mouse IgG	BD Bioscience (Franklin Lakes, NJ, USA)
Secondary Antibodies				
Target	Origin	Type of conjugate	Company	
Anti-Rabbit	Goat IgG	Peroxidase-conjugated Anti-Rabbit	Sigma Aldrich	
Anti-Mouse	Goat IgG	Peroxidase-conjugated Anti-Mouse	Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA)	

2.11. Reactive oxygen species (ROS) production analysis

The level of ROS in Mo_{NT}, Mo_{LPS} and Mo_{ET} treated with $CM_{4T1\ 50\%}$ was analysed using the H_2DCFDA (Sigma-Aldrich) staining, followed by flow cytometry analysis. The RAW 264.7 cells were seeded at a density of 5×10^5 cells/well in 6 well plate and pre-incubated for 24 h in DMEM supplemented with 10 % FBS. Then, the cells were stained with 20 µM H_2DCFDA followed by incubation for 30 min in the dark at 37 °C. Afterward, the cells were washed twice with PBS and then stimulated with LPS: twice for 48 h in the case of Mo_{ET} cells, and once for 24 h for Mo_{LPS} cells. This was followed by an additional 24 h stimulation with $CM_{4T1\ 50\%}$. After treatment, the cells were harvested, washed thrice with PBS, and the fluorescence was detected by flow cytometry using BriCyte E6 (Mindray, Shenzhen, China) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The data was presented as the ratio of the geometric mean of stimulated/control cells.

2.12. Surface markers analysis

To analyse the effect of cancer on the macrophage polarization of Mo_{ET}, the flow cytometry was performed using fluorescein isothiocyanate (FITC)-labelled anti-CD80 monoclonal antibody and allophycocyanin (APC)-conjugated anti-CD163 monoclonal antibody (Sony Biotechnology Inc., San Jose, CA, USA) staining. In this experiment, co-culture of 4 T1 and RAW 264.7 cells (Mo_{NT}, Mo_{LPS} and Mo_{ET}) was performed using well inserts of 0.4 µm of pore size (SARSTEDT, Nümbrecht, Germany). The RAW 264.7 cells were seeded at the density of 2×10^5 cells/well in 24-well plate for 24 h. The macrophages were then stimulated to obtain the Mo_{NT}, Mo_{LPS} and Mo_{ET}. After the stimulation, the media was removed, the cells were washed once with PBS and the co-culture inserts were placed gently in the wells. The 4T1 cells were then seeded at a concentration of 0.03×10^6 cells/insert. After 24 h, the inserts were removed and the monolayer of the macrophages was washed with ice-cold PBS and then harvested by gentle scraping in 1 mL of PBS. After collecting the cells, three more washes with PBS and 10 min-lasting incubation with Mouse Seroblock FcR (Bio-Rad, Hercules, CA, USA) were performed. Following the incubation, staining with anti-CD80 and anti-CD163 antibodies was performed in the dark for 30 min. The cells were then washed again with PBS thrice to remove any unbound antibodies and were finally suspended in 500 µL of PBS. The analysis was performed using BriCyte E6 flow cytometer (Mindray, Shenzhen, China).

2.13. Cell viability analysis

To assess the viability of RAW 264.7 cells after exposure to various concentrations of CM_{4T1}, an MTT assay (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma Aldrich) was conducted. The cells were plated in 12-well plates at a density of 1×10^5 cells/well and pre-incubated for 24 h in DMEM supplemented with 10 % FBS. Then, the cells were subjected to the LPS stimulation procedure to obtain

Mo_{LPS} and Mo_{ET} macrophages according to the scheme described above. These macrophages as well as control non-treated cells were then seeded in 96-well plates at a density of 3×10^3 cells/well and pre-incubated for 24 h in DMEM supplemented with 10 % FBS. Following the pre-incubation, the cells were treated with CM_{4T1} in 1 % FBS/DMEM at concentrations of 10, 25, 50, and 75 % for 24 and 48 h. After treatment, the cells were rinsed with PBS and incubated with a red phenol-free medium containing 0.5 mg/mL of MTT solution for 3 h at 37 °C. Once incubation was complete, the medium was removed, and 100 μL of DMSO was added to dissolve the formazan crystals. The plate was then placed on an orbital shaker for 15 min to ensure thorough mixing. Optical density was measured at 570 nm, with 630 nm as the reference wavelength, using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). The viability of the CM4T1-treated cells was expressed as a percentage relative to cells incubated in complete DMEM medium containing varying concentrations (10, 25, 50, or 75 %) of culture media supplemented with 1 % FBS that were treated in the same way as conditioned media.

2.14. Single Cell Energetic Metabolism by Profiling Translation Inhibition (SCENITH)

To analyse the metabolic profile of RAW 264.7 macrophages, the SCENITH was performed by seeding the macrophages at a density of 3×10^5 cells/well in 6-well plate for 24 h. To obtain Mo_{ET} cells the macrophages were primarily stimulated with LPS for 24 h and then challenged again for 2 h. Mo_{LPS} cells were treated once with LPS for 2 h. Then Mo_{NT}, Mo_{LPS}, and Mo_{ET} cells were stimulated with $CM_{4T1\ 50\%}$ for 4 h. Finally, the cells were harvested and seeded in the 96-well plate at a density of 3×10^4 cells/well. Cells in each condition were then either treated with inhibitor of glycolysis, 2-Deoxy-D-glucose (2-DG) (100 mM) (Sigma-aldridge) or inhibitor of oxidative phosphorylation (OXPHOS), Oligomycin (2 μM) (Sigma-aldridge) or both 2-DG and Oligomycin for 20 min at 37 °C. After the metabolic inhibitors, cells were treated with puromycin (10 μg/mL) (Sigma-aldridge) for 30 min at 37 °C (all compounds purchased from Sigma Aldrich). The cells were then washed in PBS supplemented with 2 % FBS thrice and then incubated in Fc blockade (BioXcel, Lebanon, NH, USA) anti-mouse/CD16/CD32 solution for 15 min at 37 °C followed by washing with PBS twice. Then, the surface staining with live cell staining dye (Thermo Fisher Scientific) was performed for 20–30 min at 4 °C, followed by washing in PBS. After the surface staining, fixation and permeabilization of these cells was performed using FOXP3 Fixation and Permeabilization Buffer (Thermo Fisher Scientific) according to the manufacturer's protocol. Finally, the intracellular staining with anti-puromycin antibody (MERCK) was performed for 1 h. Cells were then washed with permeabilization buffer twice, resuspended in PBS and stored at 4 °C till the analysis.

2.15. Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as the mean ± standard error of the mean (SEM) from three independent experiments. One-way ANOVA followed by Bonferroni's multiple comparisons test was used to assess statistical significance, with a threshold of $p < 0.05$.

3. Results

3.1. Macrophages infiltration in tumor tissue is prominent in endotoxin tolerant mice

Since it is unknown whether endotoxin-tolerant macrophages are able to infiltrate the tumor, we conducted this analysis in the mice with and without endotoxin tolerance. Using 4T1 cells, we induced breast carcinoma that, morphologically, corresponded to high-grade tubular carcinoma. The tubular differentiation was minimal, with occasional small lumina. Individual neoplastic cells have oval vesicular nuclei and a fairly extensive amount of cytoplasm. The anisocytosis and anisokaryosis were consistent. Within the interstitium the fibroblasts proliferation made a fine support stroma, with some vacuolated macrophages associated and very few scattered neutrophils and small lymphocytes. Aberrant mitosis was occasionally found. The mentioned tumor features are visible in the photographs included in Fig. S1 in Supplementary Data (SD).

The immunohistochemical analysis of the tumor sections in both the groups of mice were observed to be infiltrated with few macrophages, located mostly in the fine stromal support (Fig. 1). These infiltrated macrophages were identified by the MAC387 staining, a macrophage marker protein. MAC387 staining has been well-established in the literature as an effective tool for macrophage detection.

Considering that macrophages infiltrate tumors in both endotoxin-tolerant and non-tolerant mice, we continued our research on macrophages using an *in vitro* model of endotoxin tolerance.

3.2. Insight into the initiation and amplification of inflammation in response to single and prolonged endotoxin priming

In this research we exposed macrophages to endotoxin twice as we described in our previous paper [26] to get endotoxin-tolerant (ET) cells, which we verified by analysing IL-6 and

TNF-α expression. After the initial exposure to endotoxin, we observed a significant increase ($p < 0.001$) in mRNA expression of both cytokines; however, this effect was evidently diminished with subsequent treatments of LPS (Fig. 2a-b) ($p < 0.001$). These changes observed are in parallel to the results of protein expression levels of TNFα and IL-6 observed also in our previous studies (Fig. 2c-d).

Having endotoxin tolerant cells, we decided to analyse CD14 and COX-2 to understand the underlying mechanisms. CD14 is crucial for the initial detection of endotoxins and the subsequent activation of macrophages, leading to cytokine production. COX-2 is involved in the later stages of inflammation, where it synthesizes pro-inflammatory prostaglandins.

CD14 showed a downregulation trend in Mo_{ET} although not significantly (Fig. 3b). However, Mo_{ET} cells exhibited significantly lower production of COX-2 when compared to Mo_{LPS} ($p < 0.05$) (Fig. 3c).

Our research demonstrated that exposure of macrophages to endotoxins significantly affects factors involved in inflammation, and notably, this effect evolves over time. In Mo_{LPS}, the effect of endotoxin exposure on factors involved in inflammation was evident after 24 h. In the case of Mo_{ET}, this effect became apparent after 48 h.

3.3. Cancerogenic environment leads to a reduction in nitric oxide production by endotoxin-tolerant cells

Since it is known that cancer can significantly affect inflammation, we decided to investigate the effects of endotoxin and endotoxin tolerance in the context of cancer.

To further analyse new factors related to inflammation, we evaluated the production of nitric oxide (NO), which has been known to be regulated by similar pro-inflammatory signaling pathways, such as COX-2 involving nuclear factor (NF) κB [27,28]. We observed a significantly inhibition of iNOS expression in Mo_{ET} after prolonged stimulation with LPS when compared to Mo_{LPS} ($p < 0.001$) (Fig. 4a). Subsequently, we analysed nitric oxide production in response to endotoxin exposure and then check it within a pro-carcinogenic environment. Firstly, we observed a significant inhibition of NO production in Mo_{ET} cells after prolonged LPS stimulation compared to Mo_{LPS} macrophages in the case of culture in control media (CM_{NT}) ($p < 0.001$) (Fig. 4b). Therefore, in the next phase of the research, we examined, the production of nitric oxide (NO) by Mo_{ET} cells when stimulated with conditioned medium derived from cancer cells (CM_{4T1}) at a concentration from 10 to 50 %

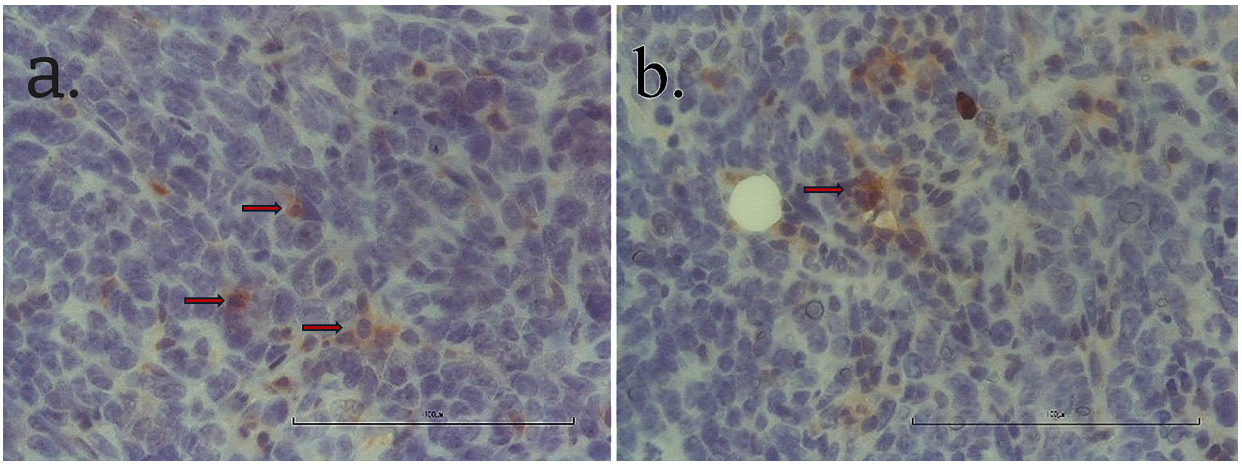

Fig. 1. Immunochemical analysis of macrophage infiltration into breast cancer parenchyma (red arrow) and stroma (MAC387, 40 × magnification) in non-tolerant mice (a) and endotoxin-tolerant mice (b). The analysis was performed in 12 individuals per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

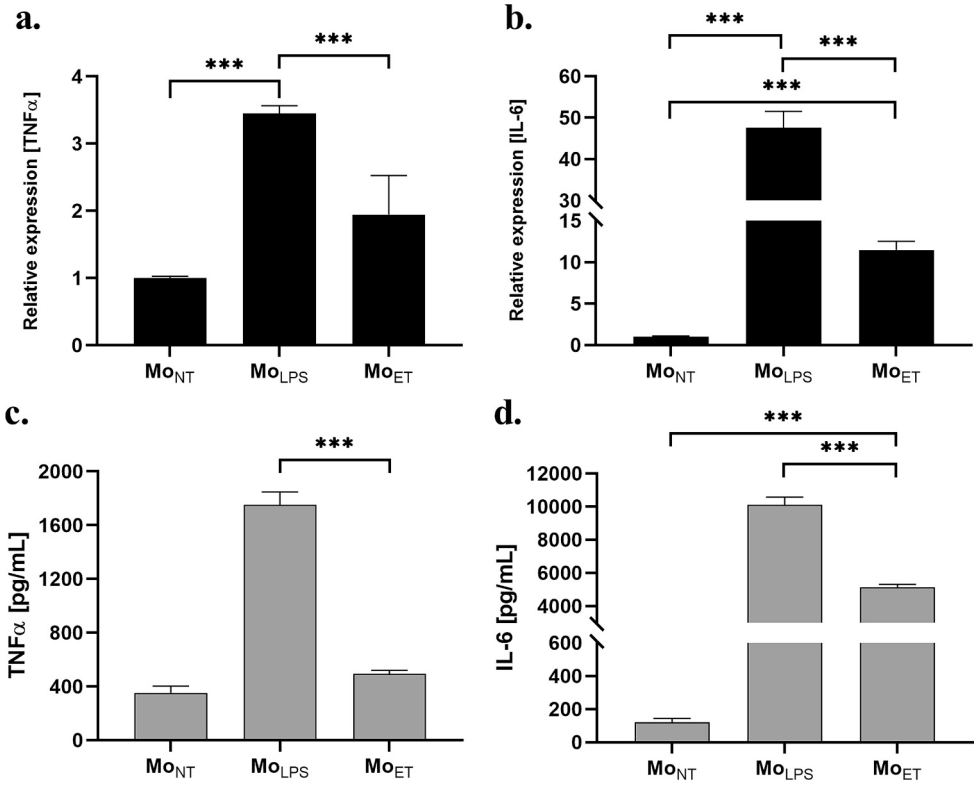

Fig. 2. The mRNA expression of TNF-α (a) and IL-6 (b) in RAW 264.7 macrophages stimulated with LPS, as well as the protein levels of TNF-α (c) and IL-6 (d) in the culture media. Mo_{LPS} – macrophages treated with LPS once; Mo_{ET} – endotoxin tolerant macrophages; Mo_{NT} – untreated macrophages. The data are shown as the means ± SEM of three independent experiments with three wells for each condition. Asterisks indicate significant differences between corresponding groups of cells as indicated (*** $p < 0.001$).

(Fig. 4b). We found that Mo_{ET} cells stimulated with CM_{4T1} produced significantly lower concentrations of NO compared to Mo_{LPS} at corresponding doses of CM ($p < 0.001$) (Fig. 4b).

3.4. The cancerous environment enhances the capacity of endotoxin-tolerant cells to produce ROS in response to endotoxin, more so than in cells treated with LPS alone

As nitric oxide production and oxygen species (ROS) are involved in the primary inflammatory response, we decided to investigate ROS production in the Mo_{ET} when they are in contact with a cancerous environment. In our study, we observed that conditioned medium from cancer cells does not affect Mo_{NT} ROS production. However, a higher level of ROS production was observed in Mo_{LPS} cells stimulated with CM_{4T1} at a concentration of 50 % in comparison with Mo_{NT} ($p < 0.001$), and this increase was even more pronounced in Mo_{ET} cells ($p < 0.001$). The CM_{4T1} at 50 % was used as a stimulant, as it represented the highest concentration that did not exert extreme toxicity on macrophages, which was dependent on the depleted nutritional content of the CM, which contained only 1 % FBS (Fig. 5).

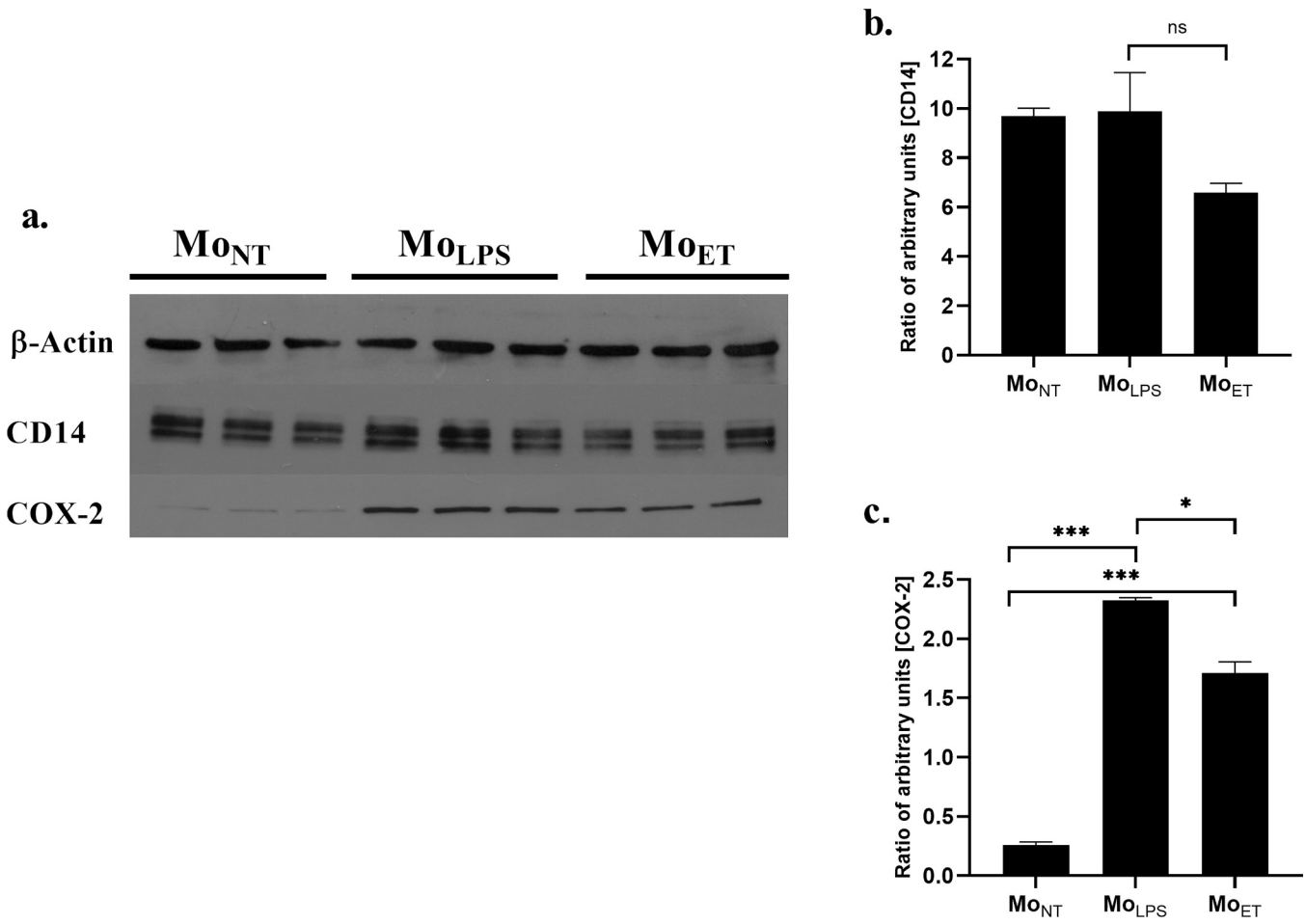

Fig. 3. Expression of CD14 (b) and COX-2 (c) in endotoxin-treated RAW 264.7 macrophages. The levels of both proteins in the cell lysates were analysed by immunoblotting relative to β-actin content (a). Mo_{LPS} – macrophages treated with LPS once; Mo_{ET} – endotoxin tolerant macrophages; Mo_{NT} – untreated macrophages. The data are shown as the means ± SEM of three independent experiments with three wells for each condition. Asterisks indicate significant differences between corresponding groups of cells as indicated (* $p < 0.05$ and *** $p < 0.001$).

3.5. Breast cancer influences the expression of surface markers on endotoxin-tolerant macrophages, shifting them toward an M2 phenotype

In our previous study, Mo_{ET} cells cultured alone predominantly exhibited the M1 phenotype, as evidenced by the increased expression of CD80 (61.6 ± 0.3 % of events). In contrast, the macrophages expressed CD163 at a much lower level (1.2 ± 0.1 % of events) [26]. Since in this research we identified numerous functional changes in the macrophages exposed to endotoxin only once compared to those that develop endotoxin tolerance, we decided to assess their surface markers related to their phenotype. Here we evaluated the impact of breast cancer cells on these macrophages in a co-culture model, which allows us to study how physical contact, in addition to soluble signals, modulates macrophage polarization and functional responses, providing a comprehensive view of macrophage-tumor cell crosstalk. When co-cultured with 4 T1 cells, Mo_{ET} cells showed significantly lower expression of CD80 compared to Mo_{NT} cells ($p < 0.05$) and MoLPS cells ($p < 0.01$) (Fig. 6), and almost similar to the expression of CD80 in monoculture of Mo_{NT}. Interestingly, a significant increase in the expression of CD163, almost 10-fold, was observed in the Mo_{ET} cells during co-culture with 4 T1 cancer cells in comparison with Mo_{NT} ($p < 0.001$) and Mo_{LPS} ($p < 0.01$) (Fig. 6). A detailed gating strategy for the flow cytometry plots is provided in Fig. S2 in SD.

3.6. Enhanced survival of endotoxin-tolerant macrophages in response to 4T1 cell conditioned media compared to macrophages with single LPS stimulation

To further assess the effect of cancer on endotoxin-tolerant macrophages, we studied the effect of the conditioned media derived from 4T1 cancer cells (at a concentration of 10, 25, 50 and 75 %) on the following types of macrophages: Mo_{NT}, Mo_{LPS}, and Mo_{ET} (Fig. 7). Mo_{ET} cells, when stimulated with the CM_{4T1}, exhibited statistically significant increased survival capacity when compared to Mo_{NT} and Mo_{LPS} at both 24 (Fig. 7a) and 48 h (Fig. 7b). Though there was a dose dependent decrease in the cell viability at 48 h the viability % still remained >50 % in case of Mo_{ET} cells stimulated with $CM_{4T1\ (50\%)}$ and was significantly higher when compared to Mo_{NT} ($p < 0.01$) and Mo_{LPS} ($p < 0.001$).

3.7. Endotoxin tolerance macrophages tend to have pronounced glycolytic activity

In this experiment, we decided to investigate the metabolic profile of these macrophages. SCENITH technique provides a way of measuring the dependence of cells on different metabolic pathways (glycolysis or oxidative phosphorylation). We observed a significant decrease in protein translation in RAW264.7 Mo_{NT} ($p < 0.001$) when treated with 2DG an inhibitor of glycolysis, when compared to the control cells not treated with the inhibitors (baseline), indicating that these cells rely heavily on

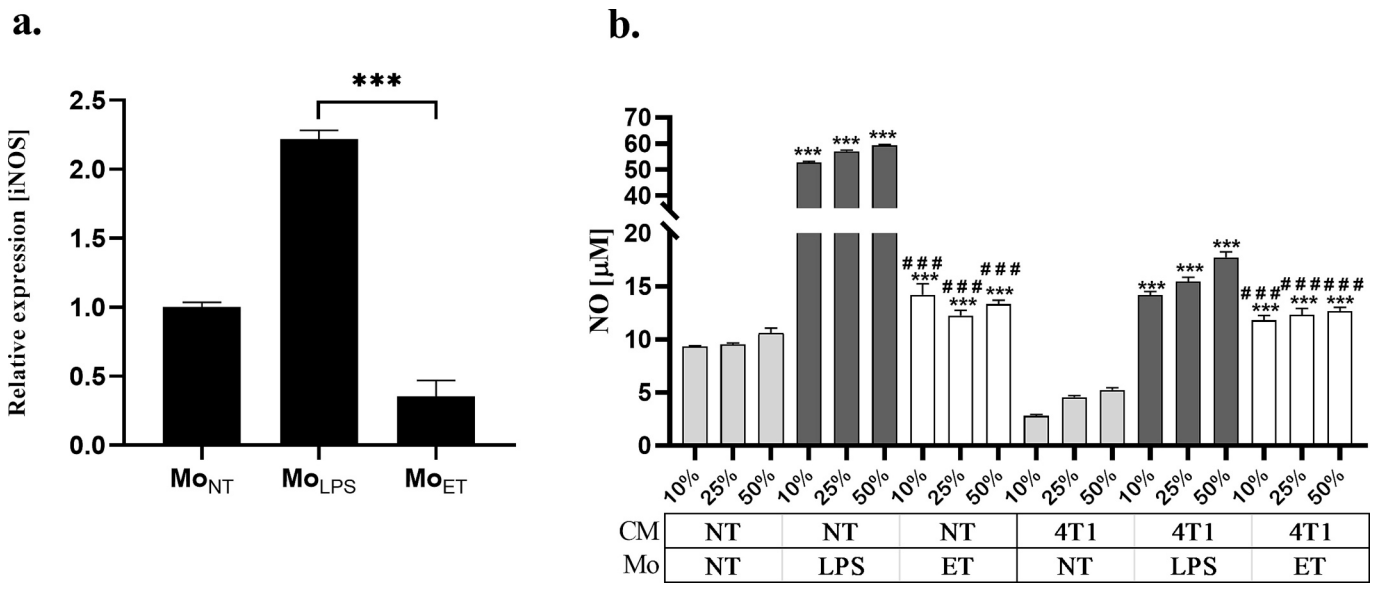

Fig. 4. The mRNA expression of iNOS in endotoxin-treated RAW 264.7 macrophages (a). Level of NO produced by endotoxin-treated macrophages stimulated with conditioned medium derived from 4T1 cancer cells (b). Mo_{LPS} – macrophages treated with LPS once; Mo_{ET} – endotoxin tolerant macrophages; Mo_{NT} – untreated macrophages; $CM_{NT\ 10–50\%}$ – control media; $CM_{4T1\ 10–50\%}$ – conditioned media from 4T1 breast cancer cells. The data are shown as the means ± SEM of three independent experiments with 6 wells for each condition. Hashes (#) indicate a significant difference between Mo_{LPS} and Mo_{ET} cells stimulated with the corresponding concentration of control CM_{NT} or CM_{4T1}, respectively (# # #$p < 0.001$). Asterisk (*) show significant differences between Mo_{NT} cultured in CM_{NT} or CM_{4T1} and Mo_{LPS} and Mo_{ET} stimulated with a corresponding concentration of CM (*** $p < 0.001$).

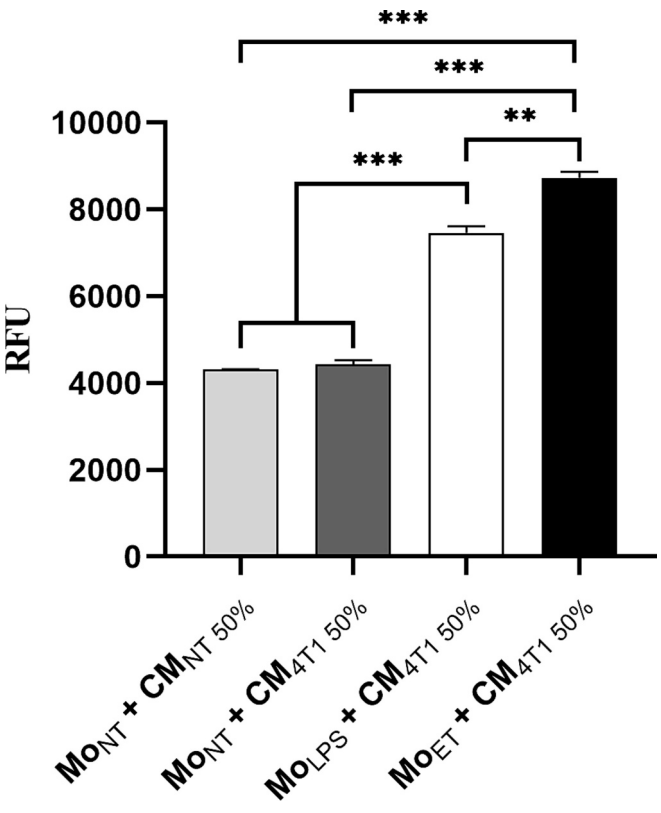

Fig. 5. Analysis of intracellular level of ROS production in RAW 264.7 macrophages treated with LPS and cultured in conditioned medium from cancer cells using H_2DCFDA staining and flow cytometry. Mo_{LPS} – macrophages treated with LPS once; Mo_{ET} – endotoxin tolerant macrophages; Mo_{NT} – untreated macrophages; $CM_{4T1\ 50\%}$ – Conditioned media obtained from 4 T1 cells. The data are shown as the means ± SEM of three independent experiments with three wells for each condition. Asterisks indicate significant differences between corresponding groups of cells as indicated (*** $p < 0.001$).

glycolysis for protein production (Fig. 8a). Conversely no significant decrease was observed in Mo_{LPS} and Mo_{ET}.

Interestingly, when treated with Oligomycin (an inhibitor of oxidative phosphorylation) Mo_{NT} ($p < 0.05$), Mo_{LPS} ($p < 0.05$) and Mo_{ET} ($p < 0.001$) exhibited a significant increase in the protein translation when compared to their respective baseline. This indicates that these ET macrophages do not strictly depend on OXPHOS and therefore, involvement of other metabolic pathway, such as the fatty acid oxidation or glutaminolysis has to be taken under consideration. Interestingly, Mo_{ET} treated with Oligomycin produced significantly higher protein when compared to Mo_{LPS} ($p < 0.001$). This finding suggests the activation of compensatory mechanisms that enhance protein production. Furthermore, this implies that endotoxin-tolerant macrophages respond differently to OXPHOS inhibition, possibly due to metabolic reprogramming associated with their tolerance state. Similarly, Mo_{NT}, Mo_{LPS} and Mo_{ET} when treated with CM_{4T1} produced similar results as on treatment with 2DG ($p < 0.001$) alone. However, when treated with oligomycin only Mo_{NT} ($p < 0.01$) and Mo_{ET} ($p < 0.001$) showed a prominent increase in the protein translation when compared to their respective baseline

(Fig. 8b). We also observed that Mo_{LPS} and Mo_{ET} when stimulated with CM_{4T1} at a concentration of 50 % also exhibited increased protein production when treated with Oligomycin than the Mo_{LPS} and Mo_{ET} alone ($p < 0.001$) (Fig. 8c).

Overall, these findings illustrate the complex interplay between metabolic pathways and protein synthesis in different macrophage. They highlight the reliance on glycolysis for protein production in Mo_{NT}, Mo_{LPS} and Mo_{ET} macrophages while suggesting that endotoxin tolerance allows for alternative metabolic adaptations. These metabolic shifts could provide tolerant macrophages with greater flexibility in adapting to LPS and different environmental challenges. Furthermore, the significant protein synthesis observed in response to oxidative phosphorylation inhibition underscores the potential for metabolic reprogramming in macrophages, particularly in the context of endotoxin tolerance, which may influence their functional roles in the tumor microenvironment.

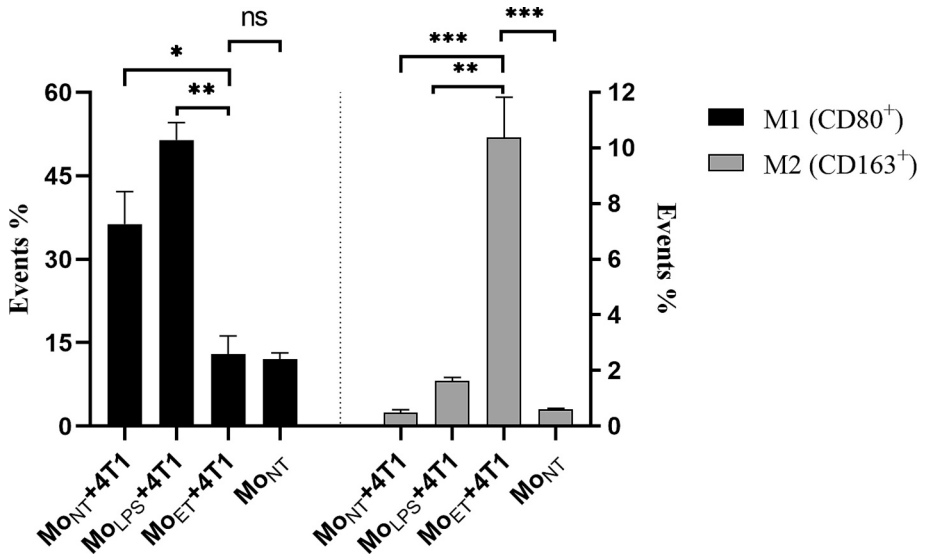

Fig. 6. Evaluation of the phenotype of the RAW 264.7 macrophages (M1 and M2 markers) when co-cultured with 4 T1 breast cancer cells. The % events of the M1 cells are plotted on the left Y-axis and the M2 cells are plotted on the right Y-axis. Mo_{LPS} – macrophages treated with LPS once; Mo_{ET} – endotoxin tolerant macrophages; Mo_{NT} – untreated macrophages. The data are shown as the means ± SEM of three independent experiments with three wells for each condition. Asterisk (*) represents the significant differences in the corresponding groups as indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and ns – not significant).

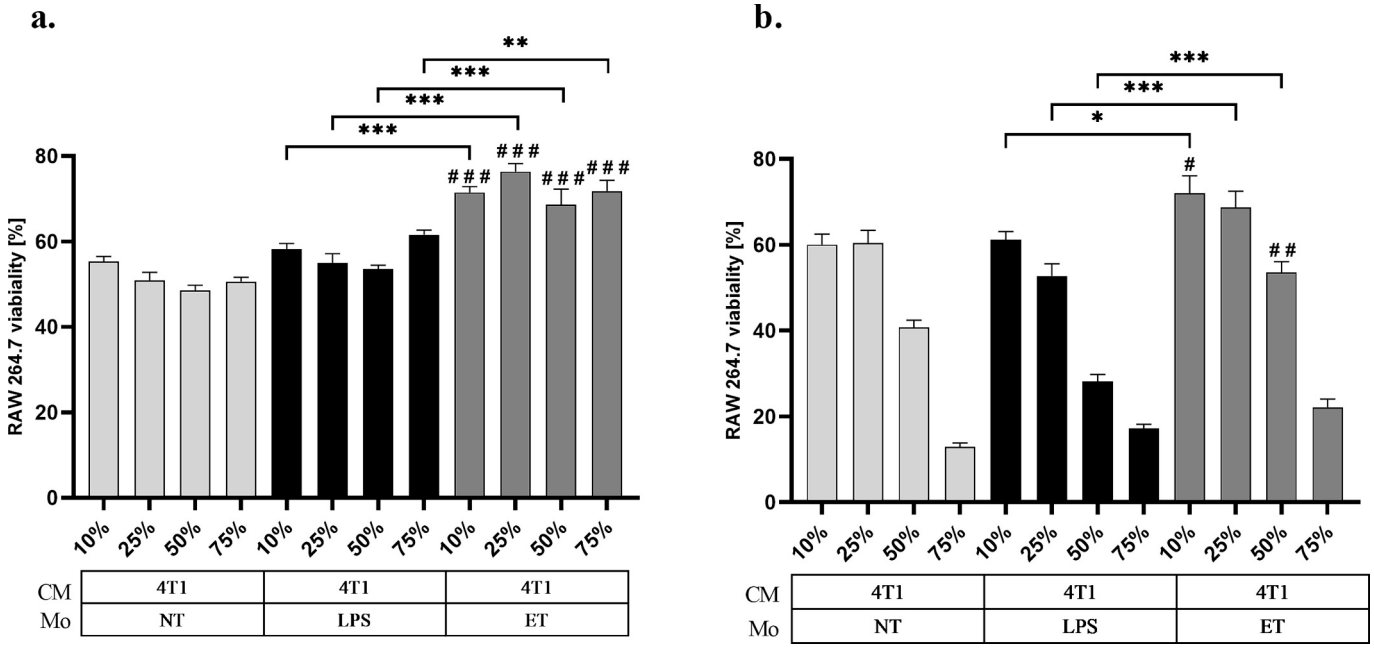

Fig. 7. Viability of RAW 264.7 cells stimulated with conditioned media (CM) obtained from 4T1 at the concentration 10, 25, 50 and 75 % for 24 h (a) and 48 h (b). Assessment of cell viability was done using MTT assay. The non-stimulated cells (which is represented as 100 %) are used as the control to evaluated the percentage viability. The data are shown as the means ± SEM of three independent experiments with 6 wells for each condition. Asterisk (*) represents the significance between Mo_{ET} and Mo_{LPS} stimulated with the corresponding concentration of conditioned medium (10–75 %) (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).
Hash (#) represents the significance of Mo_{ET} against the Mo_{NT} stimulated with the corresponding concentration of conditioned medium (# $p < 0.05$, # # $p < 0.01$ and # # # $p < 0.001$).

4. Discussion

Endotoxin tolerance (ET) occurs when the immune system becomes less responsive to inflammatory signals induced by endotoxins following prolonged or repeated exposure [29,30]. Although commonly observed in conditions such as chronic infections, sepsis, or repeated medical treatments involving endotoxin exposure [31–33], ET remains a poorly studied phenomenon. Our previous study revealed that endotoxin-tolerant macrophages foster a cancer-friendly environment [26]. This study aims to further investigate how cancer cells affect macrophages, with a particular emphasis on their sensitivity to endotoxin. After confirming that macrophages can infiltrate tumors regardless of their endotoxin responsiveness, we proceeded with *in vitro* studies. We specifically analysed cytokine production, metabolic reprogramming, and surface marker expression in macrophages cultured in a tumor-mimicking microenvironment.

Using an *in vitro* ET model, we delved into macrophage-cancer cell interactions. We observed that ET macrophages had reduced NO

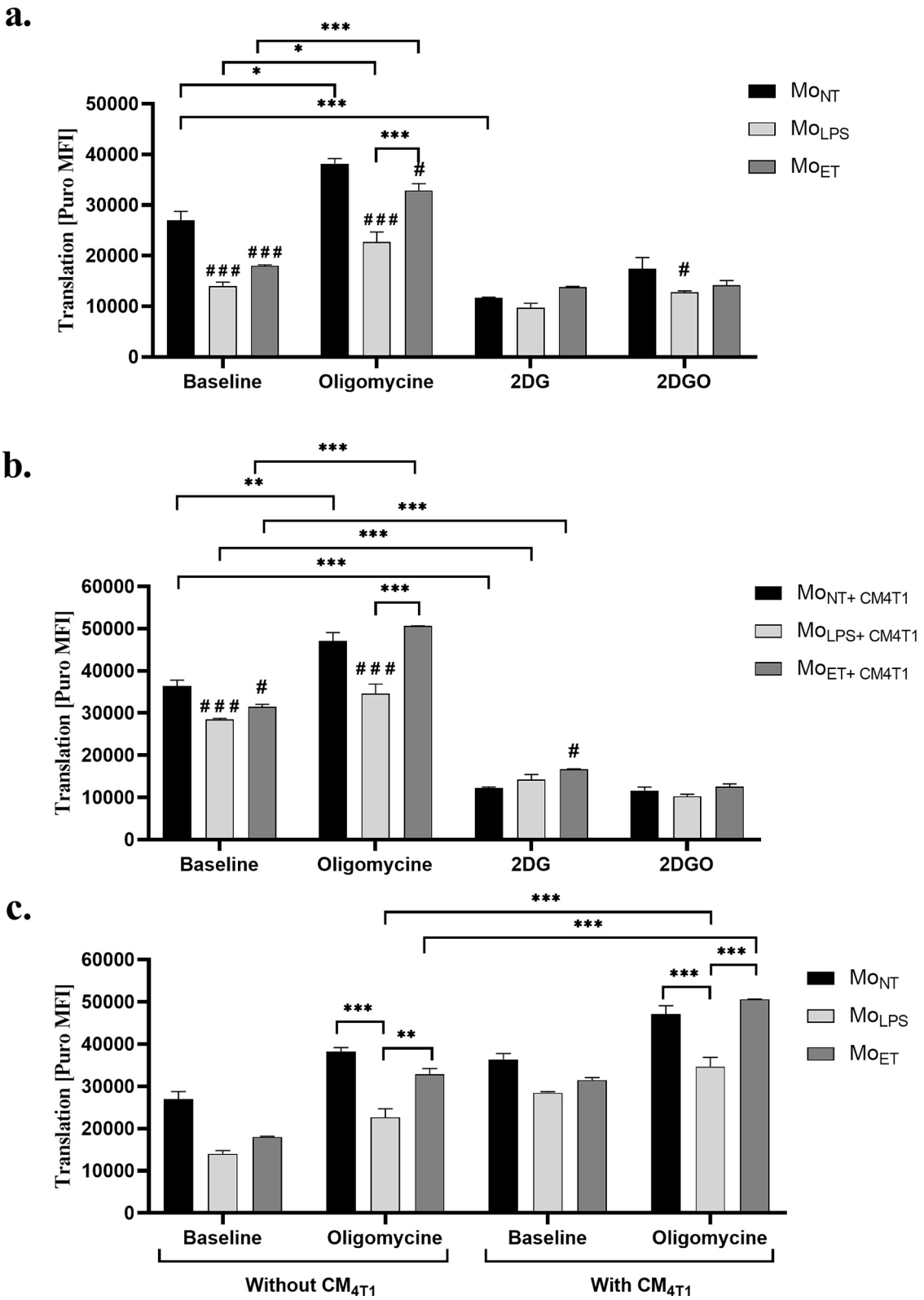

Fig. 8. Evaluation of the metabolic activity of the RAW 264.7 macrophages (Mo_{NT}, Mo_{LPS} and Mo_{ET}) stimulated with conditioned media (CM) obtained from 4 T1 at a concentration of 50 % by treating them with inhibitors Oligomycin, 2DG and puromycin. Mo_{NT} – untreated macrophages; Mo_{LPS} – macrophages treated with LPS once; Mo_{ET} – endotoxin tolerant macrophages. Flowcytometry was used to assess the protein translation MFI in Mo_{NT}, Mo_{LPS} and Mo_{ET} not stimulated with $CM_{4T1\ 50\%}$ (a), Mo_{NT}, Mo_{LPS} and Mo_{ET} stimulated with $CM_{4T1\ 50\%}$ (b) and Comparison of translation MFI on treatment with oligomycin between Mo_{NT}, Mo_{LPS} and Mo_{ET} stimulated with or without $CM_{4T1\ 50\%}$ (c). The data are shown as the means ± SEM of three independent experiments. Asterisk (*) represents the significance of Mo_{ET} against the Mo_{LPS} (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$) and hash (#) represents the significance of Mo_{ET} against the Mo_{NT} (# $p < 0.05$, # # $p < 0.01$ and # # # $p < 0.001$).

production and iNOS expression when exposed to cancer cell-conditioned medium, compared to normal and LPS-treated macrophages. This reduction in NO, which has a dual role in tumor biology promoting tumor growth at low levels and exerting cytotoxic effects at higher concentrations [34,35], suggests that ET macrophages create a tumor-favoring environment by limiting NO's cytotoxic potential [36]. In contrast, ET macrophages showed a significant increase in ROS production when stimulated with cancer cell-conditioned medium, pointing to an activation of oxidative stress pathways. This elevated ROS production aligns with tumor-promoting activities, as ROS can induce DNA damage and enhance cancer cell survival and metastasis [37–39].

These findings, *i.e.* increased ROS production (typically associated with pro-inflammatory M1 macrophages) alongside reduced NO levels (often linked to anti-inflammatory M2 macrophages) complicate the classification of ET macrophages as either M1 or M2. To further clarify, we examined surface markers, finding a shift in ET macrophages co-cultured with cancer cells, characterized by decreased CD80 expression and increased CD163 expression, suggesting an M2-like phenotype. This supports the view that tumor-associated macrophages (TAMs) often polarize toward an M2 phenotype, promoting immune suppression and tumor progression [40–42]. Additionally, ET macrophages displayed a blunted inflammatory response, with lower expression of pro-inflammatory cytokines like TNF-α and IL-6, reinforcing the notion that endotoxin tolerance shifts macrophages away from the M1 phenotype typically induced by single LPS exposure.

The paradox of increased ROS and decreased NO production in ET macrophages becomes more compelling when integrated with our SCENITH findings. Our SCENITH analysis revealed that ET macrophages are metabolically flexible, utilizing not only glycolysis but also pathways like fatty acid oxidation and glutaminolysis, especially when OXPHOS was inhibited. This metabolic adaptability may account for the elevated ROS production, possibly as a byproduct of heightened metabolic activity in response to tumor signals, while downregulating NO production due to reprogramming of metabolic pathways.

Together, these findings indicate that the metabolic and functional adaptations in ET macrophages, highlighted through SCENITH and the analysis of ROS and NO production, equip them to support tumor growth dynamically, deviating from typical macrophage polarization patterns. This interplay between metabolic flexibility, ROS production, and NO regulation underscores the need to consider both metabolic and functional changes when studying macrophage behavior within the tumor microenvironment.

To further investigate ET macrophages' role in the tumor microenvironment, we examined their survival in tumor-associated stress conditions. We found that ET macrophages, often linked to an immunosuppressive phenotype (*e.g.*, M2 polarization), displayed enhanced survival in cancer cell-conditioned media, indicating resilience to metabolic and inflammatory stresses common in tumors, such as nutrient deprivation or hypoxia. This suggests that ET not only modifies macrophage function but also confers a survival advantage, potentially allowing these cells to persist and impact the tumor's immune landscape in ways that non-tolerant macrophages cannot. This insight into ET macrophage persistence could shed light on their roles in chronic inflammation and cancer progression.

In conclusion, our findings suggest that conditions leading to ET result in, on one hand, a change in the phenotypic characteristics of macrophages, and on the other hand, a shift in how they respond to cancer cells. The capacity of ET macrophages for tumor infiltration, ROS production, NO modulation, and metabolic adaptability highlights a functional reprogramming that may enhance their pro-tumor activity. These adaptations emphasize the complex relationship between inflammation, metabolic pathways, and tumor biology, suggesting new potential targets for therapeutic intervention.

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CRediT authorship contribution statement

Konkonika Roy: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tomasz Jędrzejewski:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Conceptualization. **Justyna Sobocińska:** Writing – review & editing, Visualization. **Paulina Spisz:** Writing – review & editing, Visualization. **Bartosz Maciejewski:** Writing – review & editing, Visualization. **Nadine Hövelmeyer:** Writing – review & editing, Visualization, Funding acquisition. **Benedetta Passeri:** Writing – review & editing, Visualization, Formal analysis, Data curation. **Sylwia Wrotek:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2025.104934.

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9.3. Article #3

Roy, K.; Maciejewski, B.; Jędrzejewski, T.; Spisz, P.; Sobocińska, J.; Trzeciak, P.; Passeri, B.; Wrotek, S. Endotoxin Tolerance Enhances Breast Cancer Aggressiveness and Alters Inflammatory Marker Expression in Tumor and Spleen of Mice. *International Immunology*. (Manuscript ID: INTIMM-25-0140)

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Endotoxin tolerance enhances breast cancer aggressiveness and alters inflammatory marker expression in tumor and spleen of mice

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Title: Endotoxin tolerance enhances breast cancer aggressiveness and alters inflammatory marker expression in tumor and spleen of mice

[1]Konkonika Roy, [1]Bartosz Maciejewski, [1]Tomasz Jędrzejewski, [1]Paulina Spisz, [1]Justyna Sobocińska, [2]Melania Di Pentima, [2]Benedetta Passeri, [1]Sylwia Wrotek

[1]Department of Immunology, Faculty of Biology and Veterinary Sciences, Nicolaus Copernicus University, 1 Lwowska Street, Torun, 87-100, Poland

[2]Pathology Unit, Department of Veterinary Science, University of Parma, Strada del Taglio 10, 43126 Parma, Italy

Corresponding authors:

Konkonika Roy (konkonika.roy@doktorant.umk.pl)

Prof. Sylwia Wrotek (wrotek@umk.pl)

Abstract

Endotoxin tolerance (ET) is an immunological state in which repeated exposure to endotoxins, such as lipopolysaccharide (LPS), leads to a reprogramming of the immune system and a diminished inflammatory response. In this study, we employed a murine model to explore the role of ET in breast cancer progression, hypothesizing that ET may foster a tumor-permissive immune environment.

We compared endotoxin tolerant breast cancer-bearing mice (ETBC group) to non-endotoxin tolerant breast cancer-bearing controls (BC group). The ETBC mice exhibited significantly faster tumor progression and earlier disease onset. Hematological analysis revealed reduced leukocyte counts in the ETBC group, indicating compromised immune cell recruitment. Additionally, ETBC mice showed decreased spleen weight relative to the BC group, further supporting systemic immune suppression.

Gene expression profiling in both spleen and tumor tissues revealed marked immunological alterations in ETBC mice. In the spleen, there was a notable downregulation of key pro-inflammatory cytokines, including interleukin (IL) 6 and interferon (IFN) γ. Conversely, genes associated with immune modulation and tumor progression, such as IL-1β, inducible nitric oxide synthase (NOS2), cyclooxygenase (COX) 2, vascular endothelial growth factor (VEGF), and colony stimulating factor 1 (CSF-1) remained upregulated. Notably, IL-1β, NOS2, COX-2, IL-10, and VEGF were also found to be consistently upregulated in the tumor tissues of ETBC mice.

We concluded that ET not only impairs immune surveillance but also reshapes the tumor microenvironment in favor of cancer growth. This highlights the potential role of ET in oncology and suggests that its modulation could represent a novel avenue for therapeutic intervention.

Key words

endotoxin tolerance; cancer; immunosuppression; tumor microenvironment; inflammation

Introduction

Endotoxin tolerance (ET) is a phenomenon in which prior exposure to endotoxin, such as lipopolysaccharide (LPS), reprograms macrophages, altering their response to subsequent endotoxin challenges [1,2]. When primed with endotoxin, macrophages exhibit heightened reactivity and produce elevated levels of inflammatory mediators [3,4]. In contrast, endotoxin tolerant macrophages demonstrate a diminished response, characterized by reduced production of these mediators. Changes in inflammatory markers also lead to systemic alterations across the entire organism, with the most noticeable being the weakening or absence of fever, as evidenced by attenuated body temperature changes following the administration of a pyrogenic dose of endotoxin [5-7]. Fever itself plays a complex role in immune regulation, directly modulating immune responses by enhancing the activity of cytotoxic T cells and natural killer cells [8,9]. On the other hand, the inability to trigger fever due to ET may not only impair immune function but also obscure important warning signals of immune dysregulation, potentially affecting the body's capacity to respond to tumor development [10-12].

Breast cancer is one of the most prevalent and deadly cancers worldwide, affecting millions of people each year. Despite advances in detection and treatment, breast cancer remains a leading cause of cancer-related deaths, primarily due to its potential for metastasis and resistance to therapies [13,14]. Understanding the factors that drive breast cancer progression, particularly those linked to the immune system, is essential for developing innovative therapeutic strategies. The immune system plays a dual role in cancer by both promoting the elimination of tumor cells and, paradoxically, creating a microenvironment that supports tumor growth and metastasis [15-18]. Given the complex interplay between inflammation and cancer, exploring how ET-related immune reprogramming influences tumor development may offer valuable insights for advancing therapeutic strategies.

To date, most studies exploring the impact of ET on cancer progression have been limited to *in vitro* models [10], which fail to capture the full complexity of interactions within a living organism. To address this gap, our study investigates the *in vivo* effects of ET on breast cancer development in a mouse model. Specifically, we examined whether ET influences tumor aggressiveness and alters the expression of inflammatory markers in both tumor tissues and the spleen. By elucidating these interactions, our research aims to contribute to a deeper understanding of the interplay between immune tolerance and breast cancer.

Materials and methods

Experimental animals

Female BALB/c mice of 6-8 weeks old were purchased from the Mossakowski Medical Research Centre of the Polish Academy of Sciences (Warsaw, Poland) and allowed to acclimatize for 14 days before experimentation. The animals were housed individually in polycarbonate cages within a controlled environment. The room was maintained at a consistent relative humidity of 50 ± 10% and a temperature of 24 ± 1°C, with a 12-hour light-dark cycle, where lights were turned on at 7:00 a.m. Food and water were provided *ad libitum*. All procedures were approved by the Local Bioethical Committee for Animal Care in Bydgoszcz (Poland; permission no. LKE 50/2022). This research was conducted on 4 groups of mice i.e., untreated (NT); endotoxin tolerant (ET); breast cancer bearing (BC); endotoxin tolerant breast cancer bearing (ETBC). The Figure 1 presents a diagram illustrating the procedure conducted for each group of animals.

In vivo procedure

Temperature and motor activity measurement

Body temperature (T_b) of the mice was monitored by the temperature-sensitive miniature biotelemeters (PhysioTels model TA10TA-F40; Data Sciences International, St. Paul, MN, USA) implanted intra-abdominally in a sterile environment. Before transplantation, the mice were anesthetized via intramuscular injection of a ketamine (80 mg/kg, Biowet, Puławy, Poland) and xylazine (10 mg/kg, ScanVet, Gniezno, Poland) mixture. Then, a small area was shaved and sterilized, after which an incision was made through the abdominal skin and muscle layers. Temperature sensitive biotelemetry devices were inserted into the peritoneal cavity and the abdominal muscles and skin was sutured separately. All the surgical procedures were completed at least 10 days prior to the start of the experiment.

The motor activity of mice was monitored by tracking changes in the position of an implanted temperature-sensitive transmitter relative to a receiver board. These positional shifts resulted in variations in signal strength, which were detected by the external receiver antenna and recorded as "pulses" or "counts" of activity.

Inducing endotoxin tolerance in mice

Lipopolysaccharide (LPS) from *Escherichia coli* (0111:B4, Merck, Darmstadt, Germany) was prepared by dissolving in sterile 0.9% sodium chloride. Prior to injection, the

stock solution of LPS (2 mg/mL) was warmed to 37°C, then diluted in warm sterile saline to the desired concentration. To induce ET, mice received daily intraperitoneal (i.p.) injections of LPS (50 µg/kg) for four consecutive doses (Fig. 1). The development of ET was assessed by measuring T_b of the mice using biotelemetry.

Inducing tumor and tissue collection

On the day of the third LPS injection, mice were inoculated subcutaneously (s.c.) with 2.5×10^4 4T1 breast cancer cells on the right flank (Fig. 1). Before the experiments, 4T1 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 IU/mL penicillin (all from Merck, Darmstadt, Germany). The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Then, the mice were monitored daily for tumor growth. After approximately three weeks, the mice were euthanized through an overdose of ketamine/xylazine (240 mg/kg and 30 mg/kg, respectively). Blood, tumor and spleen samples were collected. The tumor and spleen samples were rinsed twice in sterile ice-cold PBS, and immediately flash-frozen in liquid nitrogen (Fig. 1). These tissue samples were stored at -80°C for subsequent analysis.

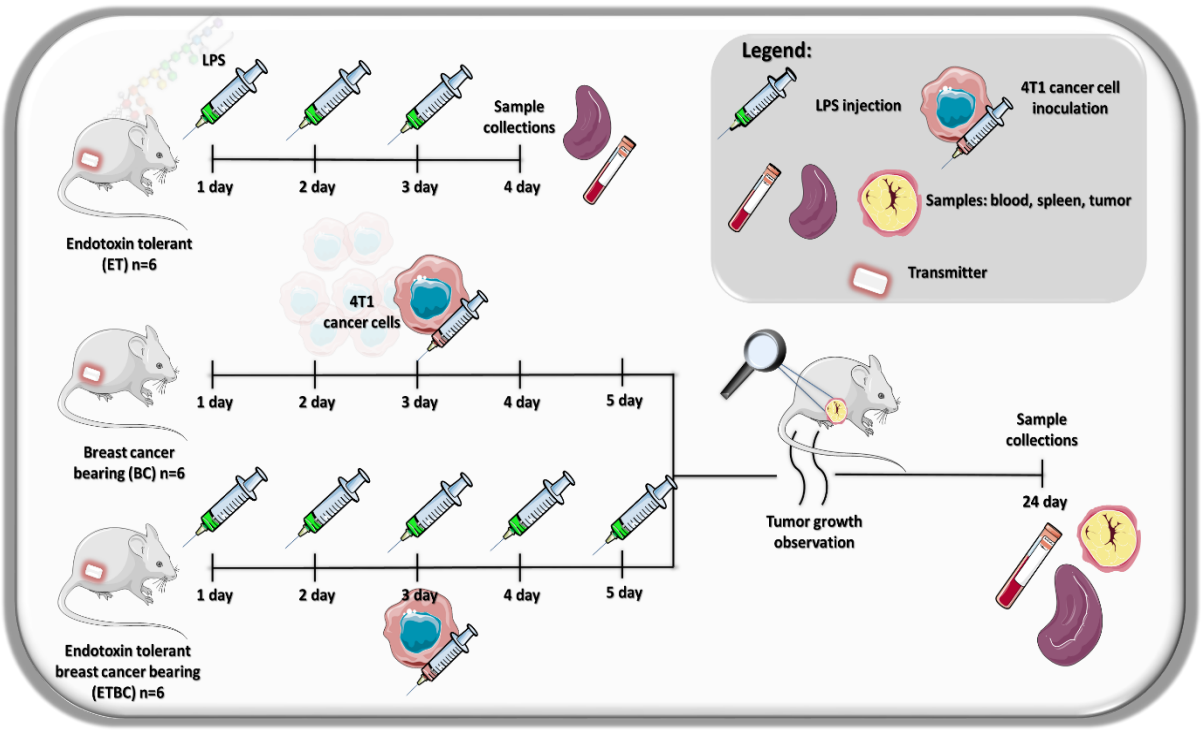

Fig 1. Scheme of experimental procedures conducted on the following groups of animals: endotoxin tolerant mice (ET), breast cancer-bearing mice (BC) and endotoxin tolerant breast cancer bearing-mice (ETBC).

Blood sample collection and blood morphology analysis

Blood samples were collected through cardiac puncture in EDTA-treated tubes from mice that were anesthetized with an overdose of ketamine/xylazine (240 mg/kg and 30 mg/kg, respectively). The total blood cell count of leukocytes, lymphocytes, monocytes, and granulocytes was evaluated using the Auto Hematology Analyzer BC-2800Vet (Mindray, Shenzhen, China).

Measurement of the spleen size

Spleens were collected following euthanasia of the mice via an overdose of ketamine and xylazine mixture. Immediately after removal, the spleens were weighed using a highly sensitive scale to ensure precise measurements.

Histological assessment

Mice mammary tumor tissues were assessed by pathologists. The tumor tissues were fixed in 10% neutral buffered formalin for 24 hours. The tissue samples were then routinely processed for histopathology and embedded in paraffin wax (FFPE). Paraffin sections of 5 μm thickness, were stained for histology with Mayer's hematoxylin and eosin (H&E). All images were captured using a Nikon Eclipse E800 microscope.

RNA extraction and RT-qPCR

Total RNA extraction from spleen and tumour tissues was performed by lysing them in PureZOL™ RNA Isolation Reagent with mechanical disruption according to the manufacturer's protocol. Then, the cDNA was obtained using 1 μg of total RNA and iScript™ cDNA Synthesis Kit following manufacturer's protocol. RT-qPCR was performed at a final volume of 10 μL with SsoAdvanced Universal SYBR® Green Supermix and PrimePCR™ SYBR® Green Assays. Table 1 lists the unique assay IDs of the primers of the immune-related genes. Test samples were analyzed in triplicate using the CFX Connect Real-Time PCR Detection System. Specificity was verified through melt curve analysis. Standard curves were generated for both the target and reference genes (actin). Calibrator-normalized quantifications were carried out using CFX Manager Software 3.1. Each reaction was conducted at least twice.

All reagents and software used in the analysis of cytokine expression were purchased from Bio-Rad (Hercules, CA, USA).

Table 1. The list of primers used for RT-qPCR

Gene Name	Protein name	Unique assay ID
actin	ACTB	qMmuCED0027505
interleukin 6	IL-6	qMmuCED0045760
interleukin 1β	IL-1β	qMmuCED0045755
cyclooxygenase 2	COX-2	qMmuCED0047314
vascular endothelial growth factor	VEGF	qMmuCED0047509
interferon γ	INF-γ	qMmuCID0006268
nitric oxide synthase 2 (NOS2)	inducible nitric oxide synthase (iNOS)	qMmuCID0023087
interleukin 10	IL-10	qMmuCED0044967
signal transducer and activator of transcription 6	STAT6	qMmuCID0006404
Colony stimulating factor 1	CSF-1	qMmuCID0019725

Survival analysis

The survival rates of mice in the BC and ETBC groups were compared. The Kaplan-Meier plot was used to assess the differences, and statistical analysis between these groups was performed using the Log-rank (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon test.

Tumour growth rate analysis

Tumour samples obtained from the group of mice with BC and ETBC were measured using Vernier calipers. The tumor volume was calculated using the formula

$$V = \frac{\pi}{6} * L * W$$

In this equation, L represents the average length of the tumors and W represents the average width of the tumors. The comparison analysis between the BC and ETBC mice groups was conducted using non-linear regression analysis.

Statistical analysis

Relative gene expression levels were evaluated and compared using the 2-ΔΔCT method. Multiple group comparisons were performed using a one-way analysis of variance (ANOVA) followed by the Sidak test. Unpaired t-test was used for the evaluation of the gene

expression between 2 groups. For the evaluation of the tumour growth rate non-linear regression was used. GraphPad Prism version 8 software (GraphPad Software Inc., La Jolla, CA, USA) was used for calculations, analysis, and results visualization. The statistical standard of significance was set at $p < 0.05$.

Results

Repeated LPS injections at a constant dose result in a progressive and sustained reduction in fever and locomotor activity, indicating the development of endotoxin tolerance

Mice are nocturnal animals revealing low day-time and high night-time T_b. Using four consecutive doses of LPS, we observed a progressive change in the response to LPS. First injection of LPS induced fever, which started within 1 hour after LPS injection. The occasional transient increase in T_b of mice at 9 a.m. (injection time) was mainly caused by stress related to the injection and handling of the mice. Fever onset was achieved and maintained for approximately one hour, with the highest body temperature reaching 38.02 ± 0.33°C, compared to 36.72 ± 0.21°C in NT mice ($p < 0.001$). This was followed by a fever duration of roughly 1.5 hours before a gradual return of body temperature to baseline levels. Repeated daily injections of LPS over several days resulted in a progressive attenuation of the febrile response, culminating in the establishment of ET model in mice. By the fourth dose of LPS, the maximum temperature in the treated group was 37.03 ± 0.33°C, compared to 38.02 ± 0.33 °C following the first dose of LPS ($p < 0.001$) (Fig. 2a).

Another important aspect of ET is sickness behavior, such as a decrease in motor activity. Indeed, a significant reduction in locomotor activity was observed in the mice exhibiting ET when compared to NT animals. The average motor activity value recorded was 2.66 ± 2.34 counts in ET mice and 2.6 ± 1.82 counts in LPS mice when compared to 4.73 ± 1.34 counts in NT mice after the LPS injection ($p < 0.05$) (Fig. 2b). The reduction in activity typically associated with fever was evident in LPS-treated mice and persisted throughout the development of ET.

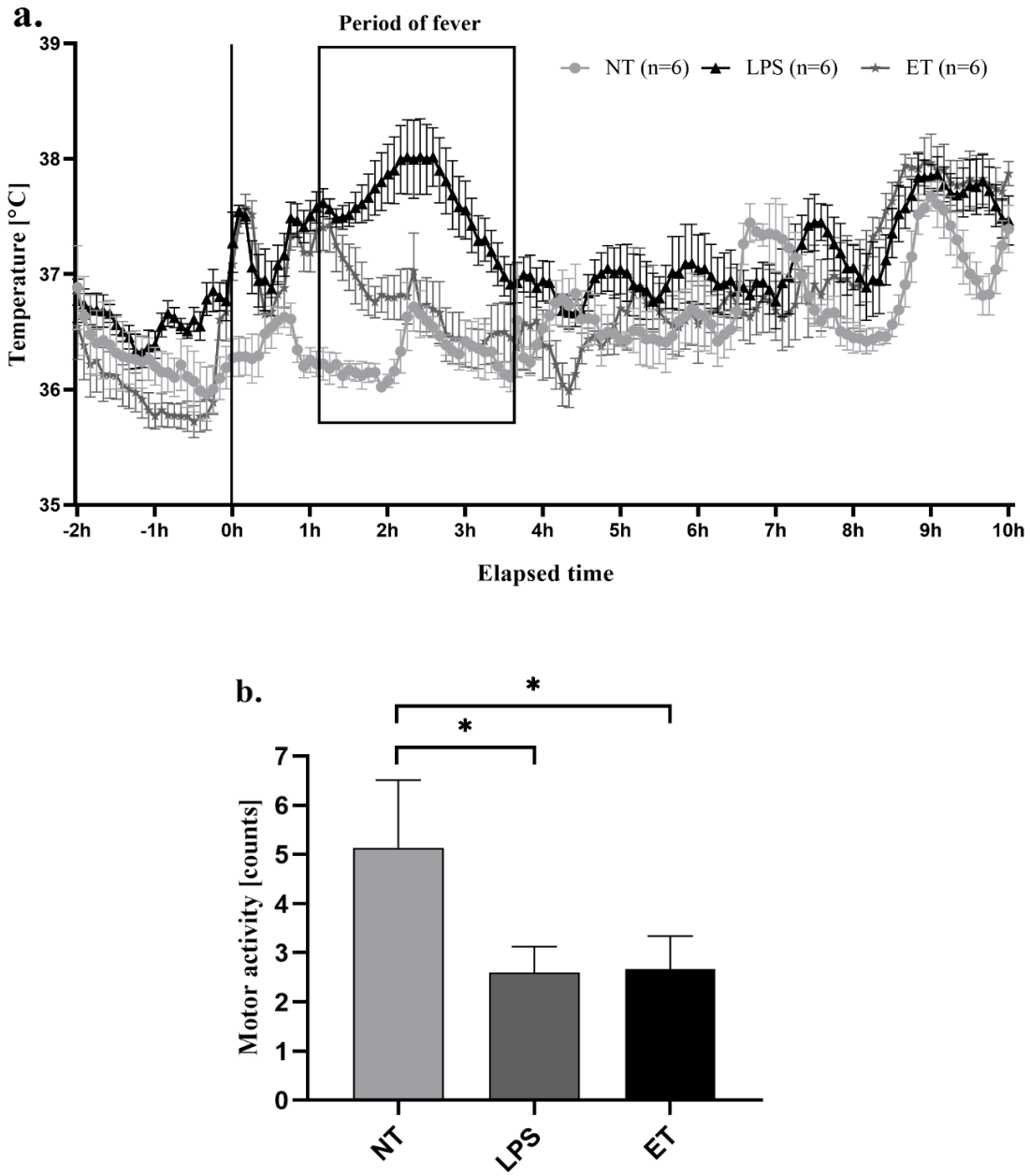

Fig. 2. Body temperature and motor activity over a 12-hour period in mice under three different experimental conditions: non-treated (control) mice (NT); mice injected with a single dose of LPS (50 µg/kg, i.p.) to induce fever (LPS); endotoxin tolerant mice (ET). Representative time-course graph depicting core body temperature changes over a 12-hour period across the three groups (a). Motor activity recorded during the same 12-hour period, demonstrating differences in activity levels between groups (b). The asterisks indicate the significant difference between the groups indicated (*$p < 0.05$). Each group consisted of n = 6 mice. Data are presented as mean ± SEM.

The absence of a systemic febrile response, a hallmark of ET, indicates successful immune reprogramming, thereby validating the robustness of the approach. In subsequent

experiments, breast cancer was induced in ET mice and its progression was compared to that in non-endotoxin-tolerant counterparts.

Microscopic evaluation of 4T1-induced mammary tumors in mice

Six days after 4T1 cells injection we observed breast tumors in mice. All the samples show solid sheets of proliferating epithelial cells, polygonal to oval shaped, with poorly demarcated margins and scant cytoplasm. Nucleus are vesicular and with coarsely chromatin and a single central basophilic nucleus. There is a very fine fibrovascular connective tissue that rarely subdivide the neoplasm into lobules. There is no tubular differentiation and the neoplastic cells are pleomorphic with numerous mitoses. The majority of ETBC mice were consistent with solid carcinoma. Both ETBC and BC groups showed numerous cells with large and small vacuoles in the cytoplasm and nucleus are often located at the periphery of the cell. These are consistent with lipid-rich carcinoma (3a-b). Necrotic areas are present in all the samples with different amount and were surrounded by a small amount of macrophages and very few neutrophils (Fig. 3c-f).

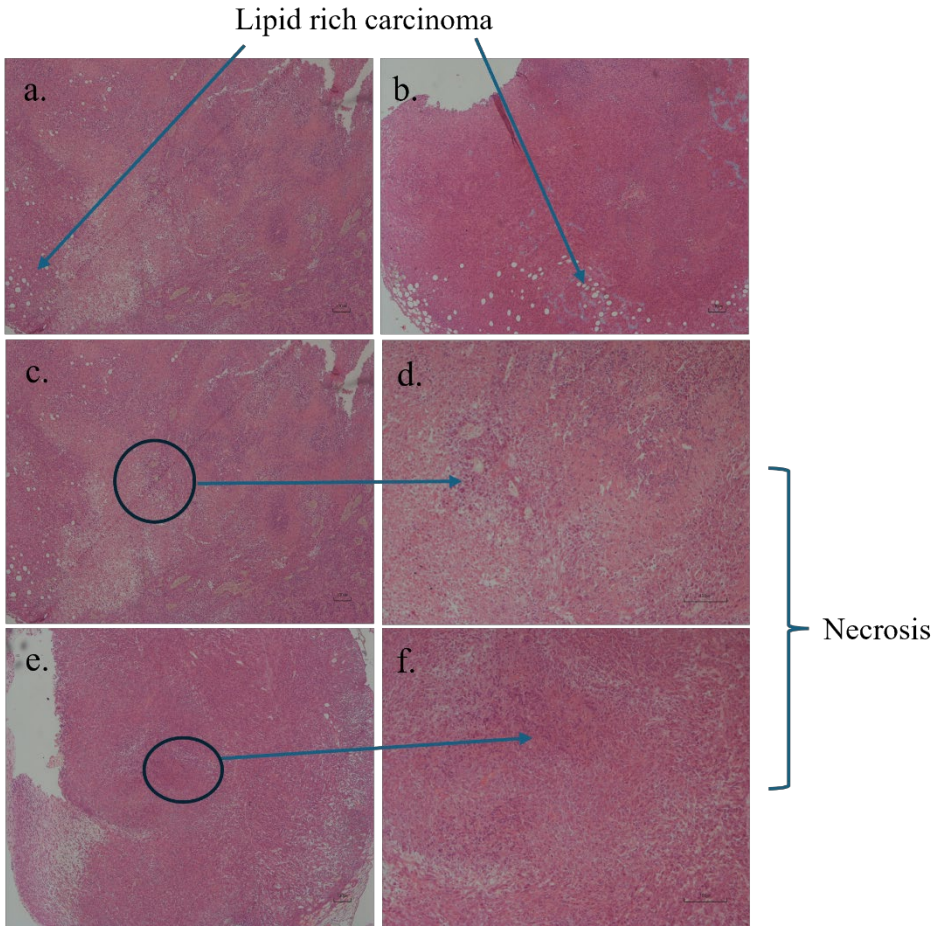

Fig. 3. Representative images of breast tumor tissues. Lipid-rich carcinoma was observed in both the breast cancer-bearing mice (BC; (a)) and endotoxin tolerant breast cancer bearing-mice (ETBC; (b)). Necrotic areas were identified in both BC (c, d) and ETBC (e, f) animals. Necrotic tissue images were

captured at 4× and 10× magnification, respectively, for both groups. Histological analysis was performed on six mice per group.

Endotoxin tolerance influences the survival and tumor growth of the mice

Having a stable model of ET manifested by the absence of fever, we studied its role in modulating tumor progression and overall survival outcomes in an experimental model of breast cancer. We observed a difference in the survival capacity of ETBC mice in comparison with BC group animals (Fig. 4a). Although the difference was not statistically significant ($p = 0.0710$), mice ETBC reached an advanced stage of the experiment more rapidly. Notably, after reaching the advanced stage of cancer, their condition deteriorated at a faster rate compared to BC mice (data not shown). The condition of none of these mice allowed for the continuation of observation beyond 40 days from the induction of breast cancer. Whereas, BC mice exhibited slower disease progression, and their condition allowed for observation for up to 45 days (Fig. 4a).

Mice in the ETBC group exhibited a more rapid increase in tumor volume, indicating that ET may enhance tumor progression. This accelerated tumor growth was accompanied by earlier onset of advanced disease stages, requiring humane termination of the experiment. In contrast, tumor progression in the BC group was slower, with some mice maintaining a stable condition for a longer duration. The following data was derived from three time points recorded during our observation study, with the final measurement being day 28. The tumor volume of the BC group on day 22 was observed to be 18.4 ± 1.8 mm and it reached 33 ± 2.8 mm³ by day 28. On the other hand, the mean tumor size in the ETBC group was 40.1 ± 7.5 mm³ on day 22, which then reached 67.4 ± 21.4 mm³ by day 28. The comparison of non-linear regression curves revealed a significant difference between groups ($p < 0.001$) (Fig. 4b). These findings suggest that ET can exacerbate tumor growth dynamics, potentially through alterations in the immune response.

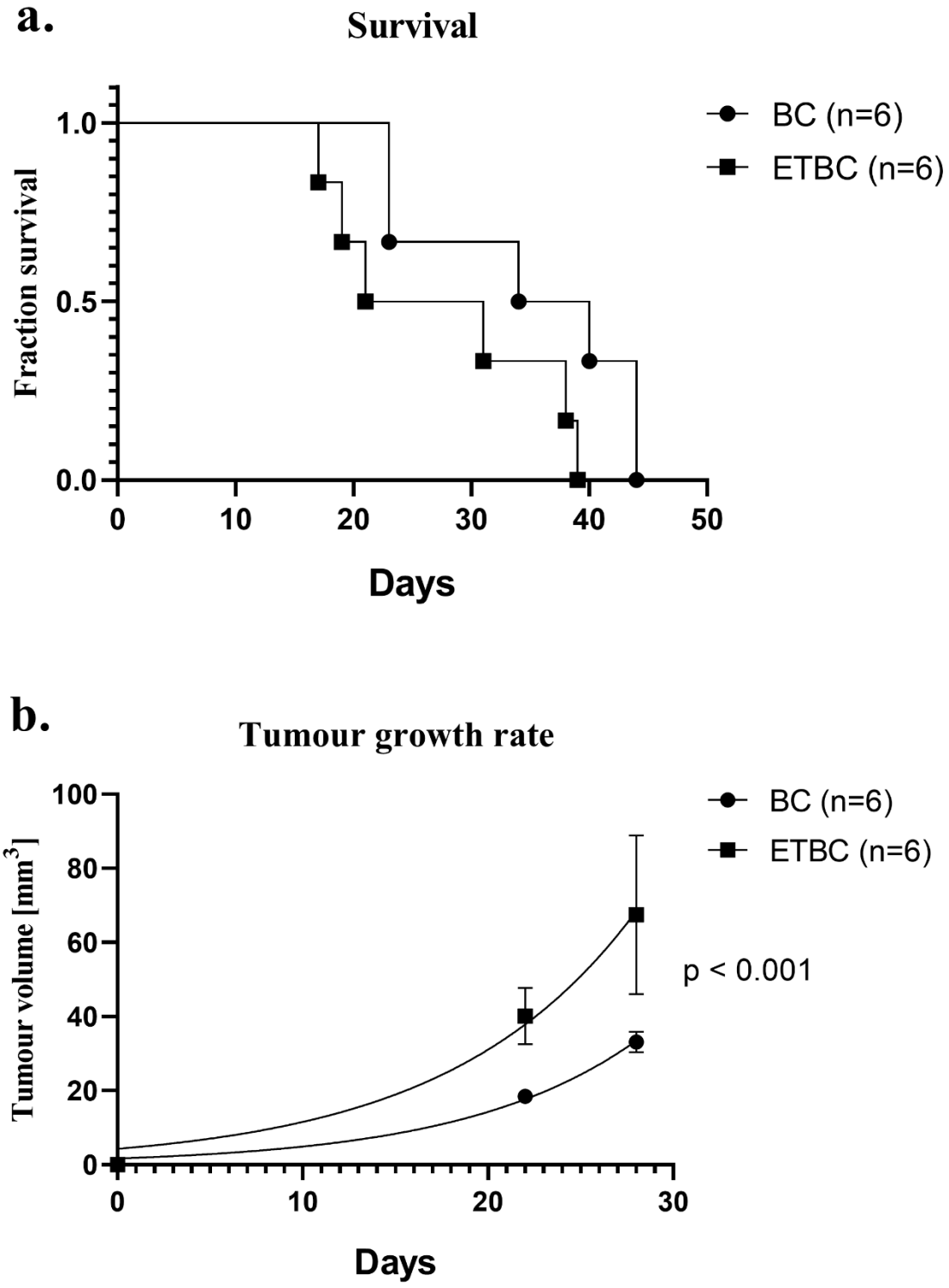

Fig. 4. Kaplan–Meier mice survival curve (a) and tumor volume (b) in cancer-bearing mice (BC) and mice with endotoxin tolerance and cancer (ETBC). The experiments were performed on 6 individuals in each group (n=6). The comparison of non-linear regression curves revealed a significant difference between groups ($p < 0.001$).

Impaired leukocyte recruitment in endotoxin tolerant mice with cancer

To better understand how ET influences immune system function, the analysis of white blood cells in four groups of mice was conducted. We observed that the general leukocyte count in NT and ET mice was almost similar ($p > 0.99$), whereas a significant increase was observed in the BC mice and ETBC animals ($p < 0.001$) (Fig 5a). A detailed analysis of individual leukocyte populations revealed that this increase was observed across all measured leukocyte populations, including lymphocytes, monocytes, and granulocytes (Fig. 5b-d, respectively). Interestingly, the leukocyte counts in cancer-bearing mice was found to be significantly lower in ETBC animals when compared to BC mice ($p < 0.001$).

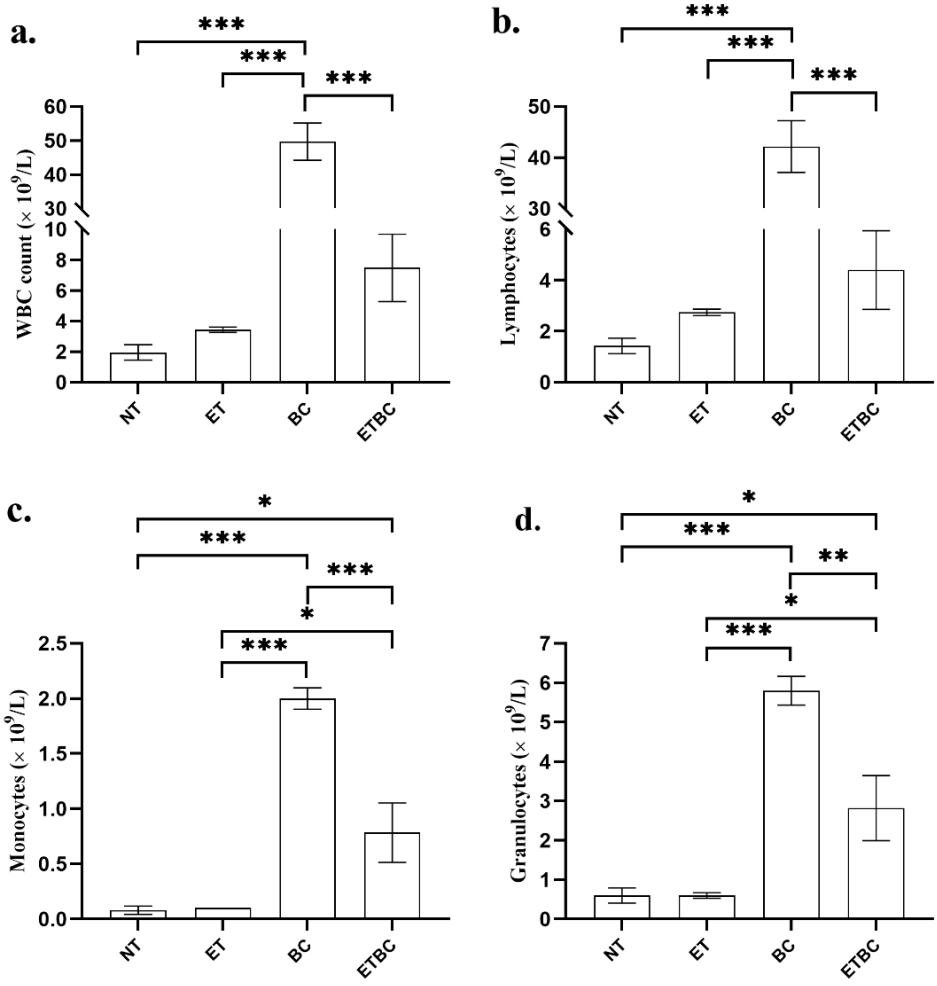

Fig 5. Effect of endotoxin tolerance (ET) and breast cancer on the total count of white blood cells (a), lymphocytes (b), monocytes (c) and granulocytes (d). Figure represents the mean ± SEM of the leukocytes, measured in mice. The asterisks (*) indicate the significant difference between the groups

indicated (***p < 0.001, **p < 0.01, *p < 0.05). Control animals (NT; n=5), endotoxin tolerant mice (ET; n=5), cancer-bearing mice (BC; n=6) and cancer-bearing mice with ET (ETBC; n=6).

Tumor-induced splenomegaly is not observed in endotoxin tolerant mice

Spleen is as a major reservoir for immune cells and plays a crucial role in the regulation of immune responses. We observed distinct differences in spleen size between the groups, which further underscored the impact of ET on immune function. Spleen samples collected from BC mice were noticeably larger, indicating splenomegaly. Notably, spleens from the ETBC animals weighed significantly less compared to those from the BC ($p < 0.01$). Additionally, the spleen weight in BC mice was significantly higher than in the NT and ET groups ($p < 0.01$) (Fig. 6).

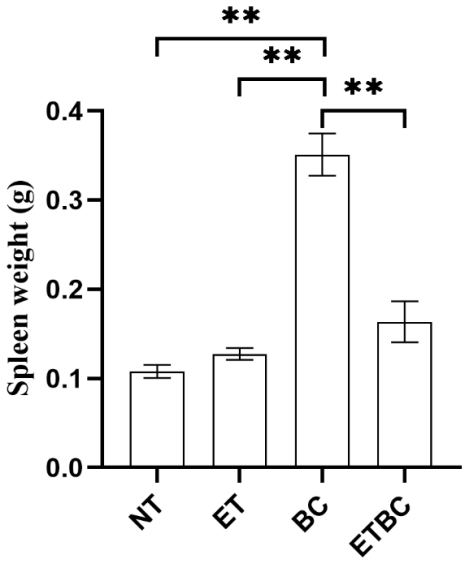

Fig 6. Effect of endotoxin tolerance (ET) and breast cancer on the spleen weight. Figure represents the mean ± SEM of the spleen weight in each group. The asterisks indicate the significant difference between the groups indicated (**p < 0.01). Control animals (NT; n=5), endotoxin tolerant mice (ET; n=5), cancer-bearing mice (BC; n=6) and cancer-bearing mice with ET (ETBC; n=6).

Endotoxin tolerance and breast cancer modulate immune-related genes in spleen

The blood morphology results show some abnormalities, therefore the gene expression analysis of immune-related genes in spleens was carried out. We examined the expression of pro-inflammatory genes, including IL-6 (Fig. 7a), IL-1β (Fig. 7b), COX-2 (Fig.7c), VEGF (Fig. 7d), INF-γ (Fig. 7e), and NOS2 (Fig. 7f). Additionally, we investigated the expression of genes associated with immune regulation or inflammation: IL-10 (Fig. 7g), STAT6 (Fig. 7h), and CSF-1 (Fig. 7i). The results showed a significant decrease in the expression of IL-6 and INF-γ

in mice with ETBC mice when compared to BC animals ($p < 0.001$, $p < 0.01$, respectively). However, when compared to ET group, the expression of IL-6 and INF-γ in the group of ETBC remained significantly increased ($p < 0.001$).

Moreover, an increase in expression of NOS2 ($p < 0.001$), IL-1β ($p < 0.001$), STAT6 ($p < 0.001$), CSF1 ($p < 0.001$) and COX-2 ($p < 0.001$) was observed in ETBC group when compared to BC group. The expression of the genes NOS2, IL-1β and COX-2 also remained significantly higher in ETBC group when compared to NT ($p < 0.001$) and ET groups ($p < 0.05$, $p < 0.001$, $p < 0.01$ respectively). Additionally, IL-10 expression, though insignificant, has been observed to be higher in ETBC group when compared to NT group ($p > 0.6$) and significantly higher when compared to ET group ($p < 0.01$). Similarly, STAT6 and CSF-1 expression was observed to be significantly higher in ETBC group when compared to NT group ($p < 0.001$) (Fig 7).

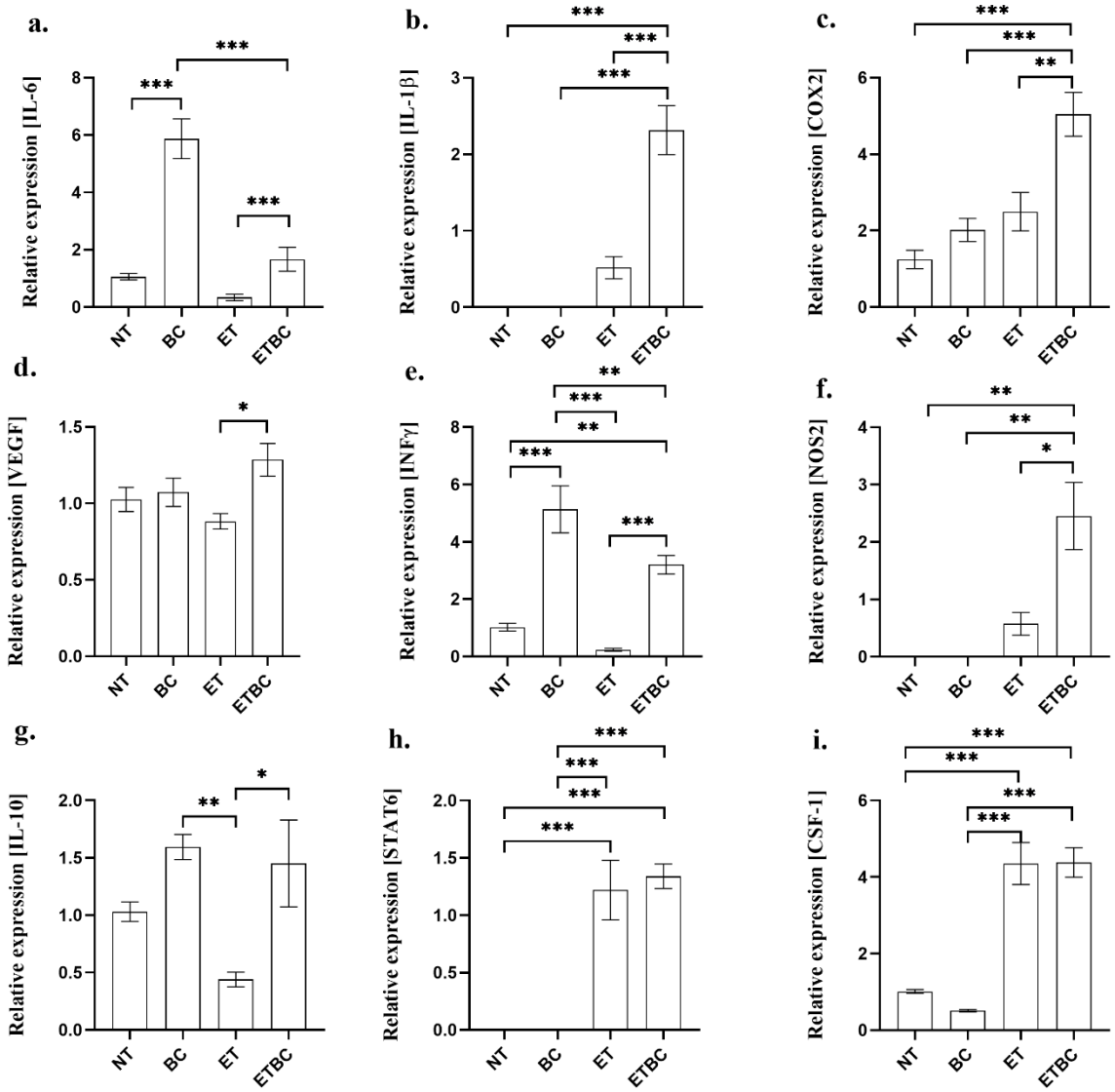

Fig 7. Expression of immune-related genes: IL-6 (a), IL-1β (b), COX-2 (c), VEGF (d), INF-γ (e), NOS2 (f), IL-10 (g), STAT6 (h), and CSF-1 (i) in spleen from the following groups of animals: control mice (NT; n=10), endotoxin tolerant mice (ET; n=10), cancer-bearing mice (BC; n=14) and cancer-bearing mice with ET (ETBC; n=14). The asterisks indicate the significant difference between the groups indicated (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

Endotoxin tolerance modulates immune-related genes in tumour tissues

Finally, we wanted to determine whether ET affects the expression of immune-related genes within the tumor itself. We analyzed the expression of IL-10, NOS2, IL-1β, VEGF, COX-2, CSF-1, CD206, STAT6 (Fig. 8a-h, respectively) in the tumor tissues from BC and ETBC mice. We observed a significant increase in the expression of IL-10, NOS2, IL-1β, VEGF and COX-2 in the ETBC mice when compared to the BC mice ($p < 0.01$, $p < 0.001$). Although statistically insignificant, the expression of CSF-1, CD206, and STAT6 was higher in the ETBC group compared to the BC group (Fig 8).

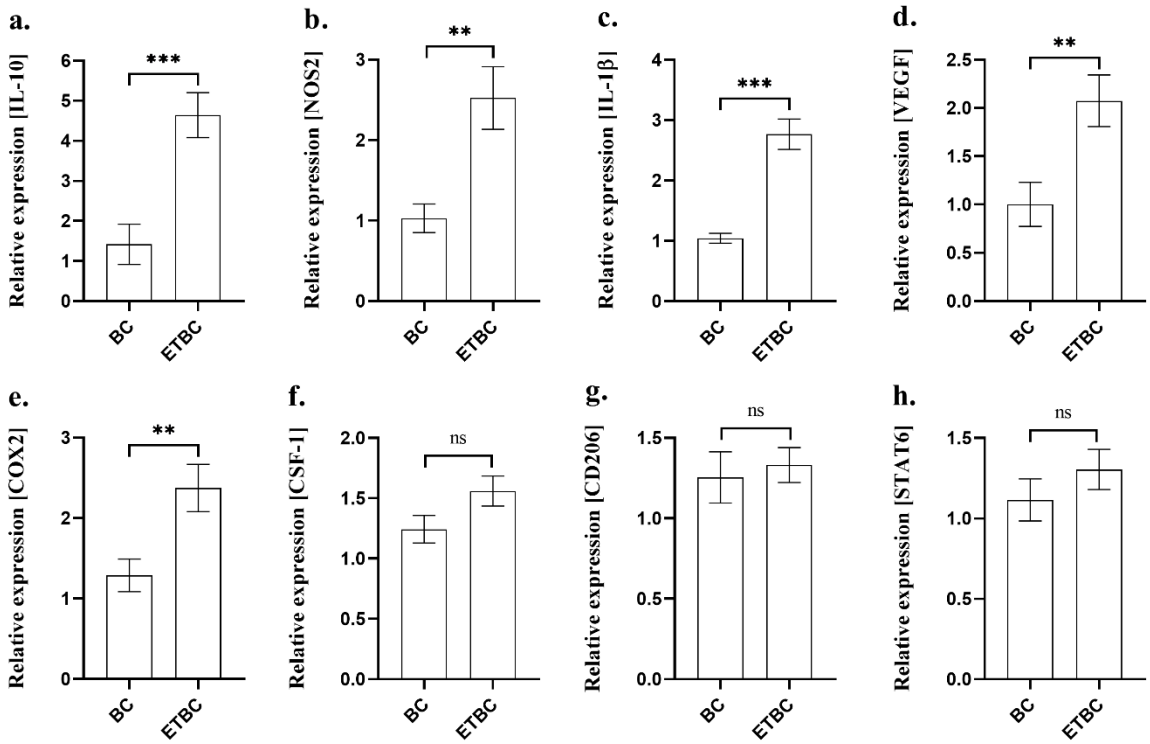

Fig 8. Expression of immune-related genes: IL-10 (a), NOS2 (b), IL-1β (c), VEGF (d), COX-2 (e), CSF-1 (f), CD206 (g) and STAT6 (h) in tumor tissues from the following groups of animals: cancer-bearing mice (BC; n=14) and cancer-bearing mice with ET (ETBC; n=14). The asterisks indicate the significant difference between the groups indicated (**$p < 0.01$, ***$p < 0.001$; ns – not significant).

Discussion

Endotoxin tolerance (ET) is a phenomenon in which the immune system becomes less responsive to endotoxins after repeated exposure, leading to alterations in immune function. Using a mouse model of ET, we confirmed the absence of fever following the administration of a pyrogenic dose of endotoxin. This is a hallmark feature of ET, indicating immune system reprogramming [19]. The inability to generate fever is particularly noteworthy, as it may indicate underlying immune alterations. These changes could have significant implications for infection management and, less commonly discussed, cancer development. Little attention has been paid to the atypical infection courses in oncology patients, often marked by rare or absent febrile episodes. In this study, we are testing on mice the hypothesis that cancer patients may have developed ET, potentially creating a tumor-friendly environment in the body.

As expected, our endotoxin tolerant mice did not develop fever in response to a pyrogenic dose of LPS. Moreover, reduced locomotor activity was observed after each LPS injection, even after the development of ET. These findings indicate that ET selectively dampens the febrile response, while sickness behavior, such as reduced locomotor activity, persists. This suggests that distinct mechanisms may underlie different components of the systemic response to LPS.

In the context of cancer, we observed that ETBC mice showed more rapid tumor progression and earlier disease onset compared to BC group. The condition of these mice deteriorated at a faster rate after reaching advanced stages of cancer, suggesting that ET creates an environment that exacerbates tumor growth. This finding supports previous studies which indicated that immune reprogramming, as a result of ET, might undermine the immune system's capacity to fight cancer, thereby promoting tumor growth [10,20-22].

In our study, we analyzed blood morphology to provide a direct measure of changes in immune cell populations and overall immune function. By analyzing parameters, such as leukocyte count and the distribution of different immune cell types, we were able to identify potential alterations in immune responses caused by ET. We found a significant difference in the leukocyte counts between ETBC and BC mice. In the BC group, there was a notable increase in leukocyte populations, including lymphocytes, monocytes, and granulocytes, which are typically recruited as part of the immune response to tumor growth. The increase in leukocytes in these mice is consistent with the tumor-associated inflammation that recruits immune cells to fight the tumor [23-27]. In contrast, ET led to a suppression of leukocyte recruitment in the ETBC group. Specifically, the leukocyte count was significantly lower in

ETBC mice compared to BC mice, suggesting that ET may impair immune cell mobilization, resulting in an ineffective immune response to cancer [28-31].

Additionally, we investigated the role of the spleen, a key organ for immune cell storage and activation. The spleen plays a critical role in regulating immune responses, especially in inflammation and infection [32–35]. We observed that in BC mice, the spleen weight was significantly increased, reflecting enhanced immune cell recruitment as part of the inflammatory response to tumor growth. However, in the ETBC group, despite the presence of cancer, the spleen weight was significantly reduced. This suggests that ET might impair immune cell mobilization or alter the immune function of the spleen, which could suppress the overall immune response to the tumor and contribute to more rapid tumor progression. The reduced spleen size and lower leukocyte count in ETBC mice further supports the notion that ET hampers immune system effectiveness in combating cancer [10,19].

To evaluate changes in gene expression related to cancer and the inflammatory response we analyzed RNA isolated from the spleens. Our findings revealed a significant reduction in the expression of IL-6 and INF-γ in the ETBC group compared to the BC group, suggesting that ET suppresses pro-inflammatory cytokines that typically contribute to immune activation. However, these levels remained significantly higher in the ETBC group compared to the ET, indicating that cancer may still modulate the immune response even in the presence of ET [36,37]. Interestingly, the ETBC group exhibited elevated expression levels of NOS2, IL-1β, and COX-2, which are associated with inflammation and immune regulation. These cytokines are involved in both promoting and resolving inflammation, suggesting that ET may lead to an altered immune environment, potentially contributing to the immune evasion of the tumor [38-40]. This complex immune modulation highlights the interaction between ET and cancer, where ET does not entirely suppress the inflammatory response but may instead redirect it in a way that enhances tumor progression.

The analysis of immune-related gene expression in tumor tissues also revealed that ET influences the tumor microenvironment by increasing the expression of IL-10, NOS2, IL-1β, VEGF, and COX-2 in the ETBC group compared to the BC group. These genes play a crucial role in tumor progression, inflammation, and immune regulation, and their elevated expression in ETBC mice suggests that ET may enhance certain inflammatory pathways within the tumor [41-43]. The expression of CSF-1, CD206, and STAT6 remained higher in the ETBC group, although these differences were not statistically significant, indicating a potential trend toward immune regulation, which requires further investigation [44-46]. Overall, these findings

suggest that ET may alter the immune and inflammatory environment within the tumor, potentially influencing cancer progression and the immune response to the tumor.

Conclusions

In summary, our study demonstrates that ET, characterized by the absence of fever, may lead to immune dysregulation, impairing the body's ability to mount an effective defense against tumors. This immune reprogramming may contribute to enhanced tumor progression, as evidenced by the more rapid growth and earlier disease onset in endotoxin tolerant mice. Furthermore, ET alters immune responses at both the systemic and local levels, influencing leukocyte counts, spleen size, and gene expression profiles related to inflammation and immune regulation. These findings highlight the complex interactions between ET and cancer, suggesting that ET may create an immune environment that favors tumor growth.

Moreover, our results increasingly point toward a possible explanation for why oncology patients often have fever-free medical histories [11,12,47]. The absence of fever may reflect an underlying state of ET, leading to immune suppression and facilitating tumor progression. Conversely, the occurrence of fever during cancer, which indicates the overcoming of tolerance, could play a crucial role in triggering immune activation and potentially driving tumor regression.

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10. Discussion

Cancer has been reported as the second leading cause of death worldwide, with lung, breast, and colorectal cancers ranking as the top three types, respectively. Despite significant advances in cancer therapies, the International Agency for Research on Cancer (IARC) predicts that by 2040, the global number of new cancer cases will rise to approximately 28 million, according to the GLOBOCAN 2020 report (141). Due to this scenario, it has become essential to develop more effective strategies for cancer prevention, early detection, and innovative treatments to address the growing burden of cancer. Even though researchers have been studying cancer for a long time, gaining a deeper understanding of the immune system's role in this disease is essential. This is particularly important due to the immune evasion mechanisms employed by cancer cells, which enable them to survive even in the presence of the immune cells.

Endotoxin tolerance is an adaptive immune phenomenon that occurs due to the repeated exposure of the body to endotoxin, such as LPS (142). This type of immune suppression can be beneficial in preventing an exaggerated immune response in certain disease conditions, such as sepsis (28,29). However, contrasting evidence suggesting that immune suppression may be harmful to patients in later and more severe cases of sepsis has been presented by Otto et al. (2011) and Schefold et al. (2008) (143,144). Given the dichotomous nature of ET and, particularly, its immunosuppressive character, we hypothesized that ET may contribute to unfavorable outcomes in the context of cancer development. Importantly, one of the major hallmarks of ET is the absence of fever. Notably, a previous study by team members with whom I conducted my doctoral research found that cancer patients had a lower fever incidence than healthy individuals (145). ET is likely associated with this phenomenon. Although the therapeutic potential of fever induction via bacterial endotoxins in cancer treatment has been explored (104,106,146), the experimental evidence supporting this approach remains inconsistent (147-149). Therefore, my thesis's general aim was to study ET's influence on cancer development. To achieve this, I focused on three key tasks during my research, which I discuss below.

Aim 1. *The impact of endotoxin tolerance on macrophages and cancer cells (Article #1)*

In my research, I have focused on macrophages, key cells of the innate immune system, which are widely recognized for their central role in the immune response to LPS (150). Depending on their polarization state, M1 or M2, macrophages can exhibit anti-cancer or pro-cancer properties. The M1 phenotype is typically characterized, among others, by the

expression of surface marker CD80, while the M2 phenotype is marked by CD163 expression (131). Additionally, cytokines, such as TNF-α and IL-6, are commonly used to assess macrophage activation status (151).

Induction of ET *in vitro* is often identified by a reduction in the expression of pro-inflammatory cytokines (i.e., TNF-α and IL-6) (152), indicating a dampened inflammatory response. For this research, I established a model of endotoxin tolerance by stimulating the macrophages with an initial dose of LPS (100 ng/mL) for 24 hours, followed by a second identical concentration for an additional 24 hours. This model is consistent with other studies reporting that a second endotoxin challenge induces a suppressed inflammatory response (153), a phenomenon also observed in our research. Specifically, we noted a significant decrease in the expression levels of cytokines (TNF-α, IL-6) in Mo_{ET} (articles #1 and #2) compared to Mo_{LPS}. I also assessed the phenotype of the macrophages to further confirm their polarization and functional response following LPS challenge. Interestingly, this analysis revealed that Mo_{ET} predominantly express CD80 rather than CD163, thus pointing towards the M1 phenotype. These results are in accordance with observations by Pena et al. (2011) (154), who reported similar dissociation between the expression of pro-inflammatory cytokines and CD206 (M2). However, it is important to consider that the macrophages in this experimental setting are not exposed to the tumor microenvironment, which plays a crucial role in influencing immune cells towards either anti-tumor or pro-tumor characteristics. Therefore, I used an experimental approach that mimicked the tumor microenvironment to understand how ET affects cancer development. During these experiments, I assessed cancer cells for their survival capacity, clonogenic potential and migration capacity following stimulation with CM obtained from the Mo_{ET}. I also evaluated the influence of ET on the 3D spheroidal cancer cell model to assess its effect on solid tumors, which are known for their increased resistance to anti-cancer therapies. This approach allows for a more comprehensive analysis, considering factors such as cell-cell interactions, cell-matrix interactions, and the three-dimensional structure of tissues (155,156). The results revealed increased survival capacity, colony formation capacity and migration capacity in both cancer models, i.e., 4T1 breast cancer cells and CT26 colon cancer cells, when exposed to CM_{ET}. These findings suggest that ET may contribute to creating a tumor-supportive microenvironment. In ET, macrophages have exhibited the pro-tumorigenic potential characterized by suppressed expression of pro-inflammatory cytokines and an enhanced anti-inflammatory cytokine profile (142,153). Thus, suggesting that ET may lead to the secretion of certain pro-tumorigenic factors, which influence the characteristics of cancer cells. Interestingly, ET exhibited different effects on

cancer spheroids obtained from breast cancer and colon cancer cells, as no significant effect was observed in colon cancer. This effect may be due to the continuous exposure of colon cancer cells to the LPS from the intestinal bacteria (157-159). Finally, the further evaluation of the influence of ET revealed that the co-culture of Mo_{ET} and cancer cells exhibited significantly reduced expression of the pro-inflammatory cytokines, indicating an immunosuppressed environment.

By considering the data obtained during my research, I observed:

- a significant suppression of TNF-α and IL-6 in both mono-cultures of Mo_{ET} and co-cultures of these macrophages with cancer cells,
- an increase in survival capacity of 4T1 and CT26 cancer cells when they were cultured in CM obtained from Mo_{ET} in comparison with those cultivated in CM from Mo_{NT},
- enhanced motility, colony formation capacity and spheroidal growth of cancer cells cultured in CM derived from Mo_{ET} compared to those cultivated in CM obtained from Mo_{NT},
- elevated expressions of CD80 in Mo_{ET}, a marker typically associated with the M1 macrophage phenotype.

Aim 2. *The effect of the tumor microenvironment on endotoxin-tolerant macrophages (Article #2)*

My research revealed the pro-tumorigenic effect of endotoxin-tolerant macrophages, as detailed in Article #1. Furthermore, my *in vivo* studies proved the infiltration of macrophages into tumor tissues in both tolerant and non-tolerant mice (articles #2). Therefore, understanding how the tumor microenvironment influences macrophages was also crucial. To explore this, I conducted a series of *in vitro* studies to evaluate how exposure to cancer-derived factors alters macrophage phenotype and function while considering their endotoxin tolerance status.

In this research, I analyzed the behavior of the Mo_{ET} in tumor microenvironment through the assessment of production of pro-inflammatory mediators, metabolic profile and the phenotypic characteristics of these cells. Building on my previous research, which established that Mo_{ET} exhibit a blunted inflammatory response, I further investigated this phenomenon by analyzing the expression of key inflammatory markers, such as CD14 and COX-2. CD14 is a co-receptor of TLR4, crucial to LPS sensing (40,160), whereas COX-2 is involved in synthesizing pro-inflammatory prostaglandins (161,162). I observed no significant changes in CD14 in the Mo_{ET}, which was similar to the observations of Martin et al. (2001) (163).

However, a significant reduction of COX-2 was noticed. Next, I evaluated NO production, a key mediator associated with the inflammatory response. NO has been reported to have a dual role in cancer: at higher levels, it exhibits cytotoxic effects, whereas at lower levels, it promotes tumorigenesis (164-166). My research further supported this finding, as Mo_{ET} produced lower NO levels upon exposure to the cancer microenvironment than control macrophages, i.e., macrophages treated once with LPS (Mo_{LPS}). This reduction in NO level was accompanied by the decreased expression of iNOS, the enzyme that catalyzes the NO production (167,168).

Given the observed suppression of NO production, I next examined the level of ROS, another critical mediator of the primary inflammatory response (169). I found elevated ROS production in Mo_{ET}, which has been reported to contribute to a pro-tumorigenic environment (170,171). These observations regarding the pro-inflammatory factors established that Mo_{ET} promoted a favorable condition for tumor development. However, one aspect that remained unclear was the classification of the M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophage phenotypes. This was due to Mo_{ET} dominant expression of CD80, a marker typically associated with the M1 phenotype (article #1), despite their overall immunosuppressive and pro-tumorigenic behavior. The observed discrepancy suggests that the traditional M1/M2 classification may not fully capture the functional complexity of macrophages in this context. Therefore, to further clarify this, I evaluated the phenotype of Mo_{ET} in the cancer environment through a co-culture assay. This revealed a shift in the Mo_{ET} phenotype from M1 to M2, as evidenced by an increased number of cells expressing CD163. The M2 phenotype is commonly associated with immune suppression and tumor progression, suggesting that the tumor microenvironment may play a critical role in modulating macrophage polarization towards a pro-tumorigenic state (172,173).

Additionally, I examined the survival capacity of Mo_{ET} within the cancer microenvironment. These macrophages exhibited increased survival capacity upon stimulation with CM_{4T1}, compared to Mo_{LPS} cells. It is suggested that ET may enable these macrophages to persist within the tumor microenvironment, potentially modulating the immune landscape, in a way that supports cancer progression. These findings provide valuable insight into how ET macrophages may contribute to tumor development and immune evasion dynamics.

The intriguing nature of ET becomes even more fascinating when considering my findings on the metabolic profile of Mo_{ET} stimulated with CM_{4T1}. The mechanisms underlying TAMs' metabolism and polarization remain poorly characterized. To gain further insight into the effects of ET, I conducted SCENITH analysis to assess the metabolic profile of Mo_{ET}. The data revealed that Mo_{ET} exhibit considerable metabolic flexibility. Although these cells were

found to be heavily dependent on glycolysis, which aligns with various studies indicating that glycolysis is the primary source of ATP for sustaining tumor cell growth (174,175), it is noteworthy that the M2 phenotype has been associated with enhanced oxidative phosphorylation (OXPHOS) (176). Interestingly, in my research, the Mo_{ET} appear to activate compensatory metabolic signaling in response to the inhibition of oxidative phosphorylation. This suggests that Mo_{ET} may rely on alternative metabolic pathways to maintain cellular function and survival, highlighting their metabolic adaptability in the tumor microenvironment. Finally, based on my findings, Mo_{ET} within the tumor microenvironment exhibit distinct features when compared to the Mo_{LPS}, which were manifested by:

- suppression of pro-inflammatory mediators, such as NO, iNOS and COX-2,
- elevation of ROS levels,
- increased survival capacity of macrophages,
- increased M2 marker (CD163) expression,
- notable metabolic flexibility.

Aim 3. *The effect of endotoxin tolerance on tumor development in mice (Article #3)*

Building on my *in vitro* research, which demonstrated ET's role in promoting pro-tumorigenic conditions, I aimed to extend these findings *in vivo* to understand better how ET influences tumor development within the context of the whole organism. In my research, I based my analysis on the fact that the absence of fever is a key indicator of ET *in vivo* (15). Fever, a hallmark of the inflammatory response, has been implicated in cancer biology. Research has shown that cancer patients tend to experience fewer febrile episodes (145,146,177). Additionally, a case study described a patient with stage four melanoma, who entered remission following the deliberate induction of fever (178), suggesting a potential link between febrile responses and anti-tumor activity. Since my *in vitro* study primarily focused on a breast cancer cell line, I decided to proceed with an *in vivo* breast cancer model to maintain consistency in my investigation.

Firstly, the important part of this study was establishing a model of ET *in vivo.* To achieve this goal, the mice were treated with four consecutive doses of LPS, and the absence of the febrile response was assessed to evaluate the development of ET. I also observed that the mice with ET (similar to mice subjected to a single LPS exposure) exhibited lower motor activity when compared to the untreated control group, which indicated a systemic effect of both fever and endotoxin tolerance. However, it is noteworthy that while endotoxin tolerance

attenuates the febrile response, sickness behaviors, such as reduced locomotor activity, remain comparable to those observed in mice exposed to LPS only once. This implies that separate regulatory pathways may govern the various aspects of the systemic reaction to LPS.

Following the successful establishment of the ET model, I proceeded to investigate how ET influences cancer development by introducing tumor cells into both ET and control mice and monitoring tumor progression. My *in vitro* research (articles #1 and #2), along with previous studies, has shown that ET may impair the immune system's capacity to combat cancer (179,180). This was further supported by my *in vivo* findings (article #3), where ETBC group of mice demonstrated an earlier onset of tumor development and significantly accelerated tumor growth compared to mice that were non endotoxin-tolerant (BC group of mice). ETBC mice also experienced a faster deterioration of health conditions, which indicated that ET was indeed playing a critical role in creating a tumor friendly environment. This impairment of the immune response was also proven by analyzing the other parameters, such as the leukocyte count, which was significantly reduced in ETBC compared to BC mice. Importantly, the increase of the leukocyte population, specifically lymphocytes, monocytes, and granulocytes, can be linked to the tumor-associated inflammation that recruits these immune cells in an attempt to combat cancer (181,182).

To gain further insight into the systemic immune response, I analyzed spleen tissues from the experimental groups, as the spleen plays an important role in inflammation (183). Analysis of this organ from the ETBC and BC groups showed a significant difference in the spleen size, with BC mice showing markedly enlarged spleens compared to the ETBC group. This data suggests that there was an enhanced inflammatory response in the BC group compared to the ETBC group, thereby implying a subdued immune reaction due to ET.

The spleen tissues were also assessed for the expression of genes related to inflammatory response and cancer progression. I analyzed the expression of IL-6, IL-1β, COX-2, VEGF, INF-γ, iNOS, IL-10, STAT6, and CSF-1. My findings revealed that while the expression levels of IL-6 and IFN-γ were suppressed in the ETBC group compared to the BC mice, they remained significantly higher than those observed in the ET group. These findings suggest that although the pro-inflammatory immune response is dampened during ET, the presence of cancer modulates this response further, resulting in a reduced, yet not entirely suppressed, inflammatory activity. On the other hand, the gene expressions of IL-10, NOS2, IL-1β, COX-2, STAT6 and CSF-1 remained significantly high in the ETBC. All these genes are involved in shaping the immune response, including STAT6 and CSF-1, which are key regulators of macrophage recruitment and polarization (121). These findings suggest that

despite the establishment of ET, key immunoregulatory pathways remain active in the ETBC group. However, their response in the tumor environment may be altered, thus leading to immune evasion of the cancer cells.

In this study, I also analyzed the expression of the immune-related genes, such as IL-10, NOS2, IL-1β, VEGF, COX-2, CSF-1, CD206, and STAT6 in the tumor tissues derived from the ETBC and BC groups of mice. This analysis revealed that the expression of IL-10, NOS2, IL-1β, VEGF and COX-2, which play an essential role in the immune landscape related to the tumor progression, inflammation, and immune regulation, remains elevated in the ETBC animals. Notably, the sustained upregulation of VEGF, a critical factor in tumor growth and metastasis (184,185), suggests the persistence of a pro-tumorigenic and immunoregulatory environment due to the induction of ET. On the other hand, there were no significant changes in the expression of CSF-1, CD206, and STAT6 among both groups of animals. Lastly, by considering all the findings I observed:

- absence of febrile response and decreased motor activity in ETBC mice in response to a pyrogenic dose of LPS,
- early tumor initiation and faster tumor growth in ETBC animals compared to BC animals,
- faster deterioration of health condition in ETBC mice compared to the BC group,
- lower leukocyte counts and smaller spleen size in ETBC mice than in BC animals,
- changes in the expression of immune-related genes in spleen and tumor tissues between ETBC and BC mice.

11. Summary and Conclusion

The studies presented in the dissertation explored the potential role of ET in cancer development. The results obtained during the research showed that:

a. ET impacts both systemic and local immune responses

The study demonstrates that ET affects immune responses at both systemic and local levels. This is evidenced by suppressed pro-inflammatory mediators, altered leukocyte populations, and changes in immune-related gene expression in spleen and tumor tissues. Additionally, ET results in the absence of fever, decreased motor activity, and other systemic signs of immune dysregulation. Interestingly, different components of the systemic response to LPS are differentially regulated during ET, with fever being suppressed while sickness behavior persist, indicating the involvement of distinct underlying mechanisms.

b. ET alters macrophage functions toward a pro-tumorigenic phenotype and modifies metabolic and inflammatory pathways

ET significantly suppresses the production of pro-inflammatory cytokines (e.g., TNF-α, IL-6, IFN-γ), leading to a weakened immune response that may facilitate tumor growth. Macrophages undergoing ET adopt an M2-like, immunosuppressive phenotype, marked by CD163 expression and altered inflammatory mediator profiles, including decreased NO and increased ROS. Additionally, ET induces a shift in metabolic activity, further contributing to cancer progression by supporting tumor-promoting processes through complex reprogramming.

c. ET enhances cancer cell aggressiveness

Cancer cells exposed to an ET-induced environment exhibit increased survival, migration, clonogenic potential, and spheroid formation, indicating that ET fosters a tumor-supportive microenvironment.

d. ET accelerates tumor progression *in vivo*

In vivo findings showed that ET leads to enhanced tumor growth, reduced leukocyte counts, and significant changes in spleen and tumor tissue gene expression, reinforcing ET's role in fostering a cancer-permissive immune landscape.

All the above results lead to the main conclusion that **endotoxin tolerance reprograms macrophages, inducing significant alterations in the tumor microenvironment that create a tumor-promoting landscape, thereby facilitating cancer progression**. These findings underscore the intricate interplay between inflammation, immune environment, and tumor biology, suggesting that targeting ET could offer novel therapeutic opportunities for cancer treatment. Definitely, this finding highlights the need for further research into ET as a potential contributor to tumor progression. It is essential to verify whether counteracting ET could create a more hostile environment for tumors. If so, efforts should focus on: 1) tests for identifying ET as a potential target for cancer prevention, and 2) exploring strategies to overcome ET as an adjuvant approach in cancer therapy.

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14. Additional achievements

14.1. Publications

- Annamaria Antona; Marco Varalda; **Konkonika Roy**; Francesco Favero; Eleonora Mazzucco; Miriam Zuccalà; Giovanni Leo; Giulia Soggia; Valentina Bettio; Martina Tosi; Miriam Gaggianesi; Beatrice Riva; Simone Reano; Armando Genazzani; Marcello Manfredi; Giorgio Stassi; Davide Corà; Sandra D'alfonso; Daniela Capello. Dissecting the Mechanism of Action of Spiperone - A Candidate for Drug Repurposing for Colorectal Cancer. *Cancers, 14 (3)*, 776, **2022**.
- Marco Varalda; Annamaria Antona; Valentina Bettio; **Konkonika Roy**; Ajay Vachamaram; Vaibhav Yellenki; Alberto Massarotti; Gianluca Baldanzi; Daniela Capello. Psychotropic Drugs Show Anticancer Activity by Disrupting Mitochondrial and Lysosomal Function. *Frontiers in Oncology*, 10, 562196, **2020**.

14.2. Conferences

- **22/06/2023 – 23/06/2023 – Wrocław, Chemistry & Biotechnology International Conference, Poland**

  Oral presentation: Endotoxin tolerance and its influence on cancer development and behaviour.

  Konkonika Roy, Henryk Mikołaj Kozłowski, Tomasz Jędrzejewski, Justyna Sobocińska, Bartosz Maciejewski, Norbert Waga, Sylwia Wrotek
- **29/06/2023 – 30/06/2023 – Toruń, XVI Kopernikańskie Seminarium Doktoranckie, Poland**

  Poster presentation: Tolerancja endotoksynowa tworzy sprzyjające warunki do rozwoju nowotworu.

  Konkonika Roy, Bartosz Maciejewski, Norbert Waga, Sylwia Wrotek
- **17/06/2019 – Florence, 2nd Workshop Differentiation and Neoplastic Transformation SIB Group in collaboration with Protein SIB Group, Florence, Italy, 2019**

"Molecular, cellular and translational approaches to differentiation and neoplastic transformation"

Presentation: The Antipsychotic Drug Spiperone Reduces Cell Viability In Colorectal Cancer Through Induction Of Endoplasmic Reticulum Stress.

Annamaria Antona, Marco Varalda, Giulia Soggia, **Konkonika Roy**, Beatrice Riva, Suresh Velnati, Matilde Todaro, Gianluca Baldanzi, Giorgio Stassi, Armando Genazzani, Daniela Capello

- **28/10/2018 – 30/10/2018 – Novara, NO CANCER International Conference, Italy**

  Presentation: Spiperone, An Antipsychotic, Induces Colorectal Carcinoma Cell Death by A Calcium Mediated Apoptosis.

  Annamaria Antona, **Konkonika Roy**, Beatrice Riva, Suresh Velnati, Marco Varalda, Gianluca Baldanzi, Armando Genazzani, D.Capello

- **22/11/2018 – 24/11/2018 – Turin, ABCD meeting "Signal Transduction in Cancer", Italy**

  Presentation: Spiperone, An Antipsychotic, Induces Colorectal Carcinoma Cell Death by A Calcium Mediated Apoptosis.

  Annamaria Antona, **Konkonika Roy**, Beatrice Riva, Suresh Velnati, Marco Varalda, G. Stassi, M. Todaro, Gianluca Baldanzi, Armando Genazzani, D.Capello.

14.3. Additional funding received

- **29/06/23 - Grants4Students -** grant awarded for the research studies within the competition funded by Nicolaus Copernicus University in Toruń through funds obtained from the "Excellence Initiative – Research University" program (IDUB)

14.4. Research internship

- **01/06/2024 – 01/09/2024 - Institute for Molecular Medicine, University Medical Center of Johannes Gutenberg-University Mainz**

Research project: Investigating the metabolic profile of the endotoxin-tolerant macrophages in the cancer environment. (Funded by NAWA PRELUDIUM BIS 2).

During my internship, under the supervision of Dr. Nadine Hövelmeyer, I evaluated the metabolic profile of endotoxin-tolerant macrophages using the Single-Cell Energetic Metabolism by Profiling Translation Inhibition (SCENITH) technique. This method enables the assessment of cellular dependence on key metabolic pathways, such as glycolysis and oxidative phosphorylation (OXPHOS), by selectively inhibiting these pathways with specific

metabolic inhibitors. Protein synthesis is then measured using anti-puromycin antibody staining, serving as a proxy for translational activity. The resulting data, acquired through flow cytometry, provides insights into the metabolic programming and adaptability of immune cells under endotoxin-tolerant conditions. The data obtained highlighted that endotoxin-tolerant macrophages are not solely dependent on glycolysis or OXPHOS and have a flexible metabolic profile.

- **01/11/2020 – 30/06/2021 - Department of Biochemistry, Università Degli Studi Del Piemonte Orientale, Novara, Italy**
  Research project: Effects of antipsychotics on adipocyte metabolism
- **01/12/2018 – 30/10/2020 - Department of Biochemistry, UPO-CAAD**
  Project 1: Identification and characterization of novel DGKα inhibitors.
  Project 2: To study the involvement of the serotonin receptors in breast cancer cell survival and migration.

14.5. Supervision and Mentorship Experience

During the course of my research work, I have actively contributed to the academic development of junior researchers by supervising a master's student as part of their thesis project. I provided guidance in experimental planning and data interpretation, ensuring the student's successful progression throughout their research. Additionally, I was involved in mentoring an ERASMUS exchange student during their internship period, supporting their integration into the laboratory environment and assisting with their assigned experimental work.