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MΦ macrophage – N0 neutrophil dialogue in the presence of TNF-α affects the endothelium

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Abstract. Tumor necrosis factor-alpha (TNF-α) plays a pivotal role in the bi-directional dialogue between macrophages and neutrophils during the pre- and post- lesional stages of atherogenesis. This pro-inflammatory cytokine orchestrates a complex interplay between these immune cells, leading to the activation and recruitment of additional leukocytes, and the modulation of endothelial cell function, which collectively drive plaque formation and progression. Elevated levels of TNF-α result in the upregulation of adhesion molecules on the surface of endothelial cells. The cross-talk between macrophages and neutrophils, mediated by TNF-α, also leads to the release of soluble factors that have profound effects on the endothelium. Notably, these factors induce endothelial cell apoptosis via mechanisms involving caspase-3 activation, further contributing to the dysfunction and eventual denudation of the endothelial layer, a hallmark of atherogenesis. At molecular level, TNF-α exposure significantly upregulates the expression of pro-inflammatory mediators in macrophages, including interleukin-1 beta (IL-1β), inducible nitric oxide synthase (iNOS), and the activation of key signaling pathways such as the activation of mitogen-activated protein kinase (MAPK) signaling pathway and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK). This bi-directional dialogue not only sustains chronic inflammation, but also amplifies the

pathogenic processes underlying atherosclerosis, suggesting that targeting TNF-α and its downstream effects could represent a therapeutic strategy to mitigate disease progression. In this study, we aimed at investigating the dialogue between macrophages and unpolarized neutrophils, by assessing the biomarkers leading to cells activation and their differentiation towards a pro-inflammatory phenotype. The effects of TNF-α were explored in the context of inflammation in the arterial wall, for a better understating of atherogenesis. The study results indicated a low intensity inflammatory response, characterized by the up-regulation of key molecules involved in cell signaling for differentiation towards an inflammatory phenotype but not in the production of significant amounts of cytokines and enzymes.

Keywords: atherosclerosis, endothelial cells, macrophages, neutrophils, TNF-α

Introduction

Atherosclerosis, a chronic inflammatory condition underlying cardiovascular disease, involves complex interactions between different types of cells in the arterial wall (Farahi *et al*., 2021). The two-way dialogue between macrophages and neutrophils is a crucial determinant of disease initiation and progression by vascular inflammation and atherosclerotic plaque development (Hansson *et al*., 2015). Macrophages and neutrophils, essential components of the innate immune system, contribute to atherosclerotic plaque formation by releasing cytokines, chemokines, and other signaling molecules (Hou *et al*., 2023; Zhang *et al*., 2023). Tumor necrosis factor-alpha (TNF-α), a key pleiotropic cytokine, plays a central role in regulating this dialogue, shaping the atherosclerotic microenvironment (Zhang *et al*., 2009; Gough *et al*., 2020).

TNF-α, a master regulator of inflammation, mediates and amplifies the macrophage – neutrophil crosstalk in atherosclerosis through its multiple effects. TNF-α modulates the adhesive interactions between these immune cells and the endothelium, promoting their recruitment to the inflamed arterial wall (Ikuta *et al*., 1991; Iademarco *et al*., 1995). TNF-α stimulates the secretion of pro-inflammatory factors and vascular adhesion molecules. In addition to atherosclerosis, this communication also plays a key role in myocardial infarction, following the hypoxic injury to the cardiac muscle (Tian *et al*., 2015).

Attracted by cytokines and cellular debris, the first cells to arrive at the site of the hypoxic injury are neutrophils, as they play an active role in promoting the initial phases of inflammation by secreting soluble factors and

reactive oxygen species (Frodermann *et al*., 2017; Horckmans *et al*., 2017; Chalise *et al*., 2021). Neutrophils sensitize the endothelium, favoring the migration of other immune cell types from the vascular compartment to the extravascular compartment (Dimasi *et al*., 2013). Circulating monocytes adhere to the endothelium and traverse the vascular compartment arriving in the subendothelial compartment, where they differentiate into resident macrophages, exposed to signaling molecules produced by neutrophils and cellular debris, further differentiating into a pro-inflammatory phenotype (Butterfield *et al.,* 2006; Čejková *et al*., 2016). The pro-inflammatory phenotype is characterized by the production of pro-inflammatory signaling molecules such as interleukin 1 beta (IL-1β), TNF-α, and inducible nitric oxide synthetase (iNOS). In addition, there are many other molecules with pro-inflammatory properties involved (Wang *et al*., 2020; Kadomoto *et al*., 2022).

The mechanisms involved in macrophage activation are intensively studied (Feng *et al*., 1999; Moore *et al*., 2010; Parameswaran and Patial, 2010), but not yet fully understood. It appears that a major role in macrophage activation is played by the transcriptional protein Nuclear Factor κB (NF-κB), which regulates gene expression of pro-inflammatory cytokines. NF-κB binds to specific DNA sequences in the promoter region of the target genes and stimulate or inhibit their transcription (Takashiba *et al*., 2010). Another key transcriptional factor involved in macrophage activation is activating protein-1 (AP-1) which is usually activated by the mitogen-activated protein kinase (MAPK) phosphorylation cascade, including the extracellular signal-related kinase (ERK) and the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (Biggs *et al*., 1999; Rao, 2001). After the differentiation of macrophages towards a pro-inflammatory phenotype, there is a sudden increase in cytokine production. More interestingly, macrophages change their shape from round-like cells to a more spindle-like morphology, suggesting that pro-inflammatory macrophages might have migratory properties, which can lead to plaque microenvironment remodeling or some degree of subendothelial remodeling (Lee, 2019).

During the dynamic dialogue, after the secretion of cytokines, an amplification of the inflammatory response takes place due to the autocrine signaling effects of TNF-α on macrophages, which become even more activated (Xie *et al*., 1998). The soluble factors released by macrophages as well as by neutrophils have different effect on the endothelium, depending on the degree of resolution of the inflammatory processes in the subendothelial microenvironment (Xu *et al.,* 2022). At high concentrations, TNF-α exhibits cytotoxic effects on endothelial cells and on other immune cell types involved in atherogenesis (Larrick *et al*., 1990). Other authors have noticed that the exposure of endothelial cells to high

concentrations of cytokines leads to cell death by apoptosis. There is evidence suggesting that endothelial cell apoptosis is the main initiator of atherosclerotic plaque rupture and thrombus formation, as the non-thrombogenic surface of the blood vessel is disrupted (Polunovsky *et al*., 1994; Kavurma *et al*., 2005).

Despite extensive research, several gaps in knowledge related to the role of TNF- α in macrophage – neutrophil signaling remain to be elucidated. The cell-specific signaling pathways, the context-specific outcomes influencing the pro-inflammatory versus regulatory effects of the cytokine, the temporal dynamics and the crosstalk with other cytokines are underexplored research pathways, important for advancing therapeutic strategies.

The dialogue between macrophages and neutrophils, mediated by TNF-α, is particularly important for endothelial cells in atherosclerosis, since it directly affects endothelial integrity, activation and function. The immortalized line of human umbilical endothelial cells Ea.hy926 proved to be suitable for research of vein or artery endothelium in different pathologies (Deng *et al*., 2017). Recent investigations have shown that the dialogue between pro-inflammatory macrophages and smooth muscle cells found within the arterial wall leads to the remodeling of the vascular wall as a consequence of up-regulation of metalloproteases and production of extracellular matrix components by the smooth muscle cells, which leads to the narrowing of the lumen and the destabilization of the atherosclerotic plaque (Macarie et al., 2025). Other studies have shown that the dialogue between macrophages and neutrophils generates different outcomes depending on the phenotype of these cells at the time of the dialogue. Neutrophils from patients with different conditions are temporally polarized into pro-inflammatory (N1) and anti-inflammatory (N2) subpopulations. When exposed to activated neutrophils type N1, macrophages acquire a pro-inflammatory phenotype with improved efferocytotic properties, while exposure to soluble factors released by N2 neutrophils leads to an anti-inflammatory phenotype (Macarie *et al*., 2018; Mihăilă *et al*., 2024).

The present study aims to explore the molecular changes that takes place during the cross-talk between unpolarized neutrophils (N0) and resting macrophages (MΦ), to ascertain whether the secretome triggers an inflammatory response in endothelial cells. Also, for mimicking the inflammatory conditions found within the vascular wall in the atherosclerotic lesion site during the cross-talk, the cells were exposed to different concentrations of TNF-α or lipopolysaccharides (LPS) to further investigate the complex interplay and cellular differentiation towards a pro-inflammatory phenotype.

Materials and methods

Chemicals and reagents

Monoclonal antibodies, anti-iNOS (#2982), anti-NF-κB (#3033), anti-SAPK/JNK (#9252), anti-phosphorylated SAPK/JNK (#9255), anti-phosphorylated ERK1/2 (#4377), anti-caspase-3 (#9662) and anti-β-tubulin (#2146) were purchased from Cell Signaling Technologies (Danvers, Massachusetts, USA). Anti-ICAM-1 (710278) was purchased from Thermo Scientific (Waltham, Massachusetts, USA), anti-IL-1β (MAB 601) and anti-ERK1/2 (MAB1576) were procured from R&D Systems (Minneapolis, Minessota, USA), anti-TNF-α (SC-52746) was purchased from Santa Cruz (Dallas, Texas, USA), anti-caspase-1 (MAB-6215) was purchased from Novus Biologicals (Centennial, Colorado, USA). Human IL-1β ELISA kit (DuoSet) was purchased from R&D Systems (Minneapolis, Minnesota, USA). Human recombinant TNF-α and LPS were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), culture media were provided by Corning Life Sciences (Corning, New York, USA) and Gibco (Waltham, Massachusetts, USA). Cell culture microplates were purchased from TPP AG (Trasadingen, Switzerland) and the co-culture inserts were from Falcon Plastics (Brookings, South Dakota). Other reagents and materials were from Sigma-Aldrich (St. Louis, Missouri, USA), Eppendorf (Hamburg, Germany), Bio-Rad (Hercules, California, USA), and Gibco (Waltham, Massachusetts, USA).

Cell cultures

THP-1, HL-60 and EA.hy926 cell lines used in this study were purchased from ATTC (American Type Culture Collection, Manassas, USA). Frequently used in cancer and drug research, THP-1 cells (ATCC TIB-202™) are immortalized human monocytes from the peripheral blood of a patient with acute monocytic leukemia. THP-1 cells were grown in suspension in RPMI-1640 culture media supplemented with 10% heat-inactivated FBS and were split 1:5 twice a week. Monocytes (1 x 10^6 cells) were plated in a 24-well plate and differentiated towards resting macrophages (МΦ) by exposure to 100 nM phorbol myristate acetate (PMA) for 3 days. As suggested by other authors (Butoi *et al*., 2016), PMA activation induces an unpolarized МΦ macrophage phenotype or a certain degree of classical activation (M1 phenotype). HL-60 (ATCC CCL-240™) is an immortalized line of pro myeloblasts isolated from a 36-year-old white woman with acute promyelocytic leukemia by leukapheresis. Briefly, HL-60 human neutrophils were grown in suspension in RPMI 1640 culture medium and were split 1:5 twice a week. Undifferentiated macrophages were co-cultured with neutrophils in RPMI-1640, without serum and antibiotics.

The entire co-culture system was exposed to different concentrations (10-100 ng/mL) of TNF-α or 1μg/mL LPS for 24h at 37°C and 5% CO_2. After 24h, the conditioned medium was collected under sterile conditions. EA.hy926 endothelial cells (ATCC CRL-2922™) were maintained in DMEM supplemented with 10% FBS and 1% PSA at 37°C under 5% CO_2. The cultures were starved of FBS 24h before exposure to conditioned medium. EA.hy926 cells were indirectly co-cultured using conditioned media resulting from macrophage – neutrophil cross-talk in the presence of TNF-α or LPS.

Experimental design: the co-culture system

Human neutrophils were plated on the filter inserts (lumenal side) in RPMI at a density of 10^6 neutrophils/insert. Human monocytes THP-1 were plated on the bottom of the transwell co-culture chamber (basolateral side) at a density of 10^6 cells/well and cultured in RPMI-1640 for macrophages differentiation, by exposure to 100 nmol/L PMA. After 3 days, the co-culture system was constructed by placing the inserts with neutrophils (N0) on the top of the microplate wells. The co-cultures were incubated for 24h at 37°C into an atmosphere enriched with 5% CO_2. Control groups were maintained in serum-free RPMI-1640. At the end of the cell co-culture period, the conditioned media resulting from the cross-talk was collected and used for further investigations and treatment of EA.hy926 endothelial cell monolayers.

The experimental groups were:

- MΦ: negative control group consisting of resting macrophages;
- MΦ + N0: resting macrophages co-cultured with unpolarized neutrophils;
- LPS: resting macrophages co-cultured with unpolarized neutrophils treated with 1 μg/mL LPS;
- LPS: resting macrophages co-cultured with unpolarized neutrophils treated with 10 ng/mL TNF-α;
- LPS: resting macrophages co-cultured with unpolarized neutrophils treated with 50 ng/mL TNF-α;
- LPS: resting macrophages co-cultured with unpolarized neutrophils treated with 100 ng/mL TNF-α.

Cell differentiation and microscopic evaluation

THP-1 cells were exposed to 100nM PMA for 72h to induce differentiation. After 24h of culture in the presence of PMA, the cells adhered to the polystyrene surface were examined by microscopy. Untreated endothelial cells and endothelial cells exposed to conditioned media from unpolarized macrophages (MΦ) were

used as negative controls. Experimental groups were exposed to conditioned media from TNF-α treated leucocytes (MΦ + N0). Cell morphology was examined under an DMi1 inverted microscope with phase contrast (Leica, Weltzar, Germany).

Western blot analysis

The protein expression of cytokines and other signaling molecules was assessed in the total extract of macrophages, neutrophils, and endothelial cells obtained by homogenizing the cells in Laemmli electrophoresis sample buffer (Sx2). Obtained cell lysates were incubated at 95°C for 5 minutes and then sonicated for 10 seconds on ice. The samples were kept on ice to inhibit protein degradation by exogenous and endogenous proteases. We determined the total protein concentration/sample by amido black staining and measured the absorbance using a microplate reader (TecanM200Pro) at 620 nm. 1µL of each sample was applied on a nitrocellulose membrane and then the membrane was washed with a fresh solution of 50% MeOH and stained for 10 seconds with 0.1% amido black solution. The obtained stained spots were collected in 400 µL 0.1M NaOH and vortexed for 10 seconds. Equal quantities of proteins were electroblotted through a 10% polyacrylamide gel and were then transferred onto a nitrocellulose membrane (Sigma-Aldrich, St. Louis, Missouri, USA). The membranes were blocked in 1% fish skin gelatin, 1% BSA, and 0.1% Tween-20 at room temperature for 1h before incubation with primary antibodies overnight at 4°C. Following washing three times with TBST, the membranes were incubated with the secondary antibody at room temperature for 1h. The following molecules were analyzed: pNF-κB, SAPK/JNK, ERK, iNOS, TNF-α for macrophages and VCAM-1, ICAM-1, vinculin, integrin αVβ5 and caspase-3 for endothelial cells, respectively. The blots were developed using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Whaltman, Massachusetts, USA) and quantified by densitometry employing gel analyzer system Luminescent image analyzer LAS-4000 (Fujifilm, Tokyo, Japan) and ImageJ software version 1.54 (National Institutes of Health, Bethesda, Maryland, USA) (Schneider *et al*., 2012).

IL-1β quantification by ELISA

IL-1β antigens were quantified in the collected conditioned medium using the ELISA kit (R&D systems, Abingdon, UK), according to the manufacturer's instructions. Briefly, strips were coated with the capture antibody overnight at room temperature. Following incubation with the capture antibody, the strips were washed 3 times with washing buffer. Then, the plates were blocked with diluent reagent for one hour at room temperature. The detection antibody and

streptavidin HRP was added, and later, the substrate. After substrate addition, the plate was incubated in the dark at room temperature for 20 minutes, and the reaction was stopped using 2N H_2SO_4. Optical density of each well was read using a microplate reader (Tecan M200 Pro) at $\lambda = 450$ nm.

Statistical analysis

All experiments were performed in duplicates and statistics was applied using the Microsoft Office 2017 Excel software. The data obtained were expressed as mean values ± standard deviation (SD). For ELISA, statistical evaluation was carried out by one-way ANOVA followed by Dunnett's multiple comparisons test using GraphPad Prism version 10.0.0 for Windows, GraphPad Software (Boston, Massachusetts, USA).

Results

The present study explored the dialogue between unpolarized neutrophils and macrophages, by assessing the biomarkers leading to cells activation and their differentiation towards a pro-inflammatory phenotype. In this regard, EA.hy926 endothelial cells were exposed to the conditioned media resulting from macrophage – neutrophil cross-talk and various concentrations of TNF-α. The experimental design was based on a co-culture system using HL-60 neutrophils and resting macrophages (MΦ) differentiated from THP-1 monocytes, respectively (Figure 1A).

Microscopic evaluation of activated leucocytes and endothelial cells

Cell differentiation and activation was assessed microscopically. THP-1 cells grow in suspension and do not adhere to the plastic surface of the culture plates. To induce terminal differentiation to unpolarized MΦ macrophages or partially polarized toward an M1 pro-inflammatory phenotype, THP-1 cells were exposed to PMA. After 24h of culture in the presence of PMA, the cells adhered to the polystyrene surface of the cell culture plate and showed a specific spindle-like morphology, suggesting the activation and change in phenotype (Figure 1B). Microscopic evaluation revealed the formation of apoptotic bodies in the experimental groups treated with conditioned media from TNF-α treated leucocytes (Figure 1C). The presence of apoptotic cells in the endothelium monolayer suggested that the conditioned medium from the macrophage – neutrophil dialogue contained soluble pro-apoptotic signaling molecules that could affect the endothelial cells by promoting apoptosis. These images

demonstrate the hallmark sequential features of apoptotic cells detected by phase-contrast microscopy, including blebs, echinoid spikes, and surface blisters (Willingham, 1999) (Figure 1D).

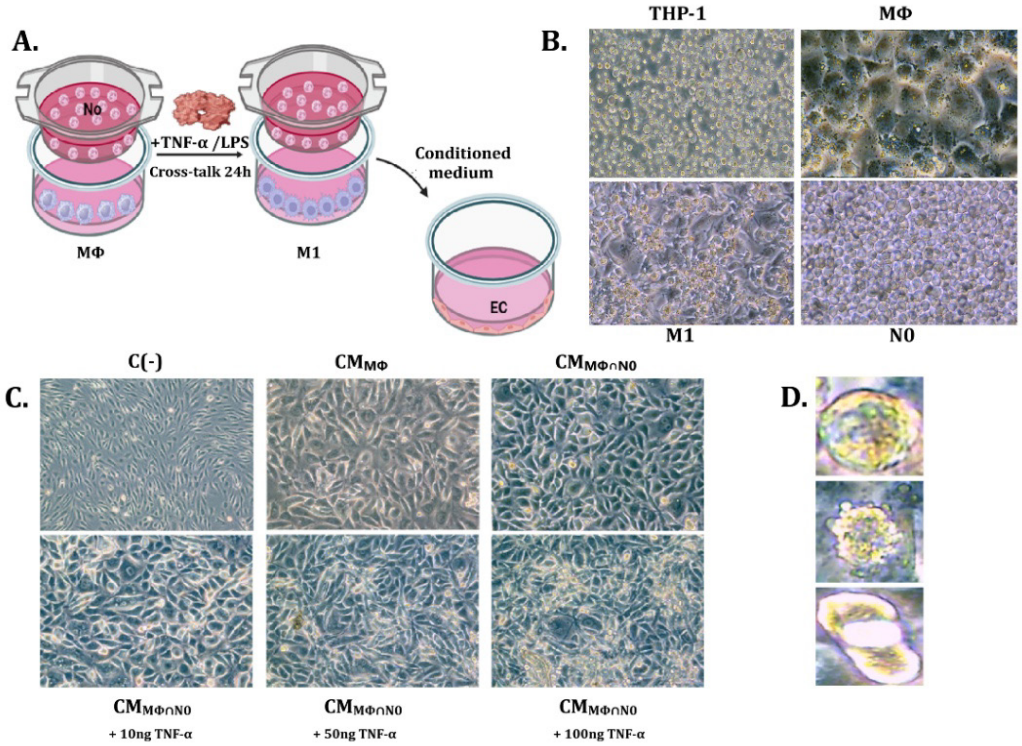

Figure 1. A. Co-culture design: endothelial cells (EC) were exposed to the conditioned media resulting from macrophage – neutrophil cross-talk and various concentrations of TNF-α or lipopolysaccharides (LPS). **B.** Induction of differentiation of THP-1 cells by PMA stimulation. THP-1 cells were incubated for 24h without or with 100 nM PMA. Undifferentiated macrophages were polarized towards an inflammatory phenotype (M1) by exposure to neutrophils (N0) and different concentrations of TNF-α, observed in phase contrast microscopy. **C.** Endothelial cells show a specific spindle-shaped-like morphology in phase contrast microscopy. Morphology of macrophages at the end of the 24h co-culture with HL-60 neutrophils, revealed polarized, spindle-shaped macrophages suggesting the activation and change in phenotype. Microscopic aspects in phase contrast microscopy showing the accumulation and presence of apoptotic cells in the endothelium monolayer, suggesting that conditioned media (CM) coming from M/N dialogue contains soluble pro-apoptotic signaling molecules that can affect the endothelial cells by promoting apoptosis. **D.** Morphology of apoptotic bodies in endothelial cell culture: the images demonstrate sequential features of apoptotic cells, including blebs, echinoid spikes, and surface blisters, detected by phase-contrast microscopy.

Macrophage activation by soluble factors released after cross-talk and TNF-α exposure

To get an insight into the molecular mechanisms involved in macrophage activation, we determined the relative expression levels of NF-κB, ERK, and SAPK/JNK, proteins involved in the activation of genes encoding cytokines.

Our results showed that, compared to basal expression, the relative expression level of the phosphorylated nuclear factor κB (pNF-κB) is upregulated upon exposure to TNF-α. This increase in expression is proportional to the TNF-α concentration, indicating a dose-dependent relationship between NF-κB activation and the levels of TNF-α. In the context of macrophage – neutrophil interaction, there is a slight up-regulation, exhibiting high variability. Additionally, LPS does not seem to induce a stronger response than MΦ. Therefore, TNF-α is a potent activator of NF-κB, whereas LPS and N0 have a weaker effect on NF-κB activation in macrophages (Figure 2A).

The SAPK/JNK pathway also demonstrated significant activity under high concentrations of TNF-α, observed in the relative expression and activation levels of the p54 and p46 isoforms. For p54, an increase in phosphorylation was observed in the presence of TNF-α and LPS, with the highest upregulation occurring at 50 and 100 ng/mL TNF-α, suggesting a dose-dependent activation. Meanwhile, exposure to LPS and 10 ng/mL TNF-α resulted in a more moderate response. For p46, phosphorylation did not appear to be affected by TNF-α exposure, as expression levels remain similar across concentrations, with a slight decrease compared to MΦ. MΦ exhibited high variability, which may indicate a spontaneous activation of p46 in some replicates. Therefore, we found that p54 is more strongly activated in the context of macrophage – neutrophil interaction compared to p46. TNF-α and LPS significantly induced p54 activation, whereas p46 activation did not appear to be affected, suggesting that this subunit is either less active in this context or is regulated by alternative signaling pathways (Figure 2B).

In the case of ERK, the relative expression and activation levels of the ERK1 (p44) and ERK2 (p42) isoforms were investigated. ERK1 phosphorylation was not influenced by the cross-talk between macrophages and neutrophils or by exposure to TNF-α. Electrophoretic analyses revealed that the relative expression level of ERK1 does not exceed basal conditions, whereas ERK2 showed preferential activation. The interaction between macrophages and neutrophils appeared to significantly upregulate ERK2 activation. Additionally, LPS induced an inflammatory response that, while weaker, is still more pronounced than the basal expression level observed in MΦ macrophages. Moreover, ERK2 activation appeared to be dependent on TNF-α concentration. ERK pathway was prominently activated by TNF-α. Phosphorylation of ERK1/2 led to downstream effects that promote pro-inflammatory cytokine production

and contributed to the broader inflammatory response. The dose-dependent activation of the ERK pathway suggested that multiple signaling cascades are converging to amplify macrophage activation in response to TNF-α (Figure 2C).

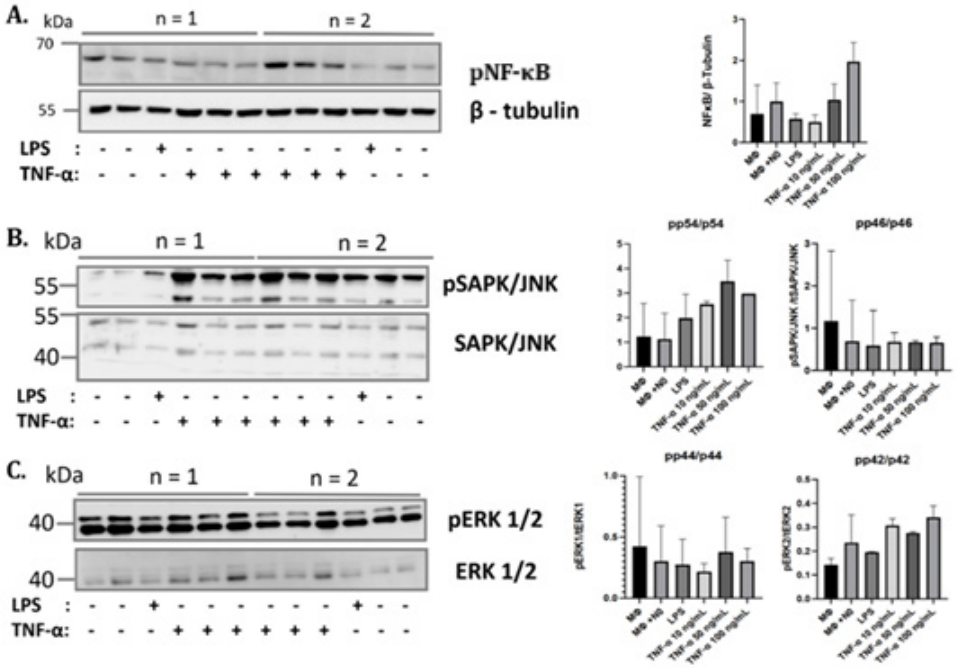

Figure 2. Relative expression levels of NF-κB (**A**), SAPK/JNK (**B**), ERK (**C**), in macrophages before and after exposure to neutrophils and TNF-α. p54 revealed a dose-dependent relationship based on the TNF-α concentrations between 10 and 50 ng/mL TNF-α. At 100ng/mL, TNF-α displayed cytotoxic effects which could interfere with the real expression level of p54. p46 did not exhibit the same pattern. ERK2 also showed the same dose-dependent pattern, while ERK1 did not. NF-κB was highly up-regulated after cross-talk and exposure to high TNF-α concentrations.

Macrophage identity and pro-inflammatory phenotype

For the analysis of macrophage activation, the relative expression levels of proinflammatory molecules characteristic to M1 macrophages were determined. Electrophoretic investigations revealed significant changes in the expression levels of key pro-inflammatory markers, specifically iNOS and TNF-α, following cross-talk with neutrophils in the presence of varying concentrations of TNF-α. A dose-dependent increase in iNOS expression was observed in macrophages co-cultured with neutrophils under stimulation with different TNF-α concentrations,

indicating a strong induction of the macrophages' antimicrobial and inflammatory functions. At low TNF-α concentrations, iNOS levels showed a modest elevation compared to the control. Low TNF-α concentrations, neutrophils and LPS failed to induce a strong activation of iNOS, comparing to basal levels. TNF-α production by macrophages significantly increased in response to the combined stimuli of neutrophil interaction and 10 ng/mL TNF-α exposure.

However, expression levels of TNF-α confirmed the autocrine signaling effect of macrophages (Figure 3). Overall, the study results revealed that macrophages, upon interacting with neutrophils and in the presence of TNF-α, exhibited a substantial upregulation of iNOS and TNF-α. The observed dose-dependent relationship with TNF-α concentration indicated that this cytokine plays a critical role in modulating macrophage identity towards a pro-inflammatory state.

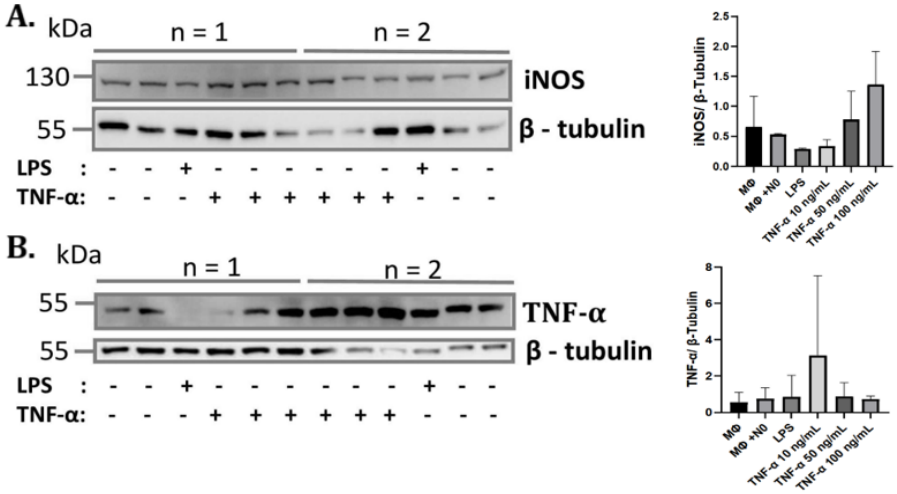

Figure 3. The relative expression levels of iNOS (**A**) are highly up-regulated in macrophages exposed to high TNF-α concentrations while TNF-α (**B**) is highly expressed in macrophages exposed to 10ng/mL TNF-α, as a peak of cytokine production after autocrine signaling effects of TNF-α.

We aimed to investigate the production of IL-1β in response to the cross-talk between macrophages and neutrophils, both in the presence and absence of TNF-α. THP-1 cells and immortalized neutrophils were co-cultured under various conditions, and IL-1β levels in the conditioned medium were quantified by ELISA assay. In the absence of TNF-α stimulation, both THP-1-derived macrophages and neutrophils did not produce IL-1β, indicating that neither cell type alone nor in co-culture without additional stimulation significantly induces IL-1β production. However, when macrophages were exposed to TNF-α, a modest increase in IL-

1β level was observed. This increase was more pronounced when macrophages were co-cultured with neutrophils (MΦ+N0), suggesting that the interaction between these cell types enhances IL-1β production, even under basal conditions. Interestingly, the production of IL-1β in response to TNF-α was not dose-dependent. Macrophages and neutrophils co-cultured and stimulated with 50 ng and 100 ng of TNF-α produced only slight increases in IL-1β levels. In contrast, the experimental group exposed to 10 ng TNF-α exhibited a substantial increase in IL-1β production, indicating a peak in cytokine release at this concentration (Figure 4). This suggested that there is an optimal concentration of TNF-α that maximally induces IL-1β production in this co-culture system.

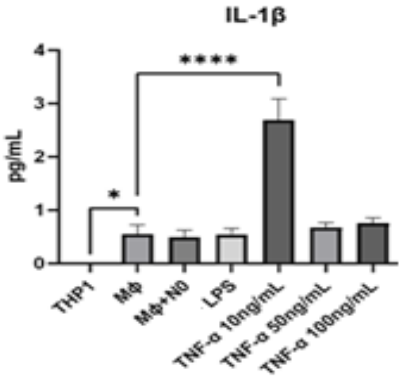

Figure 4. ELISA quantification of IL-1β revealed up-regulated levels of IL-1β in the case of macrophages co-cultured with neutrophils in the presence of 10 ng/mL TNF-α, this demonstrates a dose-dependent relation between IL-1β secretion and TNF-α concentration. Unstimulated macrophages and neutrophils showed little to no IL-1β production whereas for THP-1 cells there was no production of IL-1β. Data are expressed as the mean ± SD of three experiments for each set of experimental conditions * $p < 0.1$ and **** $p < 0.0001$ vs control.

Soluble factors released after the cross-talk enhance the adhesive properties of endothelial cells

Hypothesizing that the dialogue between the two cell types can lead to increased adhesion properties, we examined the relative protein expression levels of integrin αVβ5, VCAM-1, ICAM-1 and vinculin, well-known markers of cell adhesion. An increased expression of integrin αVβ5 in response to higher concentrations of TNF-α was observed (Figure 5A), suggesting that endothelial cells become more adhesive, promoting interactions with circulating immune cells or other cell types during inflammatory responses. VCAM-1, another critical adhesion molecule, is involved in the binding of leukocytes to the endothelium. Its upregulation was also observed (Figure 5B), further supporting the hypothesis

that TNF-α enhances endothelial cell adhesiveness through multiple pathways, reinforcing the role of endothelial cells in mediating inflammatory processes. Interestingly, ICAM-1, another adhesion molecule, did not show a significant change in expression levels despite the presence of TNF-α (Figure 5C). This could indicate a more selective or context-dependent role of ICAM-1 in TNF-α-induced adhesion processes, or perhaps that its expression is regulated by other factors or under different conditions. Vinculin also did not exhibit significant changes (Figure 5D). Therefore, we observed that while TNF-α upregulates integrin αVβ5 and VCAM-1, it might not directly affect the intracellular components linked to adhesion, such as vinculin, or that vinculin's role is more stable and less susceptible to modulation by TNF-α alone.

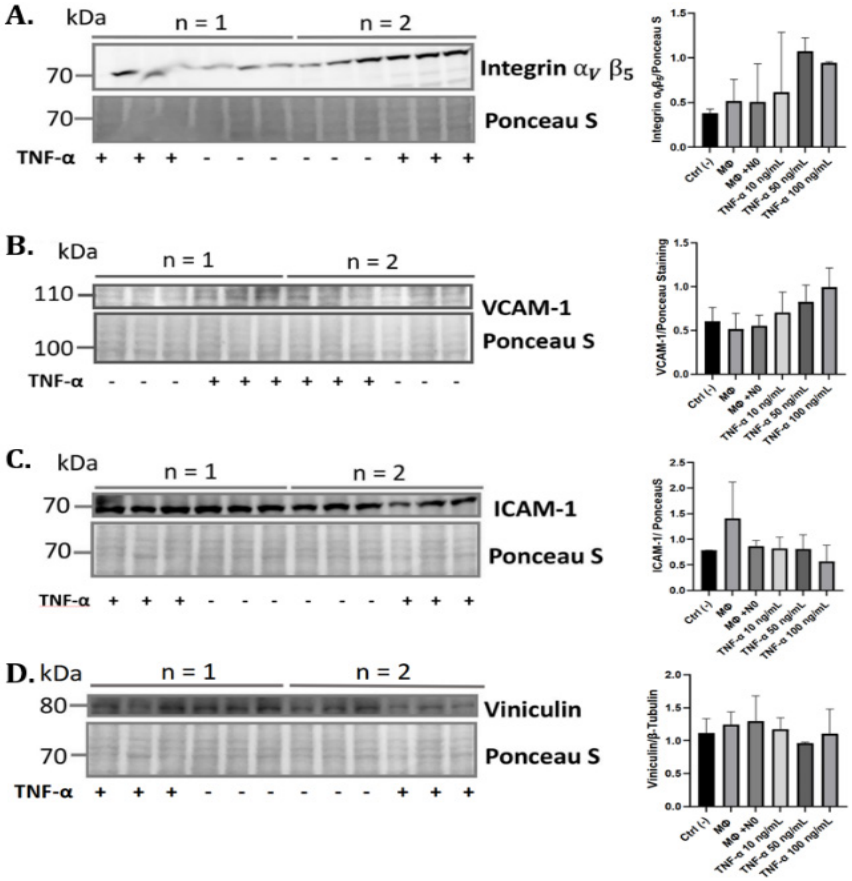

Figure 5. Electrophoretic investigations of endothelial cells exposed to the conditioned medium containing soluble factors released after the neutrophil-macrophage cross-talk revealed up-regulated levels of integrin αVβ5 (**A**), VCAM-1 (**B**), but not ICAM-1 (**C**) and vinculin (**D**).

The selective upregulation of integrins and VCAM-1 in response to TNF-α, without corresponding increases in ICAM-1 and vinculin, highlights the complex regulatory mechanisms governing endothelial cell adhesion. This selective enhancement of adhesion molecule expression may be crucial for the specific recruitment and attachment of leukocytes during inflammation, ensuring a targeted and efficient immune response. Additionally, these findings underscore the importance of considering the specific pathways activated by cytokines like TNF-α, as they may differentially regulate adhesion molecules and intracellular components involved in endothelial cell adhesion.

Soluble factors released after cross-talk induced apoptosis in endothelial cells

As a result of the bi-directional dialogue between macrophages and neutrophils, soluble factors are released by both cell types and they have major effects on the endothelium. Following this dialogue, in the presence or absence of TNF-α, these cell types undergo activation, and in particular, macrophages switch to a secretory phenotype releasing soluble cytokines. Our investigations showed that the conditioned culture medium contained TNF-α as a result of autocrine signaling of macrophages, IL-1β and iNOS. All three molecules exhibit profound effects on the endothelium. However, the dialogue between these cell types in an inactive state was not sufficient to induce a strong inflammatory response. The conditioned medium resulting from the classic dialogue still had effect on the endothelium, but its magnitude was not comparable to the effects produced by conditioned medium coming from co-cultures exposed to high concentrations of TNF-α. Endothelial cells exposed to high concentrations of TNF-α underwent cell death through a mechanism involving the activation of caspase-3 (Figure 6).

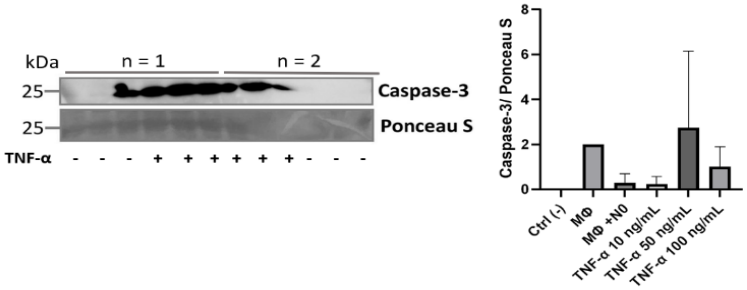

Figure 6. Electrophoretic investigations of the relative expression level of caspase-3 in endothelial cells exposed to conditioned medium derived from macrophage – neutrophil dialogue. It can be observed that upon exposure to conditioned media the endothelial cells present a sudden increase in caspase-3 levels at 50 ng/mL TNF-α, suggesting the activation of apoptotic pathways that would ultimately lead to endothelial cell death.

These results corroborate well with the microscopic observations showing an increase in the number of apoptotic bodies in endothelial monolayers, proportional with the increase of soluble factors released by the macrophage – neutrophil dialogue. Macrophage – neutrophil dialogue and TNF-α exposure induce endothelial cells apoptosis, suggesting that during the acute inflammatory stages of atherogenesis, the vessel lumen might suffer denudation leading to plaque instability and rupture.

Discussion

In this study, we demonstrated that the dialogue between macrophages with undecided fates and neutrophils leads to an inflammatory response in the presence of TNF-α, releasing pro-inflammatory molecules as a result of intracellular signaling processes that activate transcription factors controlling their expression.

The macrophages and neutrophils secretome, exerts pro-apoptotic and pro-inflammatory effects on endothelial cells. Macrophages, phagocytic cells of the innate immune system, originate from circulating monocytes that cross the vascular wall and reach the sub-endothelial space, where they differentiate (Moore *et al*., 2011). Upon exposure to stress factors, macrophages further differentiate into pro-inflammatory macrophages that release pro-inflammatory molecules into the extracellular space (Lee, 2019). These immune cells undergo morphological and functional differentiation upon exposure to soluble factors released by neutrophils and TNF-α (Parameswaran and Patial, 2010). The exposure triggers a signaling cascade that leads to macrophage activation, typically initiated by the interaction of TNF-α with the tumor necrosis factor receptor-1 (TNFR-1) expressed on the macrophage surface. Following ligand-receptor interaction, TNF receptor-associated death domain protein (TRADD) is recruited, along with an entire signal transduction complex, which interacts with NF-κB, leading to its activation (Hayden *et al*., 2014).

Our results show that NF-κB is activated upon exposure to neutrophils to LPS, and different concentrations of TNF-α, the activation being dose-dependent and proportional to TNF-α concentration. SAPK/JNK, a widely investigated kinase in the context of cellular signaling in inflammation (Dérijard *et al*., 1994; Yan *et al*., 2024) is involved in a phosphorylation cascade that activates transcription factors like c-Jun and c-Fos, which form the AP-1 complex that regulates cytokine gene expression (Yin *et al*., 2009). SAPK/JNK has two distinct isoforms, p54 and p46, encoded by different genes and presenting alternative splicing variants, that leads to high variability. p46 is more commonly associated

with fast responses, whereas p54 may have prolonged roles. Specifically, p54 is strongly activated by cellular stress, inflammation, and cytokines such as TNF-α and IL-1β, whereas p46 can be regulated by stimuli such as growth factors (EGF, NGF), cytoskeletal signaling via Rho GTPases, hypoxia, and metabolic stress (Chan *et al*., 1997). Both p54 and p46 can phosphorylate the same transcription factors (c-Jun, ATF2) but with varying efficiency. In some instances, p46 is more likely involved in cell survival, while p54 plays a greater role in apoptosis and inflammation. There is evidence that p46 may have spontaneous activity in macrophages differentiated from THP-1 cells, which may explain the high expression of p42 under basal conditions in our experimental design (Tournier *et al*., 2001). THP-1-derived macrophages express both p46 and p54, but their basal levels vary depending on the used stimuli (PMA, LPS, TNF-α). Additionally, it has been observed that p46 may exhibit higher basal activity than p54 in certain cell types, including macrophages (Tournier *et al*., 2000). Under specific conditions, p46 activation can occur without exogenous stimulation, for example, in the presence of FBS, which contains residual growth factors (Xia *et al*., 2000).

Regarding ERK, the activation of p44 (ERK1) and p42 (ERK2) isoforms is involved in a phosphorylation cascade and subsequently activates transcription factors (Wortzel and Seger, 2011). Although ERK1 and ERK2 are often considered functionally equivalent, recent findings (Wong *et al*., 2021) indicate that ERK2 plays a more dominant role in certain cellular contexts. ERK2 is more involved than ERK1 in cell proliferation and survival, which may be relevant in the context of TNF-α exposure as it induces both an inflammatory response and compensatory survival mechanisms. Higher ERK2 activity can be explained by the presence of the upstream kinase MEK1/2, which is influenced by TNF-α exposure and macrophage – neutrophil interactions (Trouba *et al*., 2004). Additionally, the lower ERK1 activity could be due to its transient presence or its degradation due to a highly regulated expression.

Our previous results showed that pro- or anti-inflammatory neutrophils can induce a pro- or anti-inflammatory profile of macrophages (Macarie *et al*., 2025). In the present study, we aimed to evaluate whether the dialogue between unpolarized macrophages and neutrophils could possibly lead to inflammation. To characterize the secretome resulting from macrophage – neutrophil dialogue in the presence of TNF-α, we evaluated the relative expression levels of hallmark pro-inflammatory molecules in macrophages, specifically iNOS and TNF-α.

iNOS is an enzyme involved in nitric oxide production, predominantly expressed in macrophages, endothelial cells, and hepatocytes in response to pro-inflammatory stimuli (Nathan and Xie, 1994). By producing NO, these enzymes contribute to vasodilation and increased vascular permeability. Besides its pro-inflammatory effects, iNOS can induce cell apoptosis through interactions with

free radicals (Nitsch *et al*., 1997). Unlike its counterparts eNOS and nNOS, which produce small, constitutive amounts of NO, iNOS generates large amounts of NO (Persichini *et al*., 2006). Our results showed that iNOS is strongly expressed by macrophages interacting with neutrophils and exposed to 50 or 100 ng/mL TNF-α, with a proportional increase in iNOS expression relative to TNF-α concentration.

TNF-α, the cytokine central to this study, is well known for both its pro-inflammatory and anti-inflammatory effects. However, in this context, a pro-inflammatory effect is evident. TNF-α expression is strictly controlled by NF-κB, which, once activated, stimulates TNF-α production and secretion, leading to autocrine signaling in macrophages that amplifies the inflammatory response via a feedback loop (Xaus *et al*., 2000). Additionally, NO activity from iNOS further enhances NF-κB activation, leading to even greater TNF-α expression. To characterize the secretome, we also quantified the relative level of IL-1β. Macrophages interacting with neutrophils and exposed to 10 ng/mL TNF-α produced the highest levels of IL-1β. However, overall IL-1β production remained low with minor variations. It was evident that N0, THP-1, and MΦ cells either did not express or expressed very low levels of IL-1β, while LPS and higher TNF-α concentrations induced a slight increase in IL-1β production. These findings indicate that the secretome resulting from macrophage – neutrophil dialogue contains pro-inflammatory molecules such as IL-1β, iNOS, and TNF-α. It is likely that this secretome includes additional pro-inflammatory molecules beyond those identified.

Based on previous determinations, we decided to explore the effects of IL-1β, TNF-α, and iNOS on the expression of cell adhesion molecules. After exposing endothelial cells to these pro-inflammatory conditions, which are also common in atherogenesis, we evaluated the relative expression levels of VCAM-1, ICAM-1, integrin αVβ5, and vinculin. Our results indicate that TNF-α plays a central role in regulating adhesion molecule and anchoring protein expression in endothelial cells exposed to macrophage – neutrophil dialogue. VCAM-1 and integrin αVβ5 were upregulated in a dose-dependent manner upon TNF-α exposure. We observed that endothelial cells treated with conditioned media from MΦ and MΦ+N0 exhibited lower VCAM expression than basal levels, possibly indicating either suppression of inflammatory effects due to the presence of anti-inflammatory molecules or a paradoxical effect of TNF-α. Unlike VCAM-1, ICAM-1 showed relatively stable expression levels, with increased expression only in response to conditioned media from MΦ, suggesting that MΦ secretes molecules that selectively induce ICAM-1 expression. Vinculin, a molecule involved in endothelial focal adhesions, interaction with extracellular matrix and cytoskeletal stability (James *et al*., 2024), exhibited relatively stable expression, suggesting that TNF-α does not dramatically affect the structure and organization of endothelial focal adhesion points. In the case of integrin αVβ5, we observed increased relative expression compared to the control and MΦ, with the conditioned

media from macrophage – neutrophil dialogue inducing a smaller increase compared to TNF-α alone. Integrins are transmembrane receptors that facilitate cell-extracellular matrix adhesion, as well as cell-cell interactions (Banerjee et *al*., 2022). However, low vinculin and ICAM-1 levels may also indicate an experimental design limitation, as decreasing expression could be artificial due to endothelial cell apoptosis induced by high concentrations of pro-inflammatory molecules and serum starvation. Recent studies also show that conventional 2D plastic culture systems are typically hypoxic, meaning that hypoxia, along with other factors, inevitably leads to cell death (Tan *et al*., 2024).

All these findings demonstrate that macrophage – neutrophil dialogue can lead to endothelial cell activation, while this cross-talk in the presence of TNF-α inevitably results in the upregulation of cell adhesion molecules. After data analysis and microscopic observations showing an accumulation of spherical, apoptotic cells, we decided to evaluate caspase-3 expression, a well-known apoptosis-related caspase. Our results indicate that caspase-3 is expressed in a TNF-α dose-dependent manner and that macrophage – neutrophil dialogue can induce apoptosis.

Conclusions

In this study, we aimed to mimic vascular inflammation conditions *in vitro* to investigate the cross-talk between macrophages and neutrophils. The secretome resulting from the interaction between these two immune cell types had significant effects on endothelial cells. Following cellular cross-talk and exposure to pro-inflammatory molecules, macrophages become activated through signaling pathways that lead to the phosphorylation of NF-κB, activation of SAPK/JNK, and ERK pathways and also differentiated into a pro-inflammatory phenotype characterized by increased expression and production of iNOS, TNF-α, and IL-1β. In the presence of TNF-α, VCAM-1 and integrin αvβ5 adhesion molecules were upregulated, while ICAM-1 and vinculin did not show significant changes. In addition to the increased expression of specific adhesion molecules, endothelial cells underwent apoptosis due to the cytotoxic effects of high TNF-α concentrations. This was confirmed both by microscopic examination and electrophoretic analysis of endothelial monolayers. Our study provides clear evidence that the cross-talk between the two immune cell types can lead to endothelial cell activation and even death. The outcomes of the study highlight the importance of the interaction and of the signaling molecule TNF-α as potential targets for the development of novel molecular therapies aimed at reducing arterial wall inflammation in various pathologies such as atherosclerosis.

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Author contributions: S.M.V. and E.B. – conceptualization, experimental design; S.M.V., A.M. and M.Ț – methodology, analysis, data curation; S.M.V – original draft preparation; A.F. – supervision, writing.

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