VER
Vascular & Endovascular Review

Mechanisms of Phagocyte Recognition of Apoptotic Cells: Implications for Vascular and Immune Homeostasis: A Review

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ABSTRACT

All phagocytic cells including (Neutrophils (PMN) and macrophages and monocytes) are the most important cellular components of the non-specific immune system. They have receptors for a variety of molecule, such as Fc for IgG, Fc for complement and certain bacterial components. Apoptotic cell removal from the tissues is thought to be dependent on their death by apoptosis, followed by their recognition and phagocytosis by macrophages. This paper will discuss the molecular and cellular aspects of phagocytosis of apoptotic cells.

KEYWORDS: Apoptosis, Phagocytosis, Immune, Cancer, Integrins, Thrombospondin.

How to Cite: Faris Q. B. Alenzi., (2025) Mechanisms of Phagocyte Recognition of Apoptotic Cells: Implications for Vascular and Immune Homeostasis: A Review, Vascular and Endovascular Review, Vol.8, No.10s, 83--89.

INTRODUCTION

Background:

Most cells including Stem cells may undergo apoptosis and not contribute to further development, alternatively they may undergo symmetric cell divisions to self-renew or undergo terminal differentiation to maintain a pool of stem cells. Apoptotic cell removal from the tissues is thought to be dependent on their death by apoptosis, followed by their recognition and phagocytosis by professional antigen presenting cells (e.g.,dendritic cells) and non- professional antigen presenting cells such as epithelial cells and fibroblasts. A key event in apoptosis are the membrane changes that identify intact senescent neutrophils for removal by phagocytes. Although, the molecular mechanisms by which the intact apoptotic cell is recognized as ready for removal remain to be fully established it is apparent that phagocytic removal of apoptotic cells may employ one or more of a wide range of potential recognition mechanisms that consist of three major processes. These will be described prior to a more detailed description of the receptor-ligand interactions responsible for monocyte-derived macrophage recognition of apoptotic neutrophils (Arandjelovic and Ravichandran, 2015, Cocco and Ucker, 2001, Zhang et al., 2018).

LECTINS

Cell surface carbohydrates on one cell binding to lectins expressed by another cell represent important mechanisms in cell-cell adhesive interactions . Lectins are proteins that recognise specific sugar residues, interactions that can be specifically inhibited by the simple sugars recognized by the lectin (Cummings, 2019, Sharon and Lis, 1989, Brandley and Schnaar, 1986, Cho et al., 2022). The importance of sugar-lectin interactions in phagocyte recognition of apoptotic cells was established by the seminal work of Duvall and colleagues. This group used mouse thymocytes induced to undergo apoptosis by glucocorticoid treatment to establish that recognition is mediated by plasma membrane expression of newly expressed sugar moieties, in particular the externalization of glycan structures normally “hidden” in the proximal regions of the sugar sidechains of membrane glycoproteins (Duvall et al., 1985, Gruver-Yates and Cidlowski, 2013, Smith and Cidlowski, 2010, Bevers and Williamson, 2016, Mittelstadt et al., 2019). Inhibition of binding of apoptotic thymocytes by elicited peritoneal macrophages observed following pre-treatment with sugars such as N-acetyl glucosamine, but direct evidence of specific sugar exposure on apoptotic cells was not obtained (Pradhan et al., 1997). Another study used neonatal rat hepatocyte cultures to demonstrate recognition of apoptotic hepatocytes by their healthy neighbours and found that this process was mediated by the sugar recognition systems of the liver, principally the asialoglycoprotein receptor ((Vinken et al., 2014& Dim et al., 1992). Fibroblasts also have the ability to selectively recognize and phagocytose apoptotic neutrophils and that this process requires co-operation betweentwo distinct pathways; one involving a mannoselfiicose-specific lectin, with the other facilitated via the vitronectin receptor (see next section) (Gregory et al., 2015, Hail et al., 1994, Drouin et al., 2020).

PHAGOCYTE $\alpha_v\beta_3$VITRONECTIN RECEPTOR (VNR) AND THROMBOSPONDIN (TSP)

Human monocyte-derived macrophage ingestion in vitro of aged apoptotic human neutrophils was specifically inhibited by amino-sugars and amino acids in a charge-dependent manner that was also directly downregulated by changes in pH implicating the involvement of negatively charged residues on the neutrophil surface (Savill et al., 1989b)(Greenlee-Wacker, 2016). Pre-incubation studies showed that amino sugars exerted their effect at the surface of the apoptotic neutrophil suggesting adhesion molecule involvement.

They were subsequently identified as macrophage surface VnR and TSP (Savill et al., 1990 and 1992a). These studies further demonstrated that during the recognition process macrophage-secreted TSP acts as “molecular bridge”, binding unknown

neutrophil surface residues to two receptors on the macrophage surface, namely the $\alpha_v\beta_3$ integrin and CD36 (Greenlee-Wacker, 2016). Indeed, later work with inhibitory monoclonal antibodies (mAb) demonstrated a wide-ranging role for $\alpha_v\beta_3$ in phagocyte recognition of apoptotic cells. This structure was implicated in macrophage recognition of peripheral blood lymphocytes, thymocytes and eosinophils undergoing apoptosis, and was also found to be important in the recognition of apoptotic neutrophils by semi-professional phagocytes including fibroblasts and smooth muscle-like glomerular mesangial cells (Gregory et al., 2009, Kourtzelis et al., 2020)(Savill et al., 1990 and 1992b; Hall et al., 1994). In addition, the CD36 mAb. MØ specifically inhibited apoptotic neutrophil phagocytosis by macrophages of, thereby displaying synergy with $\alpha_v\beta_3$ mAb, (Cockram et al., 2021, Giles et al., 2000)(Savill et al., 1992a). CD36 is thought to have a general role in the of apoptotic cell clearance , since mAb to CD36 also inhibited macrophage recognition of human eosinophils and lymphocytes undergoing apoptosis (Akbar et al., 1994).

PHAGOCYTE RECEPTORS FOR PHOSPHATIDYL SERINE (PS)

Fadok et al utilised murine peritoneal macrophages elicited with thyoglycollate to demonstrate that these cells specifically recognize phosphatidyl serine (PS) that is transposed from the internal to the external leaflet of the cell membrane on apoptosis induction. Peritoneal macrophage phagocytosis of apoptotic thymocytes was inhibited by liposomes containing phosphatidyl-L-Serine, but not by liposomes containing other anionic phospholipids such as phosphatidyl-D-Serine. These findings demonstrate that apoptotic lymphocytes lose membrane phospholipid asymmetry and that PS exposure from the internal to the outer leaflet of the plasma membrane is a vital and early event in apoptosis (Fadok et al., 1992a)(He et al., 2011). It was additionally observed that phagocytosis of apoptotic cells by elicited peritoneal macrophages was inhibited by PS, regardless of the species (human or murine) or type (lymphocyte or neutrophil) of apoptotic cell (Gregory and Devitt, 2004). In contrast, mouse bone marrow macrophages, and human monocyte-derived macrophages both used VnR for recognition of apoptotic cells, regardless of their species or type (Fadok et al., 1992b)(Fige et al., 2022). These findings demonstrate the importance of the subpopulation of macrophages studied as this determines the mechanism by which apoptotic cells are recognized and phagocytosed . Interestingly, Fadok et al reported that mouse monocyte-derived macrophages "switched" from a VnR/TSP dependent recognition mechanism to a PS-dependent process at more than 48 hours after phagocytosis of 1,3 β-gIucan particles. This switching process appeared to involve endogenously-produced TGF- β in a key autocrine/paracrine role (Moodley et al., 2003, Fadok et al., 1993).

INTEGRINS: A FAMILY OF CELL SURFACE RECEPTORS

Cell-cell and cell-substratum interactions are mediated through several different families of receptorsthat facilitate adhesion to specific extracellular matrix proteins and ligands on adjacent cells.These receptors also play important roles in many diverse functions including cell growth, differentiation, junction formation and polarity. A number of families of adhesion receptors have been described that include: the integrins, the adhesion molecules of the immunoglobulin superfamily, the cadherins and lectins such as the selectins(Singh et al., 2012, Khalili and Ahmad, 2015).

Structure

Richard Hynes (1987) coined the term "integrin" that described a family of integral membrane receptors thought to link or "integrate" the cytoskeleton of one cell with another cell or with the extracellular matrix. Integins consist of heterodimers non-covalently associated with □ subunits (Buck and Horwitz 1987; Hynes Ruoslahti and Pierschbacher 1987). To date 18□ subunits (MT 120-180 Kd) and 8 βsubunits (MT 90-1 10 Kd) have been identified by several approaches involving molecular biology and protein chemistry (Hynes, 2002, Koivisto et al., 2014)(for review see Hynes, 1992). All □ subunits have a long extracellular domain containing several cation binding regions. The β subunit also has a long extracellular domain characterised by repetitive amino acid sequences containing a high number of cysteine residues (Fig 1.3). Electron microscopic images show that integrin subunits form a globular head with two extending rod-like tails (Schnittert et al., 2018, Miyazawa et al., 2001, Clyne et al., 2002)(Nermut et al., 1988). Immunolocalization studies suggest that the extracellular amino termini of the integrin subunits are located in the globular head, while the carboxy terminal segments of the two subunits are found in the tails (Kadry and Calderwood, 2020)(Weisel et al., 1992). Biochemical analysis demonstrates that the integrin heterodimer structure involves complex associations between both subunits (Anderson et al., 2014)(Calvete et al., 1992). These receptors are thought to act as transmembrane links between the extracellular environment and the intracellular cytoskeleton (Schwartz, 2010)(Smyth et al., 1993). Integrins appear recognize specific amino acid sequences in their ligands, with the arg-gly-asp (RGD) sequence being particulary well-characterised as it is found in severalmatrix proteins including fibronectin, flbrinogen, thrombospondin, vitronectin, laminin, type I collagen and von Willebrand's factor (Lowell and Mayadas, 2012, Mrinal and Soumyabrata, 2020) (Ruoslahti and Pierschbacher 1987). However not all integrins bind to ligands via RGD-containing domains, for example other recognition sequences have been identified in the plasma protein flbrinogen namely the platelet integrin $\alpha_{IIIb}\beta_3$ (Mrinal and Soumyabrata, 2020, Sánchez-Cortés and Mrksich, 2009). The expression of integrins can be altered in the presence of growth factors and is the result of specific alterations in subunit synthesis. TGF-β1 is a potent regulator of integrin expression (Munger and Sheppard, 2011)(Heino et al., 1989; Ignotz and Massague, 1987; Ignotz et al., 1989, heino and Massague, 1989; Koli et aI., 1991). TGF- β1 treatment of several cell types results in significant upregulated expression of β1, β2 and β3 receptors (lgnotz and masssaue 1987; Heino et al., 1989; Ignotz et al., 1989) including expression of other subunits including α1-α 2 (Guo et al., 2016, Lee et al., 2011)(Heino et al., 1989; Ignotz and Massague, 1987; Ignotz et al., 1989, Heino and Massague, 1989). In contrast to TGF-,β TNF- α and IFN-y downregulate the levels of $\alpha_v\beta_3$expressed by cultured endothelial cells (Defihippi et a!., 1991), although these agents can upregulate expression of all integrins fl (Bastarache et al., 2011, Laha et al., 2021, Santaia and Heino, 1991, Lafrenie et al., 1992). In a monocyte cell line, 14-days of GM-CSF treatment upregulated expression of $\alpha_v\beta_3$ as assessed by immunoprecipitation analysis (Krissansen et al1990). Furthermore, monocytes treated with GM-CSF expressed $\alpha_v\beta_3$ but not $\alpha_v\beta_5$ following 24h of stimulation. In contrast, monocytes treated for 24h with M-CSF expressed $\alpha_v\beta_5$ but not $\alpha_v\beta_3$. After 4 days

of culture, GM-CSF-stimulated monocytes expressed $\alpha_v\beta_3$ and also $\alpha_v\beta_5$ on their surface while M- CSF-treated monocytes only expressed $\alpha_v\beta_5$ (Lotfi et al., 2020, Ushach and Zlotnik, 2016)(De Nichilo and Burns, 1993).

CD36

The membrane glycoprotein CD36 (also known as GPIIIb or GPIV) is highly expressed by human blood platelet sbearing the Naka alloantigen (Tomiyama et aI., 1990), a highly hydrophobic glycoprotein (M 88,000) that consists of a single chain protein. This hydrophobic glycoprotein is resistant to chymotrypsin degradation (Xu et al., 2014, Ghosh et al., 2011)(Poldolsak, 1977). and is expressed by a number of cell types including monocyte/macrophages, some erythrocytes, microvascular endothelial cells, mammary epithelial cells, cultured fibroblasts (Liao et al., 2022)(for review see Greenwalt et al., 1992) together with certain tumour cells such as melanomas, megakaryobastic leukaemia (MEG-01) (Imamura et al., 1989) and human erythro-leukaemic cell lines (HEL) (Guerrero-Rodríguez et al., 2022, Kieffer., 1989, Greenwalt et al., 1992). CD36 has been demonstrated to be an adhesion receptor for thrombopondin (Morandi et al., 2021)(Asch et al., 1987), collagen (Tandon et al., 1989), Plasmodium falciparum infected erythrocytes (Ockenhouse and Chulary, 1988), and platelet agglutinin protein (p37) (Lian et al., 1991), in addition to itsrole in macrophage recognition and ingestion of apoptotic neutrophils (Savill et al., 1992a).

However, despite these diverse and important functions, CD36 expression may not be essential for health. CD36 is not expressed by platelets from human subjects with the Nak^a negative (Yamamoto et al., 1990), although megakaryocytes contained the corresponding mRNA (Lipsky et al., 1991). This phenotype is present in 3-11% of the Japanese and Korean population and 0.22% of the US population (Meng et al., 2023)(Greenwalt et al., 1992). These individuals appear healthy suffering no obvious haematological problems as a result of the absence of CD36 but are at risk of developing iso-antibodies following infusion of Nak^a -positive platelets (Flesch et al., 2021).

In addition to being a receptor for collagen, CD36 is also a membrane receptor for TSP (Asch et al., 1987) that mediates the TSP-dependent adhesion of platelets to monocytes or macrophages (Silverstein et al., 1989, Febbraio et al., 2001, Rać et al., 2007, Ho et al., 2005),.These adhesive processes are inhibited by anti-CD36 mAb OKM5, suggesting functional importance for the OKM5 epitope on CD36 (Li et al., 2021)(Asch et al., 1987a; Silverstein et al., 1989). The use of synthetic peptides corresponding to various CD36 domains of suggested that the interaction between CD36 andTSP is a two-step process. Firstly; the 139-155 amino acid region of CD36 binds first to TSP, triggering a conformational change in TSP that exposes a second site, which then binds to the 93-110 region of CD36 in a high affinity manner (Li et al., 2021, Rać et al., 2007)(Leung et al., 1992). Furthermore, three functional sequences were identified within one disulfide loop of CD36, one responsible for TSP binding (87-99) and two that mediate malarial parasite cytoadhesion (8-2 1 and 97-110) (Bachmann et al., 2022). One of these peptides (87-99) is a consensus protein kinase C (PKC) phosphorylation site. The dephosphorylation of constitutively phosphorylated CD36 in non-activated platelets or a megakaryocytic cell line resulted in a loss of collagen adhesion, with a reciprocal acquisition of enhanced TSP binding. PKC-mediated phosphorylation of this ecto-domain resulted in a loss of TSP binding and a reciprocal increase in collagen binding. This report suggests that CD36 may not be in a conformation to enable it to act as a TSP receptoras some studies cast doubt on the role of CD36 as a TSP receptor. For example, COS cells transfected with CD36 adhered to P.falciparum infected erythrocytes, but did not bind TSP (Hempel et al)

Furthermore, the Ca++ depencent binding of TSP to the U937 monocytic cell line was a consequence of calcium precipitate formation in the binding medium (Diler et al., 2014) while. OKM5 did not inhibit the platelet adhesion to TSP-coated plastic.In addition, monocyte oxidative burst initiated by antibody-dependen cross-linking ofmembraneCD36 could not be induced by membrane-bound TSP due to cross-linking of CD36 Lastly, Tandon et aI found that TSP binding by Nak^a negative platelets before or after activation is unimpaired compared with normal controls (Wang and Li, 2019).

These results cast doubt on a role for CD36 as the TSP receptor (Tandon et aI., 1991). In view of the findings reported by Asch and colleagues, a potential explanation is that in these experiments CD36 was phosphorylated and therefore could not act as a TSP receptor (Liao et al., 2022)(Asch et al., 1993). In contrast, Silverstein demonstrated that stable transfection of CD36-negative human Bowes melanoma cells with CD36 cDNA induced CD36 expression confering the ability to bind TSP; conversely transfection of CD36-expressing human C32 melanoma cells with an antisense construct inhibits CD36 expression and TSP binding (Silverstein et al., 1992), thereby providing good evidence thatthat CD36 is a TSP receptor (Chen et al., 2002).

These reported discrepancies are likely to reflect variations in CD36 conformation in different experimental settings. Furthermore, alternate splicing of CD36 mRNA transcripts might result in a variation in capacity by CD36 to bind TSP. Finally, to the difficulty in confirming a role for CD36 as a TSP receptor might be attributed to the factthat CD36 function requires cooperation with other receptors which might be absent in some situations. For example, Savill et al reported that human monocyte-derived macrophages binding of TSP involved a “two-point” mechanism that required both CD36 and the VnR (Savill et al.) CD36 was expressed by capillary endothelial cells but not large vessel endothelia of. In addition, while CD36 was expressed by capillary endothelial cells of most tissues , no expression was observed for kidney glomerular capillary endothelial ce1ls . It is also ofnote that IFN-□ was found to regulate expression of CD36 by human dermal microvascular endothelial cells (Swerlick et al.,1991) (Puchałowicz and Rać, 2020, Rasti et al., 2004, Primo et al., 2005, Fu et al., 2023).

Thrombospondin

Thrombospondin (TSP) was first described as a platelet membrane associated thrombin-sensitive protein (Baeniziger et al., 1971 and 1972) that was first characterised by Lawler as a major constituent of the platelet x-granule (Lawler, 1986). TSP has since been shown to be found inn endothelial cells (McPhea.rSofl etal., 1981), fibroblasts (Jaffe et al., 1983), smooth muscle cells

(Raugi et al., 1982), granular type II pneumocytes (Sage et al., 1983), keratinocytes (Wilkner et al., 1987), glial cells (Asch et al., 198Th), osteoblasts (Robey et al., 1989), and monocyte/macrophages (Ja.ffe et al., 1985) (Leung, 1984, Bornstein, 2009, Rustiasari and Roelofs, 2022, Tabary et al., 2022). A substantial body of work has shown TSP to be a protein with affinity for cell membranes, calcium ions, several matrix macromolecules (including heparan sulfate proteoglycans, fibronectin, laminin, types I and V collagens) and plasma proteins such as fibrinogen, plasminogen and histidine-rich glycoprotein (Lawler 1986; Frazier 1987) (Campbell et al., 2010, Rusnati et al., 2010). Several cell surface molecules can act as receptors for TSP with the role of CD36 and $\alpha_v\beta_3$described above. TSP also interacts with some $\beta 1$ integrins in addition to heparan sulphate proteoglycan and sulfated glycolipids called sulfatides (Asch and nachman, 1989; Kieffer et al., 1988; Lawler et al., 1988)(Ferrari do Outeiro-Bernstein et al., 2002).

The discovery of four additional genes closely related to TSP (now termed TSP 1) designated TSP2, TSP3, TSP4, and cartilage oligomeric matrix protein (COMP), indicates TSP functions severalmolecules with homology to TSPI (Bornstein et al., 1991; Laherty et al., 1992; Vos et al., 1992; and lawler., 1993). TSP 3 is is preferentially expressed in the lung and Bornstein et al suggested that TSP3 may play have unique role in lung cell matrix interactions with functions over laping with those of TSP1 and TSP2 (Bornsteifl et al., 1993). TSP4 has a similar structure to the TSP3 and it high levels have been reportedin heart and skeletal muscle with low levels in the brain or lung with undetectable levels in the placenta, liver and kidney (Lawler et al., 1993). These observations are suggestive of the participation by TSP4 in the development and function of cardiac or skeletal muscle.

Structure of TSP

TSP is a large (450 KD) trimeric, modular glycoprotein that has a domain-like structure consisting of multiple repeating elements. The amino terminal domain binds to the heparin sulfate protoglycan and sulfatides, with the globular C terminal domain containing the associated integrin- and CD36-binding RGD sequence,. However, there is increasing evidence that the sequence CysSerVa1ThCYSOEY (CSVTCG) binds TSP as it was previously identified as an adhesion motif in several proteins (Sarrazin et al., 2011, Annaval et al., 2020)(Prater et al., 1991; Tuszysnki et al., 1992).

Function of TSP

TSP has modest growth-promoting activity when added to serum-starved smooth muscle cells and this effect is inhibited by heparin (Majack et al., 1986). TSP treatment of vascular smooth muscle cells results in greateralso plays a role in the turnover of phosphoinositides and S6 kinase activity in (Maloney et al., 2012)(Burden et al., 1988), while treatment of actively growing cultured cells with anti-TSP antibodies inhibits their growth (Majack et al., 1988). These findings suggest that TSP is an autocrine growth factor, with TSP acting as a growth promoter on cells such as dermal fibroblasts (Phan et al., 1989) with the EGF-like domain playing an important role in this function (Engel et al., 1989). However, no direct evidence exists in support of a specific role for the EGF-like domain in growth promotion. Another agent that could account for the growth promoting activity ascribed to TSP TGF-f3, which is secreted by stimulated platelets and subsequentlybindingTSP but maintaining its biological activity (Sage et al., 1989).

However, cell proliferation iduced by TSP-induced is not observed in all cells with a number of report demonsrating that exogenous TSP can induce arrest of proliferation of cultured endothelial cells derived from different species and organs (Yuan et al., 2023)(Taraboletti et al., 1990; Bagavandoss and Wilks, 1990).

Interaction with inflammatory cells

Functional studies of diverse cell types have shown that TSP induced attachment and spreading, chemotaxis, haptotaxis or proliferation, with mitogenic effects seen infibroblasts (Phan et al., 1989). In contrast, TSP inhibited endothelia1 cell mitogenesis in responses to growth and angiogenic factors (Taraboletti etal 1990). The short half-life of TSP in the cell-associated matrix together with its influence on cell activation and proliferation implicates it as a modulator of inflammation (Lerman and Hammes, 2018). TSP enhances adhesion, spreading and motility of human neutrophils (Suchard et al., 1991; Mansfield et al.,199Oand also primes their FMLP mediated chemotaxis and oxidant generation (Mansfield et al., 1990; Suchard et al.,1991). Several studies on monocytes or the monocyte-like cell line U937 have implicated TSP in platelet-monocyte or monocyte-substrate adhesion (Lawler et al., 1988; Silverstein and Nachman 1987; Varani et al., 1991). Suchard et al. reported HL-60 cell maturation along either the monocytic or granulocytic pathway resulted in upregulated TSP receptor expression (Suchard et al., 1994). In addition, undifferentiated

FIL-60 cells synthesized TSP and expressed a single class of relatively high affinity TSP receptors whose function were inhibited by heparin-. HL-60 cells differentiated to macrophage-like cells exhibited suppression of TSP synthesis, but TSP receptor expression was increased 10-fold , suggesting coordinated loss of regulation between TSP receptors with their ligand when HL60 cells are differentiated to macrophages. In contrast, HL-6O differentiation to neutrophil-like cells gave a 4-5 fold enhancement of both TSP synthesis and TSP receptor expression; observations that suggestive of differential regulation of expression of both TSP and TSP receptors during leukocyte maturation.

Regulation of TSP

Newly inflicted wounds exhibit intense staining for TSP whereas healed wounds exhibit almost no staining (Raugi et al., 1987), suggesting a requirement for the presence of TSP to permit cell proliferation and migration. Cell culture experiments bear this as proliferating endothelial cells, smooth muscle cells, and fibroblasts synthesise greater amounts of TSP than do non-dividing cells (Mumby et al 1984). TSP synthesis is induced by mediators released from platelet α -granules. For example, PDGF rapidly increases TSP synthesis () and increased TSP mRNA in growth- arrested smooth muscle cells with the induction of TSP message

paralleling PDGF-mediated mitogenesis. (Majack et al., 1985; Kobayashi and Yamamoto, 1991; Majack et al., 1987). TSP mRNA is also "super induced" by cycloheximide, a process manner similar to that seen for c-myc, c-fos and other growth-regulatory gene products (Majack., 1987). TGF- β specifically regulates TSP mRNA during proliferation of smooth muscle cells and mesangial cells by (Penttinen et al., 1988; Kobayashi and Yamamoto, 1991). In contrast treatment of human umbilical vein endothelial cells with IL- β1 and TNF- α led to a time- and dose-dependent decline in TSP synthesis (Morandi et al., 1994; Lyons-Giordano et al., 1991). Platelets induce TSP synthesis by monocytes, a process requiring platelet-monocyte contact rather than through the release of soluble products from stimulated platelets (Schwartz, 1989), indeed TSP itself might be responsible for the adhesive contact. (Silverstein and Nachman,1987).

ACKNOWLEDGMENTS

The authors extend their appreciation to Prince Sattam bin Abdulaziz University (PSAU) for funding this research work through the project number 2024/03/29361.

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