Department of Sciences

PhD Programme in “Biomedical Sciences and Technologies”

Cycle XXXII

Integrin αVβ3-dependent functions in monocytes and microglia: modulation by thyroid hormones and the environmental pollutant BPA

PhD Student: Fabio Gionfra **Tutor:** Prof. Sandra Incerpi

PhD School Coordinator: Prof. Paolo Visca **Tutor:** Prof. Maria Marino

INDEX

RIASSUNTO

Gli ormoni tiroidei (THs) L-tiroxina (T_4) e L-triiodotironina (T_3) sono importanti regolatori nei processi di differenziazione, crescita e metabolismo, sia durante lo sviluppo che nell'organismo adulto.

Le azioni del T_3 sono mediate dal suo legame con specifici recettori che possono traslocare al nucleo dove regolano l'espressione genica. Gli effetti non genomici o extranucleari dell'ormone tiroideo, mediati principalmente da T_4, iniziano a livello della membrana plasmatica e non dipendono principalmente dall'interazione dell'ormone con i classici recettori nucleari (TRs). Negli ultimi anni, la scoperta dell'integrina αVβ3 come recettore di membrana per gli ormoni tiroidei incoraggia al riesame di questi ormoni come possibili modulatori delle attività immunitarie e delle cellule del sistema immune. Sono presenti degli effetti importanti dell'ormone tiroideo che sembrano essere rilevanti nell'infiammazione ed iniziano a livello dell'integrina αVβ3.

I dati del nostro laboratorio hanno mostrato che gli ormoni tiroidei inibiscono la migrazione indotta da MCP-1 nei monociti THP-1, mostrando proprietà anti-infiammatorie. Queste informazioni ci hanno spinto a studiare la capacità degli ormoni tiroidei di modulare le attività immunitarie nelle cellule della microglia BV-2, le cellule immunitarie nel sistema nervoso centrale. In queste cellule, sono stati studiati principalmente gli effetti degli ormoni tiroidei mediati dai recettori nucleari, mentre noi abbiamo focalizzato la nostra attenzione sugli effetti extranucleari mediati da αVβ3.

Pertanto, l'obiettivo principale del mio dottorato in questi 3 anni è stato quello di studiare se gli ormoni tiroidei e i loro analoghi possano modulare alcune delle risposte tipiche delle cellule del sistema immunitario anche nelle cellule murine della microglia BV-2 e il possibile ruolo dell'integrina αVβ3.

Prima di tutto abbiamo misurato l'espressione dell'integrina mediante analisi di citometria a flusso e microscopia confocale. I risultati mostrano che l'integrina αVβ3 è molto più espressa nella microglia, rispetto ai monociti. Abbiamo poi condotto esperimenti di migrazione cellulare mediante il saggio del *wound healing* insieme a curve di proliferazione, test di citotossicità e determinazione delle ROS mediante la sonda fluorescente intracellulare DCFH-DA.

È noto che l'ormone tiroideo è in grado di modulare l'azione di fattori di crescita come EGF, TGF-α e IGF-1. Precedenti esperimenti dal nostro laboratorio hanno dimostrato che gli ormoni tiroidei sono in grado di interagire con i fattori di crescita. Al fine di caratterizzare la microglia BV-2 e la loro possibile risposta agli ormoni tiroidei, abbiamo condotto esperimenti di proliferazione

cellulare indotti dal fattore di crescita IGF-1, poiché l'integrina αVβ3 è un suo co-recettore e il suo effetto è già stato studiato in precedenza in altre linee cellulari, come i mioblasti L6 da muscolo scheletrico di ratto e i monociti leucemici umani THP-1. Le curve di proliferazione sulle cellule BV-2 stimolate con IGF-1 sono state eseguite sia in presenza che in assenza degli ormoni tiroidei. Gli ormoni tiroidei sono in grado di inibire la proliferazione cellulare indotta da IGF-1, ma questo effetto inibitorio viene completamente annullato in presenza di inibitori dell'integrina. Lo studio della trasduzione del segnale mediante specifici inibitori ha mostrato che l'effetto inibitorio dell'ormone tiroideo sulla proliferazione indotta da IGF-1 e mediato dalla MAPK.

La migrazione cellulare e la guarigione delle ferite dipendono fortemente dalla produzione di citochine e dalle specie reattive dell'ossigeno (ROS), dall'ossido nitrico (NO) e dai loro derivati. La capacità dell'ormone tiroideo di generare ROS è stata valutata anche nella microglia mediante l'uso della sonda fluorescente DCFH-DA. La produzione di ROS, indotte da T_4, veniva ridotta in presenza di inibitori dell'integrina αVβ3 come abbiamo già riportato nei monociti THP-1. E' stato ipotizzato un possibile crosstalk tra l'integrina αVβ3 e la NADPH ossidasi associata alla membrana plasmatica, ma ad oggi non è stato ancora confermato.

Il processo di guarigione delle ferite è stato studiato mediante il saggio *wound healing*. E' stato creato un taglio (scratch) nel monostrato cellulare con un puntale sterile per pipette. Successivamente le cellule BV-2 sono state stimolate con LP_S, T_3 e T_4 per 24 ore con e senza gli inibitori dell'integrina αVβ3: il tripeptide RGD e l'acido tetraiodotiroacetico (tetrac) un metabolita dell'omone tiroideo che funge anche da sonda per l'integrina αVβ3. Gli ormoni tiroidei a concentrazioni fisiologiche sono in grado di ridurre l'invasione indotta da LPS. L'utilizzo degli inibitori come RGD e tetrac non annulla l'effetto dell'ormone, come ci si aspetterebbe, ma al contrario lo potenzia. Questo insolito effetto era già stato osservato anche nei monociti THP-1.

Recentemente, è stato osservato che il bisfenolo A (BPA), un monomero presente nelle materie plastiche, lega l'integrina αVβ3 ed agisce come interferente endocrino comportandosi come antagonista dell'ormone tiroideo. Abbiamo quindi esaminato, in un ampio intervallo di concentrazione, l'effetto del BPA *in vitro* sulla linea cellulare THP-1. I risultati preliminari mostrano che nella migrazione indotta da MCP-1, il BPA a tutte le concentrazioni testate è in grado di imitare gli effetti degli ormoni tiroidei e inibire la migrazione, ma al momento non siamo in grado di confermare se questa inibizione è mediata dalla interazione tra BPA e l'integrina αVβ3.

In conclusione, i risultati della mia tesi mostrano per la prima volta che nelle cellule murine della microglia BV-2 l'effetto dei chemioattrattanti (LPS e IGF-1) modula l'espressione dell'integrina αVβ3, la migrazione cellulare e la proliferazione in modo simile a quello che abbiamo

già visto nelle THP-1 e gli ormoni tiroidei, principalmente il T_4, sono in grado di annullare i loro effetti, comportandosi come agenti anti-infiammatori.

SUMMARY

The thyroid hormones (THs) L-thyroxine (T_4) and L-triiodothyronine (T_3) are essential regulators of development, differentiation, growth and metabolism, both in the developing and the adult organism. The actions of T_3 are mediated by its binding to specific receptor proteins that may translocate to the cell nucleus where they regulate gene expression. Non-genomic or extranuclear effects of thyroid hormone, mediated principally by T_4, are initiated at the plasma membrane and do not depend primarily on the interaction of the hormone with classical nuclear receptors (TRs). In the last years, the discovery of integrin αVβ3 as a plasma membrane receptor for THs encourages reconsideration of these hormones as a possible modulators of immune activities and cells of the immune system. There are important effects of TH that appear to be relevant to inflammation and start at the integrin αVβ3.

Data from our laboratory showed that THs inhibit the migration induced by chemokine in THP-1 monocytes, showing anti-inflammatory properties. All this information prompted us to study the capability of THs to modulate immune activities in the BV-2 microglia, the resident immune cells of the CNS. In these cells, in fact, the effects of THs have been studied mainly as to the nuclear effects, whereas we focused our attention to the extra-nuclear effects of THs possibly mediated by αVβ3.

Therefore, the main goal of the three years PhD is to study whether THs and their analogues might be able to modulate responses typical of immune cells also in murine BV-2 microglial cells and the possible role of integrin αVβ3.

First we evaluated the expression of the integrin by flow cytometry analysis and by confocal microscopy. Results show that the integrin αVβ3 shows a high expression in murine microglia, higher than in THP-1 leukemic monocytes. We carried out experiments of cell migration by wound healing together with proliferation curves, cytotoxicity assay by MTT and ROS determination by the intracellular fluorescent probe DCFH-DA.

TH is known to be able to modulate the actions of growth factors such as EGF, TGF-α and IGF-1. Previous experiments from our laboratory have shown that THs are able to crosstalk with growth factors. In order to characterize BV-2 microglia and their possible response to THs, we carried out experiments of cell proliferation stimulated by a growth factor, IGF-1, since integrin αVβ3 is a co-receptor and its effect has been already studied with and without THs in different cells, L6 myoblasts from rat skeletal muscle and THP-1 human leukemic monocytes. The proliferation curves of BV-2 cells induced by IGF-1 were carried out both in the presence and absence of THs. THs are able to inhibit the proliferation stimulated by IGF-1 and inhibitors of the

integrin fully reverted the inhibitory effect of the hormone. Inhibitors of the transduction signaling show that the effect of TH on IGF-1-stimulated proliferation is mediated by the MAPK pathway.

Transmigration and wound healing are strongly dependent on cytokines production and reactive oxygen species (ROS), nitric oxide (NO) and derived products. The capability of THs to give rise to ROS was assessed also in microglia by the fluorescent probe DCFH-DA. ROS induced by T_4, were blocked by the inhibitors of integrin αVβ3 as reported in THP-1 monocytes. A possible crosstalk of integrin αVβ3 with the plasma membrane NADPH oxidase can be hypothesized, but at present has not been verified.

The wound healing was carried out by a scratch assay. Confluent BV-2 monolayer were scraped to form a wound. Cells were stimulated with LPS, T_3 and T_4 for 24 hours with and without the inhibitors of integrin αVβ3: RGD peptide and tetraiodothyroacetic acid (tetrac) a metabolite of TH and a probe for integrin αVβ3. THs at physiological concentrations were able to prevent the invasion induced by LPS. The inhibition was not reverted by the use of RGD or tetrac, as expected, but in some cases was potentiated. This unusual effect was already observed in THP-1 monocytes.

Recently, it was shown that bisphenol A (BPA), a monomer of plastic materials, binds integrin αVβ3 interfering with the actions of THs as a hormone antagonist. We then examined, over a wide concentration range, the effect of BPA exposure *in vitro* on the THP-1 human cell line. Preliminary results on the migration induced by MCP-1 show that BPA at all concentrations tested was able to mimic the effects of thyroid hormones and inhibit the migration induced by MCP-1, but at present we are not able to confirm that this inhibition is mediated by interaction between BPA and αVβ3 integrin.

In conclusion, the results of my thesis show for the first time that BV-2 murine microglial cells give a response to chemoattractants (LPS and IGF-1) evaluated as expression of integrin αVβ3, cell migration and proliferation similar to that found in THP-1 and THs, mainly T_4, are able to inhibit it, indicating that THs also in these cells behave as anti-inflammatory agents.

1. INTRODUCTION

1.1 Integrins

1.1.1 Structure and function

Integrins are a family of cell surface receptors expressed in all multicellular organisms and different cell types, that mediate adhesion to a wide range of ligands present within the extracellular matrix or on the surface of opposing cells. Integrin activation controls extracellular matrix assembly, thereby contributing to processes such as angiogenesis, tumor cell metastasis, inflammation, the immune responses and homeostasis (Ruoslahti, 1999; Allen *et al*., 2002; Hynes, 2002). In addition, integrins also play key roles in the assembly of the actin cytoskeleton as well as in modulating signal transduction pathways that control biological and cellular functions including cell adhesion, migration, proliferation, cell differentiation and apoptosis (Schwartz *et al*., 1995; Geiger and Yamada, 2011). Structurally, integrins exist as two noncovalently bound α and β subunits, which pair to form heterodimers. There are 18α and 8β known subunits which combine to form at least 24 distinct integrin heterodimers (Hynes, 2002). Structurally, the largest part of the integrin heterodimer receptor is ligand binding globular extracellular domain. Both α and β subunits also have a single transmembrane domain and short cytoplasmic tail. Although short, the integrin tails are known to associate with many proteins and also with the cell cytoskeleton (Figure 1). Thus, integrins function as a linker between ECM and the cell body (Campbell and Humphries, 2011). Mice that lack integrin expression either constitutively or in specific cell types exhibit a wide range of phenotypes. These knockout mice have provided much insight into the functions of specific integrin heterodimers, reflecting the unique roles of the various integrins. In general, each of the 24 integrins has a specific, nonredundant function (Chen and Sheppard, 2007).

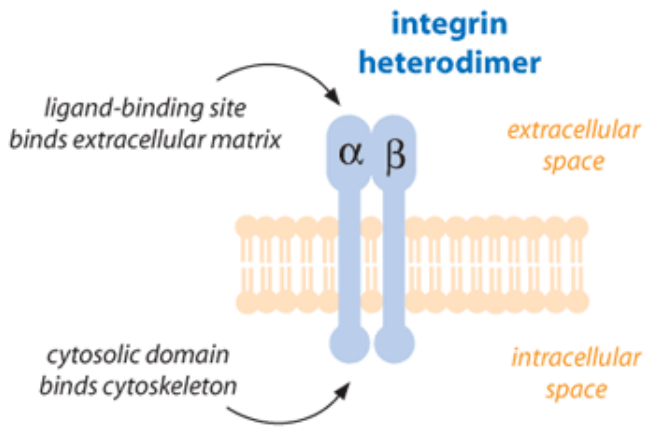

Figure 1. The basic structure of the integrin, a cell surface protein receptor (Eslami, 2005).

Each heterodimer pair has a preference to bind specific extracellular matrix (ECM) proteins or cell surface ligands. Based on ligand specificity, integrin heterodimers can be classified to four main families: collagen, RGD (arginine-glycine-aspartic acid motif), laminin and leukocyte-specific receptors (Barczyk *et al*., 2010) (Figure 2).

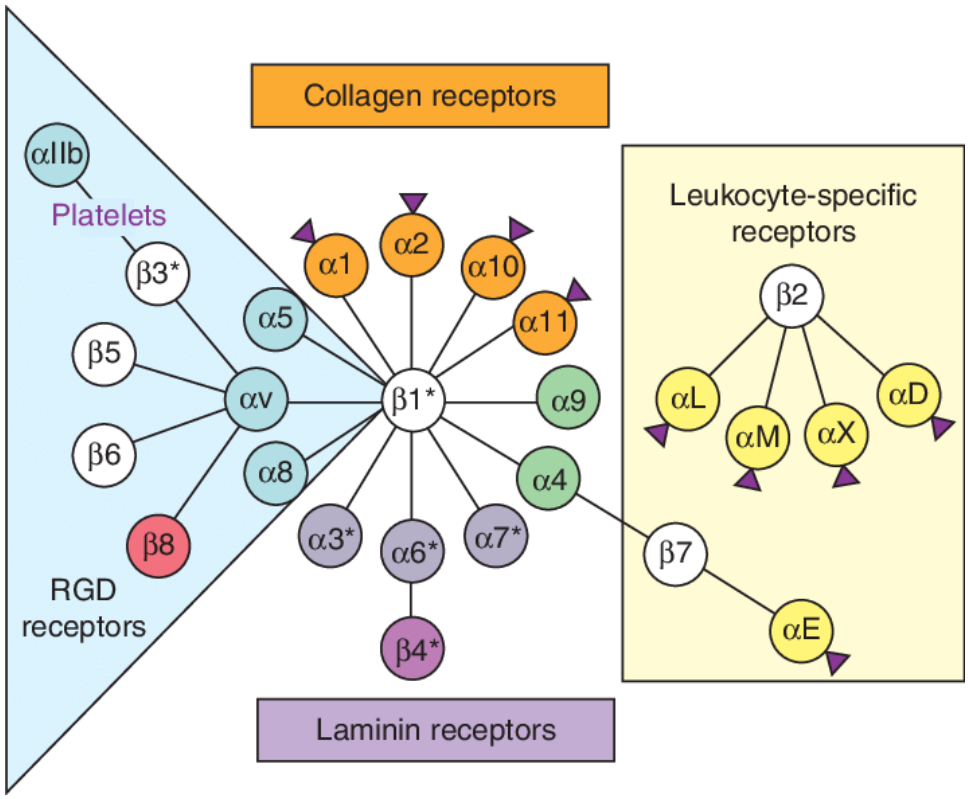

Figure 2. Different pairs of integrin heterodimers and their ligands (Hynes, 2002).

Depending on the cell type, integrins can be either basally activated as with most adherent cells that are attached to a basement membrane, or basally inactive as with platelets or leukocytes that freely circulate until activated to undergo platelet aggregation or mediate an inflammatory response, respectively. The function of integrins is regulated by activation and inactivation of the receptor complex. Integrin activation involves a large conformational change mainly in the extracellular domain and since integrins have only short cytoplasmic tails the signalling processes are carried out by intracellular tail binding proteins.

In the model of outside-in signalling the extracellular ligand binding results in activation of the receptor. The activation leads to conformational change, tail separation and allows cytoplasmic regulators bind the tails. Ligand binding also clusters the integrins together to form multimeric complexes of several heterodimers. On the other hand, integrins can also be activated from inside of the cell. The inside-out signalling is triggered by an intracellular activator such as talin or kindlin that bind to the β tail. Talin binding results in a conformational change that increases the integrin

affinity towards extracellular ligands (Shattil *et al*., 2010). In the "switch-blade" model integrins change their conformation upon activation. Integrin receptors are either in a bent or extended conformation and the headpiece (ligand binding domain) is either closed or open (Takagi *et al*., 2002; Luo *et al*., 2007). Integrin receptor conformation fluctuates between different conformation states that can be (Figure 3):

1) closed headpiece (bent). Low affinity.
2) closed headpiece (extended). Intermediate affinity. Also called primed.
3) open headpiece (extended). High affinity. Also called active or ligand-bound.

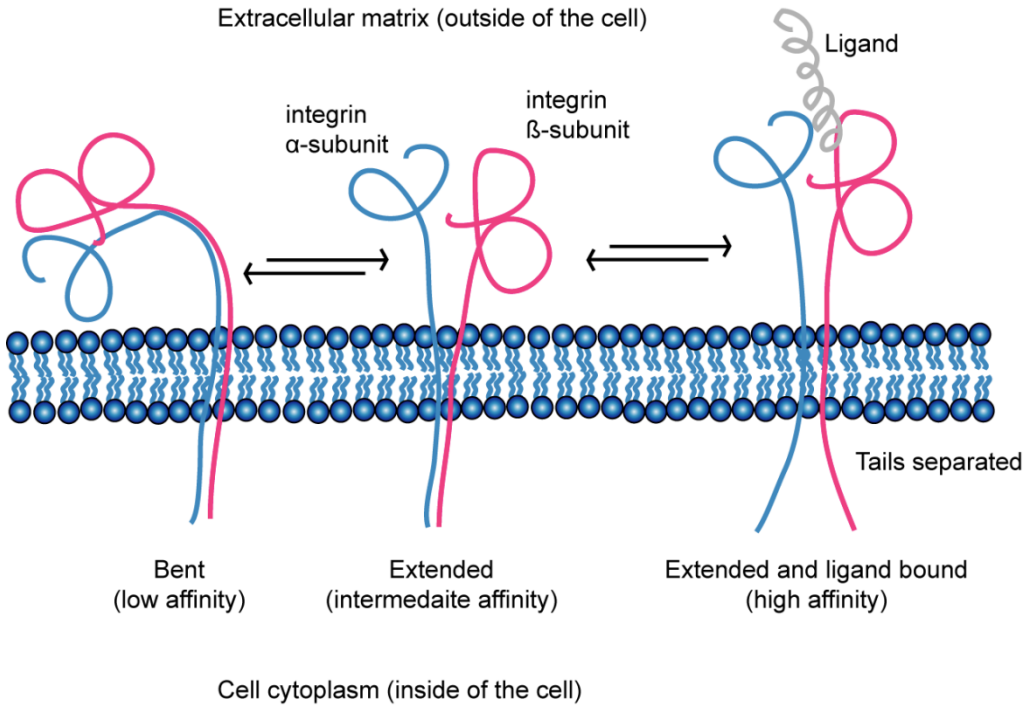

Figure 3. Artistic view of different integrin receptor conformations during activation (Arjonen, PhD thesis).

It is believed that in the bent conformation the ligand binding pocket is facing towards the plasma-membrane and there is no room for ligand binding. Integrin extension would thus allow ligand binding to take place. In another controversial model of integrin activation called "deadbolt" also the bent conformation could bind the ligand (Xiong *et al*., 2003).

Integrins themselves have no kinase activity but instead provide a connection between the extracellular matrix and the actin cytoskeleton. This connection allows integrins to regulate cytoskeletal organization and cell motility as well as to alter fluxes of many intracellular-signaling pathways. The extracellular domains can bind a variety of ligands, whereas the intracellular

cytoplasmic domains anchor cytoskeletal proteins. This linkage between the cell exterior and interior allows for bidirectional signaling across the plasma membrane.

Integrin signaling is closely associated with the concept of "integrin activation" where rapid, reversible changes occur in the conformation of the extracellular domains and the relative positioning of the transmembrane and cytoplasmic domains of the integrin heterodimer (Tadokoro *et al*., 2003; Shattil *et al*., 2010; Su *et al*., 2016). These conformational changes increase the ligand binding affinity of the integrin and initiate integrin signaling (Shattil *et al*., 2010). Integrins are able to transduce signals intracellularly following ligand binding ("outside-in" signaling). However, unlike most other cell receptors, integrins can shift between high- and low-affinity conformations for ligand binding ("inside-out" signaling). During inside-out signaling, activator proteins such as talins and kindlins bind to the β-integrin cytoplasmic tail, leading to a conformation shift in the integrin dimer that results in higher ligand binding affinity. Functionally, inside-out signaling can control the adhesion strength and modify cell migration behavior (Shattil, 1999; Takagi *et al*., 2002; Shen *et al*., 2012).

Since their discovery approximately 20 years ago, significant progress has been made in the integrin biology field that has resulted in a greatly improved understanding of their structure and function.

1.1.2 Integrin αVβ3

The αVβ3 is a member of the integrin superfamily of adhesion proteins. This class of receptors was named in 1986 by Hynes to emphasize their role in integrating the intra- cellular cytoskeleton with the external milieu. The term "vitronectin receptor" was first applied to αVβ3 as it bound the plasma protein vitronectin; it was first purified from placenta and defined as a vitronectin receptor by Pytela in 1985, and cloned and sequenced by Suzuki in 1986. Its name is a misnomer, as it clearly is not selective for vitronectin or its sole receptor (Pytela *et al*., 1985; Suzuki *et al*., 1986; Hynes, 1992).

αVβ3 is the most abundant integrin expressed in mammalian cells (Humphries, 2000). The site of highest expression *in vivo* is the osteoclast. Lower levels are also seen in platelets and megakaryocytes, kidney, some vascular smooth muscle, endothelium and placenta. αVβ3 is up-regulated in certain pathologies, such as malignant melanoma, and in numerous *in vitro* cultured adherent cell lines (Horton, 1997). Integrin αVβ3 has diverse roles in several distinct processes, such as osteoclast-mediated bone resorption, angiogenesis, pathological neo-vascularization and tumor metastasis (Wilder, 2002). For angiogenesis, αVβ3 appears to be the most important one among all the integrins (Brooks *et al*., 1994; Kumar, 2003).

αVβ3, originally described as the vitronectin receptor, is one of the most promiscuous integrins. It binds to a variety of plasma and extracellular matrix (ECM) proteins including vitronectin, fibrinogen, fibronectin, von Willebrand's factor, thrombospondin, osteopontin and bone sialic protein 1 (Denis *et al*., 1993; Sonnenberg, 1993; Krutzsch *et al*., 1999). It has also been reported to serve as a receptor for adenovirus, foot and mouth disease virus, coxsackievirus A9, and hantaviruses (Wickham *et al*., 1993; Neff *et al*., 1998; Triantafilou *et al*., 2000). It binds to tat (a regulatory protein from HIV), fibroblast growth factor 2 and a fragment from the disintegrin ADAM-15, suggesting roles in the control of soluble factors as well (Rusnati *et al*., 1997; Barillari *et al*., 1999; Nath *et al*., 1999). In addition to interacting with a number of ECM proteins, integrin αVβ3 has been shown to be associated with metalloproteinase MMP-2, activated PDGF, insulin and VEGF receptors, facilitating the optimal activation of cell proliferation, invasion and preventing apoptosis (Kumar, 2003). Most of these protein ligands encode an RGD (Arg-Gly-Asp) sequence that is presumed to represent the binding site for αVβ3 (Liu *et al*., 2008) (Figure 4). More importantly, the inhibition of αVβ3 integrin activity by monoclonal antibodies (mAbs), cyclic RGD peptide antagonists, and peptidomimetics has been shown to induce endothelial cell apoptosis (Meerovitch *et al*., 2003) and to inhibit angiogenesis (Kumar, 2003).

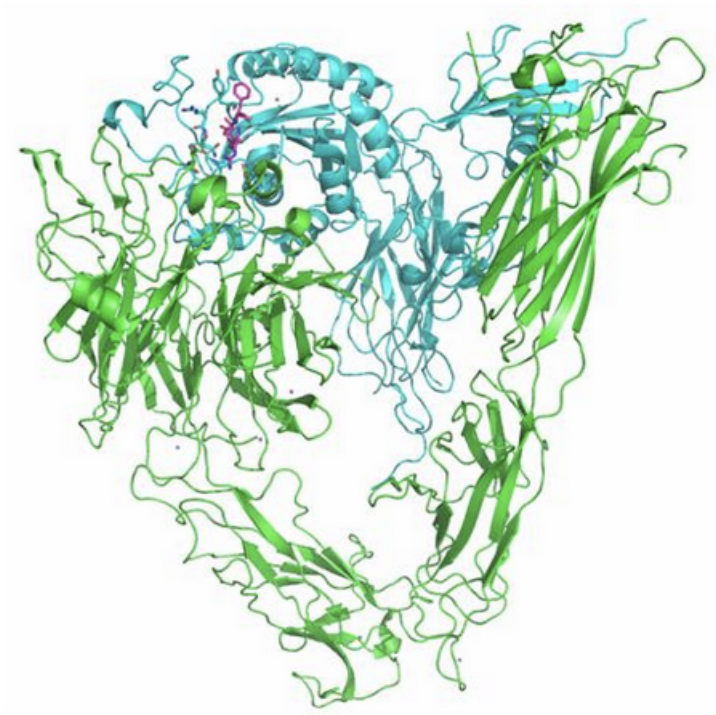

Figure 4. Crystal structure of αVβ3 with αV (green) and β3 (cyan). Also shown is the RGD cyclic peptide (violet) (Xiong *et al*., 2002).

Interestingly, αVβ3 has been shown also to mediate cell-cell interaction by binding to the membrane-associated glycoproteins of the immunoglobulin superfamily, CD31/PCAM-1 and the L1 adhesion molecule, the latter via an RGD sequence in one of its immunoglobulin domains

(Buckley *et al*., 1996; Ebeling *et al*., 1996). In addition, αVβ3 was demonstrated to bind an RGD sequence in the adenoviral penton base coat protein (Wickham *et al*., 1993). These latter findings suggest that this receptor may mediate a wide range of physiological and pathological processes.

1.1.3 Binding by the integrin αVβ3 of thyroid hormones and analogues

In the last years, several studies have identified the integrin αVβ3 as the membrane receptor also for thyroid hormones in normal tissues, but also in several types of cancer cells (Yalcin *et al*., 2010, 2013; Mousa *et al*., 2012; Lin *et al*., 2016; Gionfra *et al*., 2019). Small molecules have not been previously seen to be ligands of the integrin (Plow *et al*., 2000), and because the principal ligands of the integrins are proteins, the concept that a receptor for a small molecule like thyroid hormone existed on integrin αVβ3 was surprising, if not heretical. Subsequently, however, it has been appreciated that receptors for the stilbene, resveratrol (Lin *et al*., 2006, 2011, 2019) and for dihydrotestosterone (DHT) (Lin *et al*., 2009a) are present on αVβ3.

Evidence suggesting that a cell surface receptor site for thyroid hormone existed on an integrin accumulated from several sources before identification of the receptor on integrin αVβ3. However, in a series of studies published a decade or more before integrin αVβ3 was shown to bear a thyroid hormone receptor, Lin and collaborators showed that tetraiodothyroacetic acid (tetrac), a deaminated derivative of L-thyroxine (T_4), inhibited certain thyroid hormone actions, the onset of which was sufficiently rapid to exclude participation of nuclear Thyroid Receptors (TR) and gene expression or transcription (Mylotte *et al*., 1985; Lin *et al*., 1998, 1999a; Davis *et al*., 2000).

The thyroid hormone-binding site on integrin αVβ3 is located near this RGD recognition site and comprises two distinct domains that are related neither structurally nor functionally to TRs (Cody *et al*., 2007a; Lin *et al*., 2009). In order to understand how thyroid hormones and analogues interact with integrin αVβ3, models of their potential interactions were carried out (Cody *et al*., 2007a). In the case of tetrac-binding, the acetic acid moiety was mapped to that of Asp in the RGD cyclic peptide. In this model, most of the hormone interactions are with the βA domain of the integrin. Similar models were made with T_4 and T_3, and with the stilbene, resveratrol, and estradiol. These modeling data indicated that there was sufficient space in the cavity for the hormones to bind (Xiong *et al*., 2002). In the case of the more planar steroid-like molecules, the modeling data indicate a second, smaller binding pocket present near the RGD recognition site (Cody *et al*., 2007a). The binding site S1 exclusively binds T_3 and causes activation of the phosphoinositide 3-kinase (PI3-K) cascade, while S2 binds both T_3 and T_4 and results in MAPK (ERK1/2) pathway activation (Lin *et al*., 2009) (Figure 5). Thus, in contrast to the TR/thyroid hormone axis, both T_4 and T_3 act as active hormones at the integrin receptor. In fact, as the affinity for T_4 is far higher than

that for T_3, and lies in the physiologic concentration range that is comparable to the T_3 affinity for nuclear receptors, T_4 may well be the main hormone signalling at the integrin, thus transcending its role of pro-hormone for T_3 (Davis *et al*., 2013). Tetrac binds to both thyroid hormone-binding sites on the integrin and is a specific inhibitor of integrin-mediated thyroid hormone action (Wu *et al*., 2005; Mousa *et al*., 2006c).

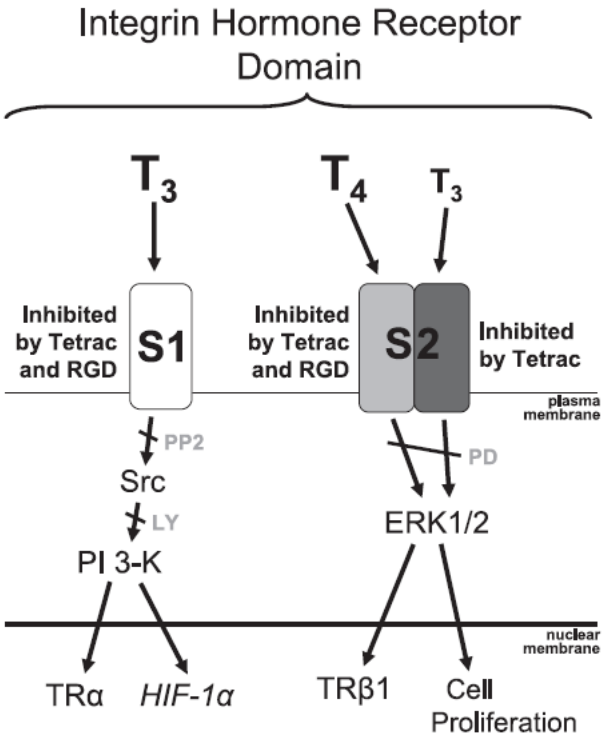

Figure 5. Two hormone-binding sites: S1 and S2 are proposed within the iodothyronine receptor domain on integrin αVβ3. T_3 interacts with S1 to activate the PI3-K signal transduction pathway and T_3-initiated action at S1 is inhibited by tetrac and the RGD peptide. T_4 and T_3 both bind to S2 to activate ERK1/2 and this effect is blocked by both tetrac and RGD peptide, but the action of T_3 on S2 is inhibited only by tetrac. This may reflect allosteric changes in the integrin site that are distinctive for the liganding of T_3 and T_4 (Lin *et al*., 2009b).

To more quantitatively model potential interactions of αVβ3 integrin with thyroid hormone (T_4, T_3) and the analogue tetrac, molecular docking experiments using quantum chemical calculations (QM/MM) were carried out in the presence of Ca^{2+} or Mg^{2+} near the RGD recognition site (Cody *et al*., 2007b), as observed in their crystal structures (Xiong *et al*., 2002). These computational results indicated a strong electronic contribution to the binding energies by the presence of Ca^{2+} or Mg^{2+} near the active site that impacts ligand-binding. These computational results showed that the preferential binding of T_4 and tetrac to the RGD recognition site was similar. Computational data reveals that the phenolic ring of T_3 (T_3-Ca^{2+}) occupies an alternate binding pocket near the RGD peptide site. These data support the binding kinetics data that are consistent with the presence of two discrete binding sites for T_3 that control distinct downstream signal transduction pathways (Davis *et al*., 2011).

Integrin αVβ3 is known to transduce signals via mitogen-activated protein kinase (MAPK) (Illario *et al*., 2005; Rucci *et al*., 2005). Thyroid hormone has been shown in a variety of human cell lines to activate MAPK (ERK1/2) (Davis *et al*., 2005, 2006; Mousa *et al*., 2005, 2006a) and it is now appreciated that such activation is integrin-dependent (Bergh *et al*., 2005). Cellular events consequent to activation of MAPK by thyroid hormone include angiogenesis and tumor cell proliferation (Bergh *et al*., 2005; Davis *et al*., 2006). Also intracellular protein trafficking can be regulated from the thyroid hormone receptor on αVβ3. For example, nuclear import of TRβ1 that is resident in cytoplasm is stimulated by thyroid hormone; the hormone-induced TR trafficking requires phosphorylation of TR by MAPK (Davis *et al*., 2000, 2008; Lin *et al*., 2003) and uptake of TR by the nucleus probably occurs with the receptor in a complex with the kinase in thyroid hormone-treated cells (Davis *et al*., 2008; Cao *et al*., 2009). Estrogen receptor-α (ERα) is similarly caused to move into the nucleus from the cytoplasm by αVβ3-mediated thyroid hormone action. Signal transducer and activator of transcription-1α (STAT1α) is involved in the conversion of cytokine signals into cellular actions and its nuclear uptake is also promoted non-genomically by T_4 via the integrin. The potentiation by thyroid hormone of the STAT1α-dependent action of interferon-γ on HLA-DR expression may be a function of the effect of the hormone on the trafficking of STAT1α (Lin *et al*., 1998). Like TR, ERα (Tang *et al*., 2004) and STAT1α (Lin *et al*., 1998) are both subject to phosphorylation of specific serines by MAPK (extracellular regulated protein kinase 1/2, ERK1/2). Other proteins whose shuttling into the nucleus occurs under the direction of thyroid hormone include the oncogene suppressor protein, p53 (Shih *et al*., 2001), STAT3 (Lin *et al*., 1999b), Trip230 (Chen *et al*., 1999) and the internalized αV monomer. The insertion of Na, K-ATPase protein into the lung alveolar cell plasma membrane is induced non-genomically by thyroid hormone and involves PI3-K (Bhargava *et al*., 2007), a signal transducing enzyme that can be controlled from the cell surface by T_3 at the αVβ3 receptor for the hormone (Lin *et al*., 2009b).

Competition data reveal that RGD peptides block cell surface thyroid hormone-binding and cellular actions of the hormone (Bergh *et al*., 2005), suggesting that the thyroid hormone receptor site on the integrin is at or very near the RGD site (Davis *et al*., 2005) (Figure 6). Tetraiodothyroacetic acid (tetrac) is a deaminated analogue of L-thyroxine (T_4) that is not an agonist at the integrin receptor site, but blocks thyroid hormone-binding at the integrin and inhibits the ability of thyroid hormone analogues to activate MAPK via this site (Bergh *et al*., 2005). Interestingly, tetrac can also inhibit the cellular effects of certain vascular endothelial growth factors that contain an RGD sequence and act via integrin αVβ3 on endothelial cells (Mousa *et al*., 2006b). That the RGD and hormone receptor sites can be functionally distinguished is shown also by the

ability of T_4 and a vascular growth factor, FGF2, to act concurrently to activate MAPK in endothelial cells (Davis *et al*., 2004). This further supports the proximity proposed for hormone receptor and the RGD recognition sites.

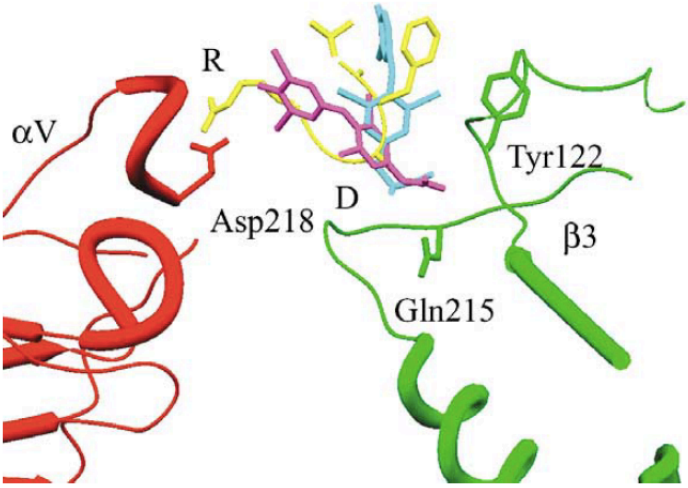

Figura 6. Model of interaction along the integrin αVβ3, T_4 (light blue), tetrac (purple) and the RGD peptide (yellow) (Davis *et al*., 2005).

1.2 Thyroid hormones

1.2.1 Synthesis and effects on target tissues

The thyroid produces and releases two hormones, L-thyroxine (tetraiodothyronine, T_4) and L-triiodothyronine (T_3) (Figure 7). Iodine is an indispensable component of the thyroid hormones, comprising 65% of T_4's weight, and 58% of T_3's. The thyroid hormones (THs) are essential regulators of development, differentiation, growth and metabolism, both in the developing and the adult organism (Brent, 2012; Davis *et al*., 2018).

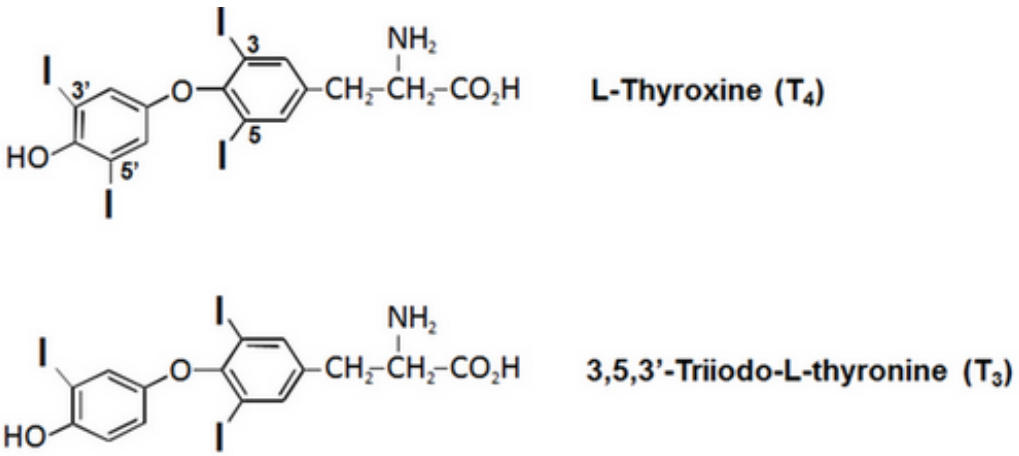

Figure 7. Chemical structures of thyroid hormone analogues L-thyroxine (T_4) and 3,5,3'-triiodo-L-thyronine (T_3) (Lin *et al*., 2011).

The thyroid gland is part of the endocrine system and is responsible for producing and releasing THs into the bloodstream. Accordingly, TH concentrations determine the extent of hormonal regulation and generate downstream effects in peripheral cells. Classical regulation of the thyroid gland involves the hypothalamic-pituitary-thyroid axis, whereas low TH concentrations trigger a negative feedback that results in the release of both thyroid releasing hormone (TRH) from the hypothalamus and thyroid stimulating hormone (TSH) from the pituitary gland (Fekete and Lechan, 2014; Ortiga-Carvalho *et al*., 2016). However, besides the capacity of the thyroid gland to produce the correct amount of THs, the periphery can modify the TH signal in time and space. Indeed, while plasma concentrations of TH are relatively stable, tissues can coordinate TH levels through the cell-autonomous regulation of TH transporters, deiodinases and TH receptors (Gereben *et al*., 2008a).

The iodothyronine deiodinase family of selenoproteins is constituted by three enzymes, D1, D2, and D3. These enzymes are present in specific tissues, and regulate TH activation and inactivation (Dentice and Salvatore, 2011; Luongo *et al*., 2019). The differential expression of deiodinases enables close control of T_3 and its prohormone, T_4, by removing iodine moieties ("deiodination") at different sites of the phenolic or tyrosylic ring of the THs (Gereben *et al*., 2008b).

The essential role of thyroid hormones in tissue growth and development, both accompanied by metabolic and physiological maintenance, is well-understood (Cheng *et al*., 2010; Brent, 2012; Davis, 2012). Thyroid hormones play a crucial role in augmenting the metabolic rate of cells and tissues through the upregulation of protein synthesis and by the increase of mitochondria size and so, their bioenergetic function (Goglia *et al*., 2002). Among the consequences of such changes are: increase in oxygen, nutrient and glucose consumption (Davies, 1972), and organ system-specific changes, such as increases in blood pressure and heart rate and striated muscle contraction (Danzi and Klein, 2014; Salvatore *et al*., 2014).

Some of these events are regulated by non-genomic mechanisms that are initiated at the surface/plasma membrane of cells, through interactions of thyroid hormones with transmembrane proteins, for example, the integrin αVβ3 (Davis *et al*., 2005) or modified nuclear thyroid hormone receptors that function at the plasma membrane (Kalyanaraman *et al*., 2014). Long-term actions, on the other hand, require binding of thyroid hormones to nuclear receptors (TRs), that once activated, modulate transcription of many genes involved in a variety of functions (Cheng *et al*., 2010; Incerpi *et al*., 2016). The different modes of action may be coupled, and several reports have recently shown that several TH metabolites act accordingly (Moreno *et al*., 2008; Senese *et al*., 2014a, 2014b; Gnocchi *et al*., 2016).

1.2.2 Non-genomic actions of thyroid hormones

In the 1970's and early 1980's, extranuclear (or non-genomic) effects of thyroid hormones were described by Sterling (Sterling *et al*., 1978, 1980) demonstrating that applications of T_3, in low concentrations to isolated mitochondria, acutely enhanced adenosine triphosphate (ATP) production and oxygen utilization. Activity of thyroid hormone was also described on transporters and enzymes located at cellular membrane such as: Na^+/K^+ and Ca^{2+}-ATPase, Na^+/H^+ exchanger, glucose transporters, ion channels and acetylcholinesterase (de Mendoza *et al*., 1978; Segal and Ingbar, 1979, 1989; Galo *et al*., 1981; Davis *et al*., 1983; Incerpi *et al*., 1999; Scapin *et al*., 2009).

Initially, the mechanisms by which thyroid hormones might influence cellular functions without activating nuclear TRs, were not clear. Explanations were offered about the existence of putative thyroid hormone receptor molecules on the plasma membrane that could trigger complex signal transduction inside the cell (Bergh *et al*., 2005; Davis *et al*., 2005). For example, phosphorylation of Ser-142 on TRβ1 isoform (Davis *et al*., 2000) and of Ser-118 on estrogen receptor-α (ERα) (Tang *et al*., 2004), were shown to be regulated by thyroid hormone acting at the cellular surface. A receptor that mediated these plasma membrane-initiated actions of thyroid hormone was found to belong to a structural protein of the membrane surface whose extracellular domain interacted with proteins of extracellular matrix (ECM). This membrane protein was integrin αVβ3 (Bergh *et al*., 2005; Davis *et al*., 2005). Acting at the thyroid hormone receptor on αVβ3, either T_4 (in physiological concentrations) or T_3 (in supraphysiological amounts), affected intracellular protein trafficking, activities of certain signal transducing kinases (MAPK and PI-3K) and phosphorylation of nuclear receptors; the latter included TR and ERα (Davis *et al*., 2016) (Figure 8).

Useful probes in the studies of non-genomic actions included: T_4-agarose (which does not enter the cell (Lin *et al*., 2009b; Scapin *et al*., 2009)), tetraiodothyroacetic acid (tetrac, a deaminated analogue of T_4 that antagonizes thyroid hormone effects and which is thyromimetic inside the cell) and an antibody specific for integrin αVβ3 both acting at the cell surface receptor (Bergh *et al*., 2005; Yoshida *et al*., 2012; Schmohl *et al*., 2019). Other studies have documented the existence of non-genomic actions of thyroid hormone, although it is not known yet whether the majority of these effects may be initiated at αVβ3 location (Davis *et al*., 2016).

Additional non-genomic actions of T_4 and T_3 include also the induction of actin polymerization turnover (transition from soluble to fibrous actin) and therefore the modulation of microfilament composition of the cell (Siegrist-Kaiser *et al*., 1990; Davis *et al*., 2016). T_3 has no effects on the state of actin (Cheng *et al*., 2010; Davis *et al*., 2016).

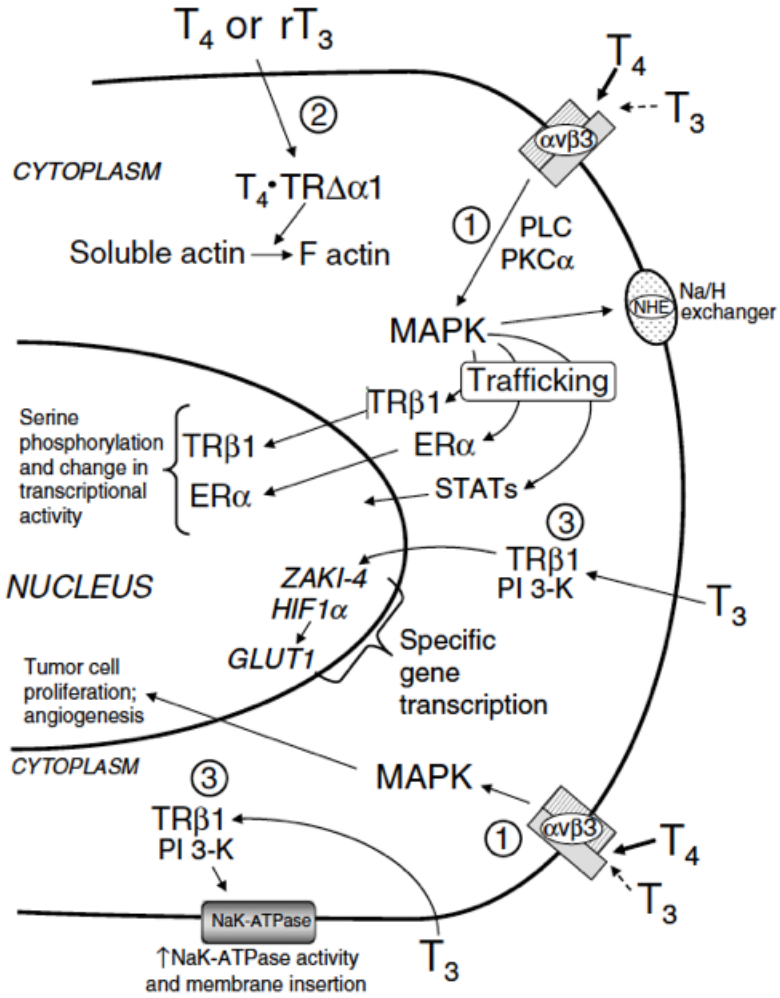

Figure 8. Schematic representation of non-genomic cellular actions of thyroid hormones (Davis *et al*., 2008).

1.2.3 Thyroid status and immune system: the crosstalk

The critical regulation of the functions of the endocrine system is mediated by hypothalamic-pituitary-gonadal, -adrenal or -thyroid axes. Alternatively, the immune system can modulate hormonal release, either centrally from endocrine glands or peripherally from tissues or organs that are targets of the hormones. Solid experimental evidence documents the existence of neuro-endocrine and immune systems linkages that are bidirectional and in which hormones and cytokines represent the molecular players (Barreiro Arcos *et al*., 2006; De Vito *et al*., 2011, 2012). Interestingly, the sensitivity of immune cells to hormones and neurotransmitters (De Vito *et al*., 2010, 2013), and of endocrine cells to several cytokines such as: interleukin-1 (IL-1), IL-6, IFN-γ and tumor necrosis factor-α (TFN-α), reflects the bilateral expression of hormonal and immune receptors, respectively (Klecha *et al*., 2000, 2008). The participation of T_3 and T_4 through MAPK pathway in the activation of STAT1α, as the last step in thyroid hormone-induced cytokine release (enhancing IFN-γ action), confers on these molecules an important role in the modulation of immune system (De Vito *et al*., 2012).

Previous reports indicate that alterations in TH levels can affect the immune system (De Vito *et al*., 2012). Abnormal thyroid hormone secretion-hyperthyroidism of several etiologies, autoimmune thyroiditis, hypothyroidism with or without autoimmune (Hashimoto's) thyroiditis affects various immunological functions. In hyperthyroidism an increase of humoral and cellular immune responses was observed, compared to control patients (De Vito *et al*., 2011) (Figure 9). Opposite immune responses were found in hypothyroid patients (Klecha *et al*., 2008). However, in some cases, contrasting results have been reported and therefore it is difficult to establish a clear correlation between immune function and hyper- or hypothyroid conditions (Foster *et al*., 2000; Fabris *et al*., 2005; Marino *et al*., 2006). It appears to be of particular importance to study thyroid hormone-immune cell crosstalk during immune disease and immune pathophysiology because the hormone concentrations appear to be correlated with immunological reactivity (Hodkinson *et al*., 2009). Recently, it has been shown that T_3 can also control the activity of lymphocytes T involving the dendritic cells development and function (Alamino *et al*., 2019).

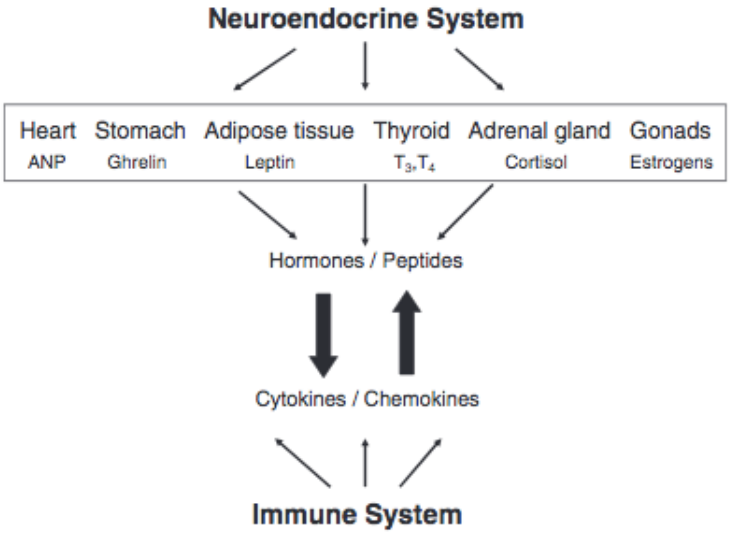

TABLE 1. EFFECTS OF HYPERTHYROIDISM AND HYPOTHYROIDISM ON COMMON IMMUNE FUNCTION PARAMETERS

Immune function	*Hyperthyroidism*	*Hypothyroidism*
Immune response	↑ or ↓	– or ↓
Antibody production	↑ or ↓	↑ or ↓
Migration/chemotaxis	↑ or –	– or ↓
Proinflammatory markers	↓	↑
Lymphocyte proliferation	↑	↓
Reactive oxygen species	↑	↑ or ↓
Antioxidant capacity	↓	↑

↑, increase; ↓, decrease; –, no change.

Figure 9. Neuroendocrine and immune system crosstalk (De Vito *et al*., 2011).

A fascinating observation is that T and B lymphocytes can produce and release TSH. The source of this endocrine modulator from non-pituitary cells could explain how the immune system would be connected in the production of thyroid hormones, either in health or during immune disturbances, e.g., cellular stress, infections or inflammation (Klein, 2006). Initial reports of TSH and immune cells appeared more than 20 years ago (Kruger & Blalock, 1986; Smith *et al*., 1983). Bacterial toxins or *in vitro* TRH exposure enhances TSH production and release from leukocytes (Smith *et al*., 1983). Work form Blalock *et al*. showed that TSH induces a strong immunological response followed by antibody production, potentiating the phytohemagglutinin-mediated proliferation of lymphocytes (Blalock *et al*., 1984). *In vivo* experiments performed in mice lacking the pituitary gland and so unable to produce central TSH, showed a clear increase of thyroid hormones levels during inflammatory conditions (Bagriacik *et al*., 2001).

On the other hand, impaired or unbalanced immunological response may be linked to low levels of thyroid hormone in the plasma, since TSH fluctuations might alter T_3 and T_4 release from thyroid gland. Moreover, infectious conditions indirectly influence thyroid hormones through the action of inflammatory molecules on hypothalamus, thus increasing the levels of T_3 and T_4 in the circulation and in turn minimizing the TSH action on thyroid. Such a mechanism lowers systemic energy expenditure during illnesses, offering an alternative pathway to HPT axis control, for central neuroendocrine-immune and metabolic adjustment in the body (Klein, 2006).

Thyroid hormones are possibly involved in the regulation of vitamin D balance (Mackawy *et al*., 2013) and *vice versa* (Nettore *et al*., 2017). Vitamins C and D have been also recognized as key modulators of immune response (Baeke *et al*., 2010; Sorice *et al*., 2014) and recently low circulating vitamin D levels have been shown to be associated with hypothyroidism (Mackawy *et al*., 2013). Moreover, vitamin C is a well-known antioxidant molecule capable of reducing ROS activity increased by thyroid hormone via non-genomic actions, for example, during chronic inflammations (Incerpi *et al*., 2007).

TSH production by T and B cells can be expected to affect normal and abnormal (cancer) thyroid cells that express the TSH receptor, but it may be noted that the TSH receptor is expressed by ovarian cancer cells (Gyftaki *et al*., 2014) and by normal extrathyroidal cells (hepatocytes, adipocytes, striated muscle cells) (Moon *et al*., 2016). TSH may be proinflammatory in any of these settings, since it is clear that this peptide hormone is capable of stimulating the production of endogenous inflammatory factors such as interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) (Gagnon *et al*., 2014).

To provide detailed insights into the effects of THs and TSH on immune system functions, the next sections describe the effects of T_3, T_4 and TSH on various immune cell types, such as macrophages and microglia.

1.2.4 Effects of thyroid hormones on immune system cells

Macrophages are key immune system cells derived from monocytes in response to infections or to the accumulation of damaged or dead cells (Yang *et al*., 2014). Two distinct polarized activation states have been defined for macrophages: the classically activated (M1) macrophage phenotype and the alternatively activated (M2) macrophage phenotype. Classically activated (M1) macrophages act as effector cells in the TH1 cellular immune response, whereas alternatively activated (M2) macrophages appear involved in immunosuppression and tissue repair (Mosser, 2003)

THs positively affect macrophage bactericidal effector functions, consequently improving wild-type mouse survival in a number of disease models, such as meningococcal infection (Chen *et al*., 2012). Specifically, when the RAW264.7 macrophage cell line is treated *in vitro* with T_3 or T_4 for 24 hours before *Neisseria meningitidis* infection, this cell line can capture significantly more bacteria than untreated macrophages (Chen *et al*., 2012). Here, THs enhance iNOs-mediated nitric oxide production in a membrane receptor integrin αVβ3-dependent manner (i.e. via non-genomic stimulation) through pathways involving PI3-K and ERK1/2 (Chen *et al*., 2012). Mouse and human studies suggest that a T_4-induced inhibition of the macrophage migration inhibitory factor (MIF) could improve the treatment of sepsis treatment (Bozza *et al*., 2004; Al-Abed *et al*., 2011). In greater detail, the MIF molecule contains a hydrophobic pocket important for many proinflammatory activities. Several small molecules can inhibit the catalytic activity of this pocket, thereby reducing MIF activity. The dose-dependent inhibitory effects of T_4 on MIF and subsequently improved survival rates suggest a clinically relevant interaction between T_4 and MIF (Al-Abed *et al*., 2011).

Macrophages significantly contribute to immune system surveillance by sensing and adapting to local stimuli and microenvironment signals (Murray and Wynn, 2011). Regarding macrophage differentiation, T_3 was recently shown *in vitro* to negatively contribute to the differentiation of bone marrow-derived monocytes into non-polarized macrophages (Perrotta *et al*., 2014). T_3 promotes the generation of M1 macrophages, even after the differentiation and activation of monocytes into M2 macrophages (Perrotta *et al*., 2014). Nevertheless, while T_3 increases the number of resident macrophages in the peritoneal cavity, the THs can also reduce monocyte-derived cell recruitment (Perrotta *et al*., 2014). In an *in vivo* model of endotoxemia, as induced by

intraperitoneal lipopolysaccharide injection, T_3 protects mice from developing endotoxic shock (Perrotta *et al*., 2014). While low T_3 levels increase inflammatory cell recruitment into tissues, an opposite phenomenon occurs when T_3 levels are restored (Murray and Wynn, 2011; Perrotta *et al*., 2014).

Microglia is another cell type with innate immune properties in central nervous system (CNS), which also can be modulated by THs (Lima *et al*., 2001; Noda, 2018). It has been reported that hypothyroid rat pups present defects in the morphology, microglial process formation and diminution in the number of these cells until the 3rd week after birth, as compared to controls. However, these defects could be reverted by the administration of T_3 (Lima *et al*., 2001). In addition, *in vitro* microglial cultures showed that T_3 can promote the survival and growth of the culture. In addition to modulating the microglial phenotype, the lack of T_3 also affects the phagocytic capacity of these cells *in vitro* and *in vivo*, which is one of the principal functions of microglia in the CNS (Mori *et al*., 2015). Taking into account the microglial function modulation by THs, Perrotta *et al*. recently reported the role of T_3 inducing the proliferation of malignant glioma cell line GL261, only through microglial cocultures. The cellular changes observed in microglia after stimulation with T_3 (Perrotta *et al*., 2015) were an increase in the levels of pSTAT3 pathway and the induction of CXCL9 and CXCL10 chemokine expression, which directly impacted on glioma cell line proliferation (Perrotta *et al*., 2015).

These data suggest a novel homeostatic link between THs and the pathophysiological role of macrophages, thus providing new perspectives on interactions between the endocrine and immune systems.

1.3 Bisphenol A

1.3.1 BPA as thyroid-disrupting factor

The endocrine system is composed of glands that secrete hormones produced in the body to regulate the activity of cells or organs. Hormones control growth, development, and reproduction as well as the electrolyte composition of body fluids and the metabolism of body (Hiller-Sturmhöfel and Bartke, 1998). According to European Union, Endocrine Disruptors (ED) are exogenous substances that cause adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function. Endocrine Disruptor Compounds (EDCs) regroup a large variety of substances such as those used in multiple industrial processes, including solvents/lubricants (polychlorinated biphenyls (PCBs)), plastics (bisphenol A (BPA) and phthalates), pesticides (dichlorodiphenyltrichloroethane (DDT)) or pharmaceuticals (such as estradiol (E2)) (Diamanti-Kandarakis *et al*., 2009).

Recently, the potential influence of BPA on human health at environmentally relevant low-dose has attracted much concern (Rochester, 2013; ECHA, 2015; EFSA, 2015; ANSES, 2018). BPA is known as 2,2-bis(4-hydroxyphenyl)propane, a phenol derivative composed of a rigid planar aromatic ring and a flexible nonlinear aliphatic side chain. BPA is mainly used as intermediate for the production of polycarbonate plastic, epoxyresin and other polymer materials, which has been widely used in food and beverage packaging materials (such as bottles) and liner, digital media (such as CDs and DVDs), automobile, electronic devices, sports safety equipment and medical instruments (such as dental sealants) (Jiao *et al*., 2008). In addition, a small amount of BPA is also used in the manufacture of phenolic resins, unsaturated polyester resins, thermal paper additive, flame retardants (such as tetrabromobisphenol A), heat and carbonless paper coating (ANSES, 2013; ECHA, 2015; EFSA, 2015).

A large number of *in vivo* and *in vitro* studies have indicated that BPA has adverse effects on the reproductive function, mammary gland development, cognitive function and metabolism (ANSES, 2018; Beausoleil *et al*., 2018). BPA is known to have mixed estrogen agonist/antagonist properties (Acconcia *et al*., 2015). In addition to its estrogenic activity, there is some evidence that BPA binds to TR, acting as a TH antagonist by preventing the binding of T_3 (Moriyama *et al*., 2002) (Figure 10). However, other studies have been unable to duplicate these results, finding that BPA does not competitively inhibit the binding of labeled T_3 to the TR or induce TH-dependent production of growth hormone (GH) in GH3 cells (Kitamura *et al*., 2002, 2005).

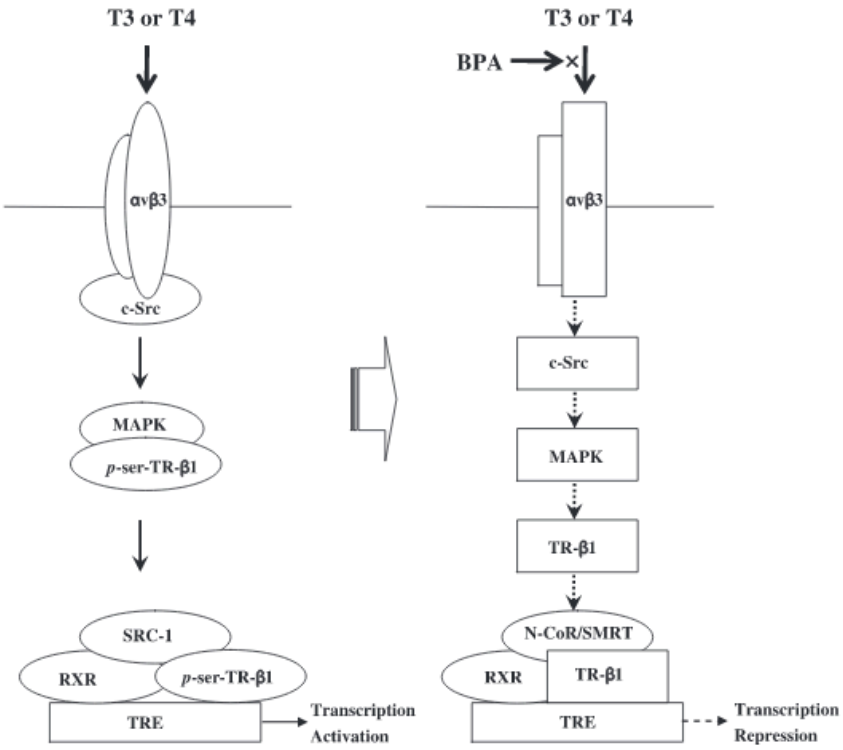

Figure 10. A proposed mode by which low concentrations of BPA suppress the TR transcription (Sheng *et al.*, 2012).

In vivo studies examining the effects of low dose BPA on TH signaling have suggested that perinatally exposed rats have elevated T_4 levels on postnatal day (PND) 15 and up-regulation of a TH responsive gene in the brain (Zoeller *et al*., 2005). Furthermore, perinatal exposure to low dose BPA in rats has been implicated in the abnormal brain development, characterized by hyperactivity and impaired cognition (Kubo *et al*., 2001; Carr *et al*., 2003). It was also observed in Medaka fish that the acceleration in embryonic development and time to hatch induced by BPA are blocked by a TR antagonist, suggesting that BPA is acting through a TH pathway (Ramakrishnan and Wayne, 2008). However, the mechanisms associated with endocrine disrupting effects of low concentration BPA on the TH system are currently ill-defined and warrant further detailed investigation.

Several studies have shown also that EDCs directly affect innate immune system. Ohnishi *et al*. showed also that some agrochemicals and resin-related chemicals could potentially inhibit macrophage function (Ohnishi *et al*., 2008). In addition, Roy *et al*. found that maternal exposure to BPA modulates innate immunity in adult offspring but not adaptive immune responses to influenza A virus infection in mice (Roy *et al*., 2012). Another work by Watanabe *et al*. showed that BPA enhances neutrophilic maturation of the leukocytes through estrogen receptor-independent pathway, suggesting that BPA affects the innate immunity of mammals (Watanabe *et al*., 2003). Epidemiological studies have also uncovered the possible link between EDCs and immune disorders; for example, the exposure to phthalates is associated with increased risks to develop allergies and asthma, even though the lack of accurate exposure information limits the interpretation (Kimber and Dearman, 2011).

There is a growing number of studies that have evaluated the effects of BPA on immune responses with contradictory findings and little consensus (Rogers *et al*., 2013). For instance, lymphocyte proliferative responses have been reported to be either enhanced (Youn *et al*., 2002; Yoshino *et al*., 2003; Goto *et al*., 2004) or suppressed (Jontell *et al*., 1995; Yamazaki *et al*., 2000; Sakazaki *et al*., 2002) by BPA treatment. Moreover, some studies demonstrated that BPA exerted inhibitory effects on macrophage function by suppressing the production of nitric oxide and tumor necrosis factor alpha (Kim and Jeong, 2003; Byun *et al*., 2005). In contract, stimulatory effects of BPA on macrophage function were also reported as evidenced by increased production of nitric oxide and elevated expression of costimulatory molecules and MHCII (Hong *et al*., 2004; Kuan *et al*., 2012). In addition, the reported effects of BPA on humoral immune responses have been inconsistent, as some studies found enhancement of antibody responses by BPA treatment (Han *et al*., 2002; Yoshino *et al*., 2003; Yurino *et al*., 2004) while others showed no effects (Takahashi *et al*., 2002) (Figure 11).

The reported contradictory effects of BPA on immune responses can putatively be attributed, at least in part, to one or more of the following explanations: (1) differences in biological models spanning a variety of animal species, strains, and cell lines; (2) differences in concentrations/doses of BPA, duration of exposure, and developmental stages when the exposure occurs. In fact, many of the aforementioned studies were conducted using high BPA concentrations/doses that greatly exceed estimated exposure experienced by people (US Food and Drug Administration, 2017). Due to the above limitations, it has been challenging to establish a comprehensive immunotoxicological profile for BPA.

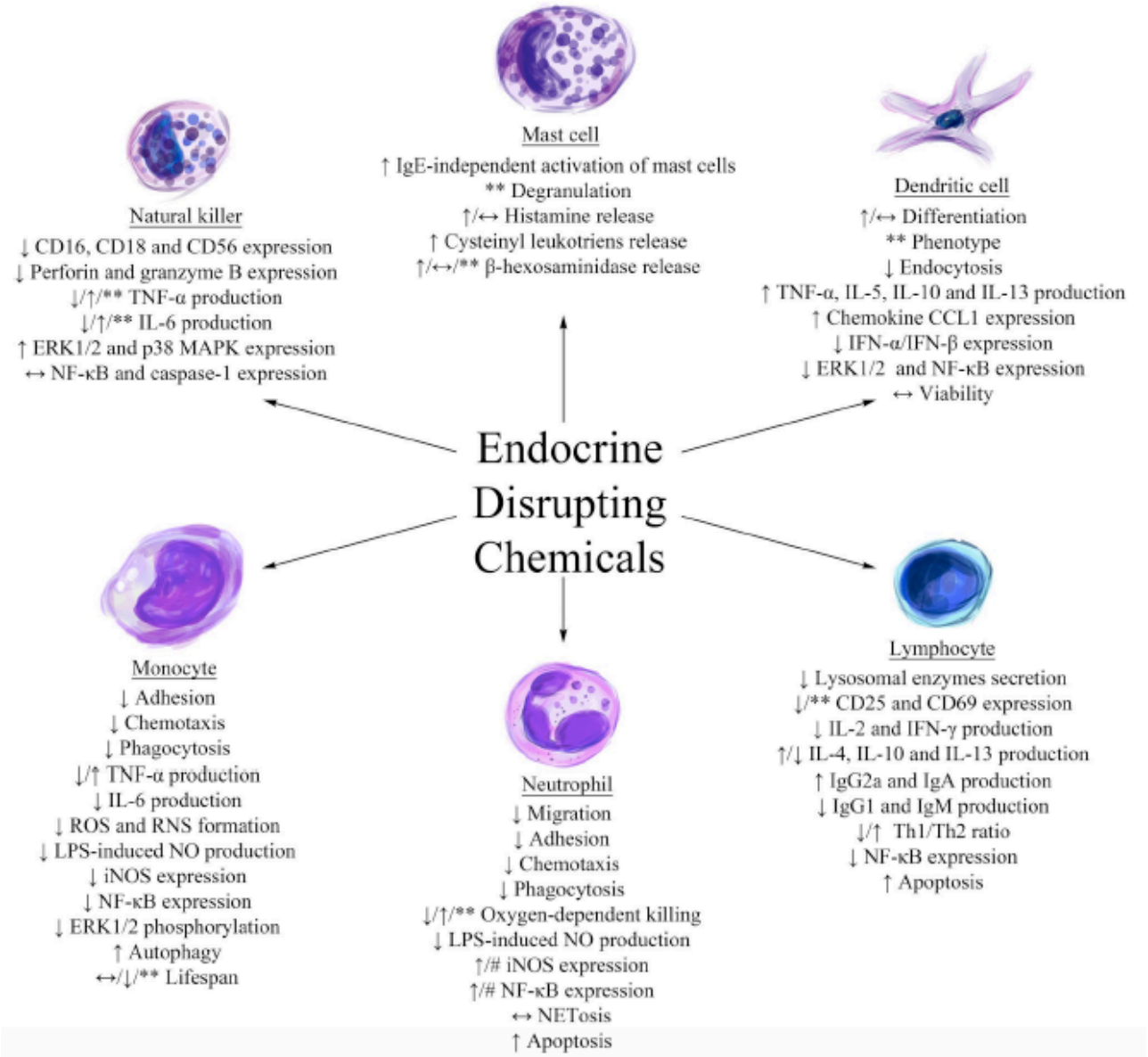

Figure 11. Immunological effects of endocrine disrupting chemicals (EDCs). EDCs affect functions and lifespan of immune cells. Legend: ↑ - increase; ↓ - decrease; ↔ ↔ - no effect; ** - dose-dependent effect; # - sex-dependent effect. (Nowak *et al*., 2019).

2. AIM

Non-genomic effects of thyroid hormones (THs) have been reported for many years and involve mainly the plasma membrane modulation of membrane transport systems, but also cytosolic responses. Bergh *et al*. in the 2005 identified a plasma membrane receptor for THs in the integrin αVβ3 with a downstream activation of MAPK pathway leading to angiogenesis and tumor cell proliferation. Integrin αVβ3 is an extracellular matrix protein involved in many physio-pathological processes and also a co-receptor for the Insulin-like growth factor -1 (IGF-1). In L6 myoblasts from rat skeletal muscle TH in the physiological range is able to inhibit responses to IGF-1 such as glucose uptake and cell proliferation. The effect is mediated by integrin αVβ3, since the RGD tripeptide, the disintegrin echistatin, tetrac, a metabolite of TH and a probe for the integrin αVβ3, the antibody for αVβ3 are able to prevent the inhibition by TH of IGF-1- mediated effects, both in short term and long-term, showing the crosstalk between non-genomic and genomic effects (Incerpi *et al*., 2014).

In recent years it has been shown by several groups that there is a crosstalk between THs and immune system (Klein, 2006; De Vito *et al*., 2011, 2012). This comes mainly from observations that patients affected by hypo- or hyperthyroidism show alterations of the antibody responses, but not always in a predictable way, that is in hyperthyroid patients there is mainly and increased immune response, but not always.

Dellacasagrande *et al*. showed that αVβ3 integrin and LPS are involved in the production of TNF-α in THP-1 leukemic monocytes stimulated by *Coxiella burnetii* (Dellacasagrande *et al*., 2000). All this information prompted us to study in THP-1 human leukemic monocytes the possibility of the modulation of immune responses by TH through integrin αVβ3. It was found that THs are able to inhibit the migration induced by MCP-1 and the effect is mediated by integrin αVβ3 (Candelotti, PhD thesis).

The project of my PhD thesis has been the study of the capability of THs to modulate immune activities in the BV-2 microglia, the resident immune cells of the central nervous system (CNS). In these cells, in fact, the effects of THs have been studied mainly as to the nuclear effects, whereas we focused our attention to the extra-nuclear effects of THs possibly mediated by αVβ3.

Recently, it was shown that bisphenol A (BPA), a monomer of plastic materials, binds integrin αVβ3 interfering with the actions of THs as a hormone antagonist (Moriyama *et al*., 2002). We then examined, over a wide concentration range, the effect of BPA exposure *in vitro* on immune cells in culture.

3. MATERIALS AND METHODS

3.1 Cells in culture

Human leukemic monocytes THP-1, from American Type Culture Collection (Rockville, MD, USA), were grown in a suspension containing RPMI-1640 medium with 10% FBS, 100 µg/ml streptomycin and 100 U/ml penicillin, in a humidified atmosphere of 5% CO_2 at 37°C. These cells show a large, round, single-cell morphology. The THP-1 monocytes were passaged twice a week by 1:8 dilutions and re-seeded in T25 culture flasks; only cells from passages n. 7-23 were used for the experiments (Pedersen *et al*., 2007; Lombardo *et al*., 2013). For differentiation of macrophages, the monocytic cells were grown for 24 hours in conditioned medium from THP-1 stimulated with 100 nM PMA (Lombardo *et al*., 2013).

The immortalized murine microglial cell line BV-2 was a kind gift from Dr. Tiziana Persichini (Università degli Studi Roma Tre, Rome, Italy). BV-2 cells, derived from primary murine microglial cells immortalized by transduction with v-raf and v-myc expressing J2 retrovirus (Blasi et *al*., 1990). The cells were maintained in the logarithmic phase of growth in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics at 37°C in a humidified atmosphere of 5% CO_2. Confluent cultures were passaged twice a week by trypsinization and re-seeded in T25 culture flasks.

3.2 Flow cytometry analysis

BV-2 murine microglia cells and THP-1 human leukemic monocytes ($1x10^6$ cells) were washed twice with PBS/BSA 5%, centrifuged and incubated with a rabbit anti-integrin αV + β3 polyclonal antibody (diluted 1:50), for 30 min on ice. After washing twice, cells were further incubated with a goat anti-rabbit Alexa Fluor® 488 secondary fluorescent antibody (diluted 1:50); after 30 min on ice the integrin αVβ3 cell population fluorescence profile was acquired using a CytoFlex Flow Cytometer (Beckman Coulter). Unstained sample (autofluorescence) and a sample incubated with only secondary antibody were included as negative controls.

3.3 Immunofluorescence

BV-2 microglia cells and THP-1 leukemic monocytes were seeded in glass microscope slides and fixed with 4% formaldehyde, permeabilized and stained using the rabbit anti-integrin αV + β3 polyclonal antibody (diluted 1:50). Nuclear DNA was counterstained with 5 µg/ml DAPI in PBS. A goat anti-rabbit cross-adsorbed Alexa Fluor® 546 was used as secondary antibody (diluted

1:250). Samples were observed with a CLSM Leica TCS SP5 attached to a Leica DMI6000 inverted microscope (Leica Microsystem). To analyze the fluorescence of the samples, laser wavelength Diode (405 nm) and HeNe (543 nm) were used. The confocal microscope was controlled by the Leica LAS software AF version 1.6.3 (Leica Microsystems).

3.4 Long-term proliferation assay

Cells were seeded in 60×15 mm Petri dishes with DMEM and stimulated with RGD, T_4 and IGF-1 the day after the seeding. Cells were counted every 24 hours. The role of integrin αVβ3 on the proliferation of BV-2 cells was studied using RGD peptide (10 μM), tetrac (10 μM) and echistatin (100 nM) as integrin αVβ3 inhibitors. RGD peptide, tetrac and echistatin were pre-incubated 20 min before adding T_4 (100 nM) and IGF-1 (10 nM). Moreover, we studied PI3-K and MAPK pathways by the use of wortmannin (100 nM) and PD98059 (10 μM) were pre-incubated 20 min before adding T_4 and IGF-1. Cells were counted with an optical microscope with the Neubauer chamber after mild trypsinization (Incerpi *et al*., 2014).

3.5 Intracellular ROS determination

The method used was a standard assay based on the intracellular fluorescent probe DCF (Pedersen *et al*., 2007). For BV-2 cells, the medium was discarded and cells were washed twice with phosphate buffered saline (PBS) containing 5 mM glucose (PBS-glucose) at 37°C. Cells were gently scraped off with PBS-glucose and centrifuged at 1200 rpm for 5 min, the supernatant was discarded and the pellet re-suspended in PBS-glucose with a plastic Pasteur pipette. Incubation with the probe DCFH2-DA at a final concentration of 10 μM (from a stock solution of 10 mM in DMSO) was carried out for 30 min in the dark at 37°C. The cells were gently re-suspended every 10 min; at the end of the incubation cells were washed twice, centrifuged at 1200 rpm for 5 min and the final cell pellet was re-suspended in PBS-glucose. Before the experiments cells recovered at 37°C for 1 h in the dark. Intracellular fluorescence was measured with a LS-50B Luminescence Spectrometer (Perkin-Elmer, Norwalk, CT). Excitation and emission wavelengths were set at 498 nm and 530 nm respectively, using 5 and 10 nm slits for the two light paths. Cells were suspended in PBS containing Ca^{2+} (1 mM) and Mg^{2+} (1 mM) under continuous gentle magnetic stirring at 37°C; they were pre-incubated with the inhibitors of integrin αVβ3 for 30 min before the addition of hormone. Cumene hydroperoxide diluted in DMSO was used as radical generator (final concentration 200 μM); DMSO at the concentrations used did not affect the fluorescence signal. ROS production induced by THs and role of integrin αVβ3 was determined by the variation of intracellular DCF fluorescence, measured as ΔF/10 min, and calculated relative to the fluorescence

change induced by 200 μM cumene hydroperoxide alone (100%). None of the compounds tested gave rise to fluorescence on their own.

3.6 Migration studies

Migration experiments were carried out by the use of transwells (Corning) with an 8 μm polycarbonate membrane, 6.5 mm insert 24-wells plate with serum-free RPMI-1640 medium containing 0.2% BSA in both chambers, for 4 hours at 37°C. THP-1 cells (about 200.000 cells/well) were placed in the upper chamber with DMSO, BPA (10^{-5} M - 10^{-9} M) and MCP-1 (100 ng/ml) were added to the bottom chamber. All αVβ3 inhibitors were pre-incubated for 20 min at 37°C before starting the experiment. After 4 hours of incubation, at 37°C, cells migrated (from the upper part to the bottom part of the chamber) were counted with the Neubauer chamber (De Vito *et al.*, 2012).

3.7 Scratch wound assay

To analyze microglial migration we performed a scratch wound assay as previously described with minor modifications (Liang *et al.*, 2007). The confluent BV-2 cell monolayer in 24-well plates was scraped with a P200 pipette tip to create a wound, followed by a wash with serum-free DMEM to remove debris and replaced with 1 ml serum-free DMEM to avoid proliferation and the FBS-mediated activation of the cells (Laurenzi *et al.*, 2001). The cells were stimulated with LPS (1 μg/ml), RGD (10 μM), tetrac (10 μM), T_3 (10^{-7} M - 10^{-9} M) and T_4 (10^{-6} M - 10^{-8} M) for 24 hours. To determine the migration of the cells, we acquired images at defined time points. Images were taken through a light microscope in a marked sector as reference point. Scratch widths were measured before the treatment and wound closure was calculated by dividing widths measured after incubation using ImageJ software.

3.8 MTT assay

Cell viability and the possible cytotoxic effect of THs and BPA were assessed by the MTT assay. BV-2 cells were seeded in 96-wells plates at 10.000 cells/well in 200 μl DMEM containing 10% serum. The day after seeding the medium was discarded and 100 μl of new medium containing cumene hydroperoxide (200 μM), THs and BPA at different concentrations were added to each well. Then MTT solution (5 mg/ml in PBS) was added at the final concentration of 10% with respect to the total volume, and incubation was carried out at 37˚C for 3-4 hours in the dark. During the incubation, there was a conversion of the yellow MTT to purple formazan by the mitochondrial succinate dehydrogenase of living cells. Then lysis buffer (DMSO containing ammonia (Wang *et*

al., 2012)) was added and further incubation at 37˚C for 30 min in the dark was carried out. Cells were then re-suspended and the optical density was read with an ELISA-reader at 550-570 nm.

3.9 Materials

Roswell Park Memorial Institute medium (RPMI-1640), Dulbecco's modified Eagle medium (DMEM), sodium pyruvate (100 mM), L-glutamine (200 mM), streptomycin (100 mg/ml), penicillin (100 U/ml), phosphate buffered saline (PBS, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl dissolved in 500 ml of distilled water, pH 7.4), D-glucose (5 mM), O-(4-hydroxy-3-iodophenyl)-3,5-diiodo-L-tyrosine sodium salt (3,3′,5-triiodo-L-thyronine, T_3), 3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]-L-alanine sodium salt (L-thyroxine, T_4,), tetraiodothyroacetic acid (tetrac), human recombinant insulin-like growth factor-1 (IGF-1), Arg-Gly-Asp (RGD) peptide, lipopolysaccharide (LPS, from *Escherichia coli* 0111:B4), DAPI (4′,6′-diamidino-2-phenylindole), diphenylene iodonium (DPI), phorbol 12-myristate 13-acetate (PMA), bisphenol A (BPA), cumene hydroperoxide and dimethyl sulfoxide (DMSO) were supplied by Sigma-Aldrich (MO, USA). Sterile plasticware for cell culture was purchased from Falcon (3V Chimica S.r.l., Rome, Italy), fetal bovine serum (FBS) was obtained from GIBCO (NY, USA). Monocyte chemoattractant protein (MCP-1) was purchased from PeproTech (NJ, USA). Sterile PBS, D-PBS, (Dulbecco's phosphate buffered saline, without calcium and magnesium) was obtained from EuroClone (Milan, Italy). PD98059 (a selective inhibitor of MAP kinase kinases (MAPKK), MEK1 and MEK2), wortmannin (a selective irreversible inhibitor of phosphatidylinositol 3-kinase) were purchased from Alexis Biochemicals (Laufelfingen, Switzerland). Rabbit anti-integrin alpha V + beta 3 polyclonal antibody (bs-1310r) was obtained from Bioss Antibodies (MA, USA). Goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody Alexa Fluor® 488 (A-11008) and Alexa Fluor® 546 (A-11010) were obtained from ThermoFisher Scientific (MA, USA).

3.10 Solutions

3,3',5-triiodo-L-thyronine (T_3) sodium salt (MW 673 g/mol) and L-thyroxine (T_4) sodium salt (MW 776.9 g/mol) were both dissolved in 0.1 N NaOH to obtain 10^{-3} M stock solutions. The aliquots were maintained frozen and used during 30 days. Bisphenol A (BPA) was dissolved in DMSO to obtain 1 M stock solution and kept at -20°C. The vial containing insulin-like growth factor 1 (IGF-1) was reconstituted using 10 mM HCl, and for stock solutions of less than 1 mg/ml, a carrier protein, bovine serum albumin (BSA), was added to obtain the final concentration of 0.1 mg/ml. The aliquots of IGF-1 (2×10^{-5} M) were kept at -80°C and used during 3 months. The vial

which contained lyophilized monocyte chemoattractant protein-1 (MCP-1) was centrifuged at 1200 rpm for 5 min. Then MPC-1 was reconstituted in sterile ultrapure water containing 0.1% BSA to the final concentration of 1 mg/ml and the aliquots were maintained at -80°C. Cumene hydroperoxide was diluted in DMSO from stock solution (1:1000) and the aliquots stored at -20°C. RGD tripeptide (MW 346.34 g/mol) was dissolved in distilled water at the final concentration of 1 mM and kept at -20°C. 3-3'-5-5'-tetraiodothyroacetic acid (MW 747.8 g/mol) was dissolved in 0.1 N NaOH to obtain 10^{-3} M stock solution maintained at -20°C. Rabbit anti-integrin alpha V + beta 3 polyclonal antibody (bs-1310r) was diluted in PBS (1:10) and stored at 2-8°C. DAPI stock solution was diluted to 300 nM in PBS. PD98059, a MEK inhibitor (MW 267.3 g/mol) was dissolved in DMSO at the final concentration of 10 mM. The long-term storage was at -20°C, and during the use, it was protected from light. Wortmannin, a PI3-K inhibitor, (MW 428.4 g/mol) was dissolved in DMSO at the final concentration of 1 mM and kept at -20°C. Phorbol 12-myristate 13-acetate (PMA) was dissolved in DMSO to obtain 20 nM stock solution maintained at -20°C. Rabbit anti-integrin alpha V + beta 3 polyclonal antibody (bs-1310r) was diluted in PBS (1:10) and stored at 2-8°C.

3.11 Statistical analysis

The results are reported as means ± SD and analyzed by one-way ANOVA, followed by post-hoc Bonferroni's multiple comparison test or by the Student's t-test; these were carried out using the Prism 7 statistics program (GraphPad, San Diego, CA). Differences were considered significant at $P < 0.05$.

4. RESULTS

4.1 Evaluation of cell viability of thyroid hormones

Since THs are essential for normal growth and development and exert profound effects on cellular metabolism in almost all tissues, before starting all experiments it is important to assess whether the hormones at the concentrations used might give rise to some toxicity *in vitro*.

The measurement of the cell-stimulating effect of THs is based on the capacity of mitochondrial enzymes of viable cells to transform the MTT tetrazolium salt into MTT formazan. A dose-response of the MTT signal was observed within range from 10^{-5} M to 10^{-10} M in the culture medium.

The MTT assay carried out in BV-2 cells on a wide concentration range did not show any cytotoxicity both for T_4 and T_3.

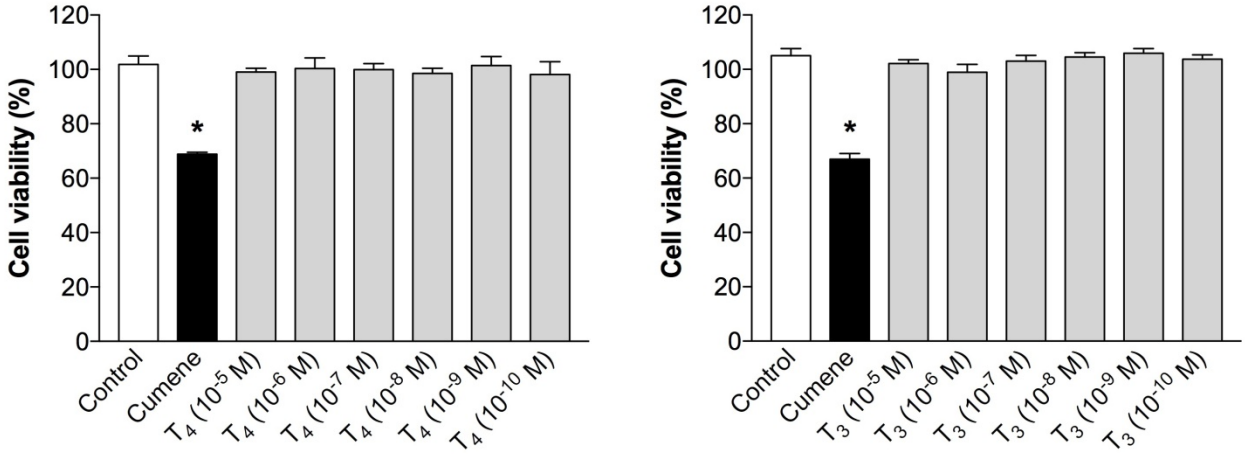

Figure 12. Effects of T_4 and T_3 on cell viability in BV-2 cells. Cumene hydroperoxide (200 μM), an inductor of oxidative stress, was used as positive control. Results are reported as mean ± SD of 3 different experiments carried out in triplicate. * $p<0.001$ *vs* all.

4.2 Expression of αVβ3 and its modulation by thyroid hormones

Microglia express several different integrins, and although it has been shown that expression of some of these receptors is regulated in different pathological conditions, and also modulated by LPS and some cytokines *in vitro* (Kloss *et al.*, 2001), what remains to be determined is which specific factors exert the major influence on microglial integrin αVβ3 expression.

THP-1 human leukemic monocytes show high expression of integrin αVβ3, already known from the literature (Dellacasagrande *et al.*, 2000; De Vito *et al.*, 2012). Since THP-1 monocytes can be considered similar as to functional role to the BV-2 microglial cells, as representing cells of the immune system, our first aim of this study was to perform a comprehensive investigation to examine the influence of individual cytokines and THs on integrin αVβ3 expression. To address this question, we quantified cell surface expression levels of integrin αVβ3 by flow cytometry analysis in physio- and pathological conditions, stimulating cells with MCP-1 and LPS to generate a model of inflammation.

It appears that integrin αVβ3 is well expressed in the plasma membrane of both cells but BV-2 shower a higher expression with respect to THP-1 (Figure 16). LPS gave a higher expression of the receptor in BV-2, partially reverted by T_3 treatment but even more by T_4. On the other hand, in THP-1 the expression of integrin was not modified by MCP-1; then the combination with T_4 decreased its expression, whereas in case of T_3 combined to MCP-1 there was only a trend to a reduction of the expression after 24 hours.

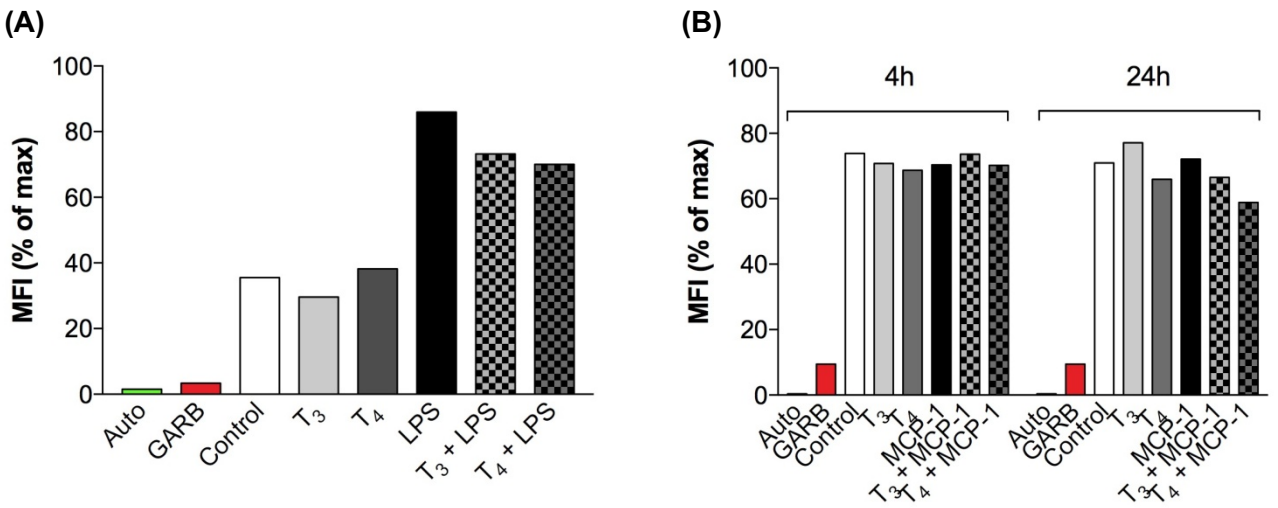

Figure 13. Expression of αVβ3 integrin in (A) murine microglia BV-2 and (B) human acute monocytic leukemia THP-1 cell lines measured by flow cytometry analysis. Cell were treated with T_3 (10^{-7} M), T_4 (10^{-7} M), LPS (1 μg/ml) and MCP-1 (100 ng/ml) for 4 and 24 hours. Unstained sample (autofluorescence) and a sample incubated with only secondary antibody (goat anti-rabbit) were included as negative controls.

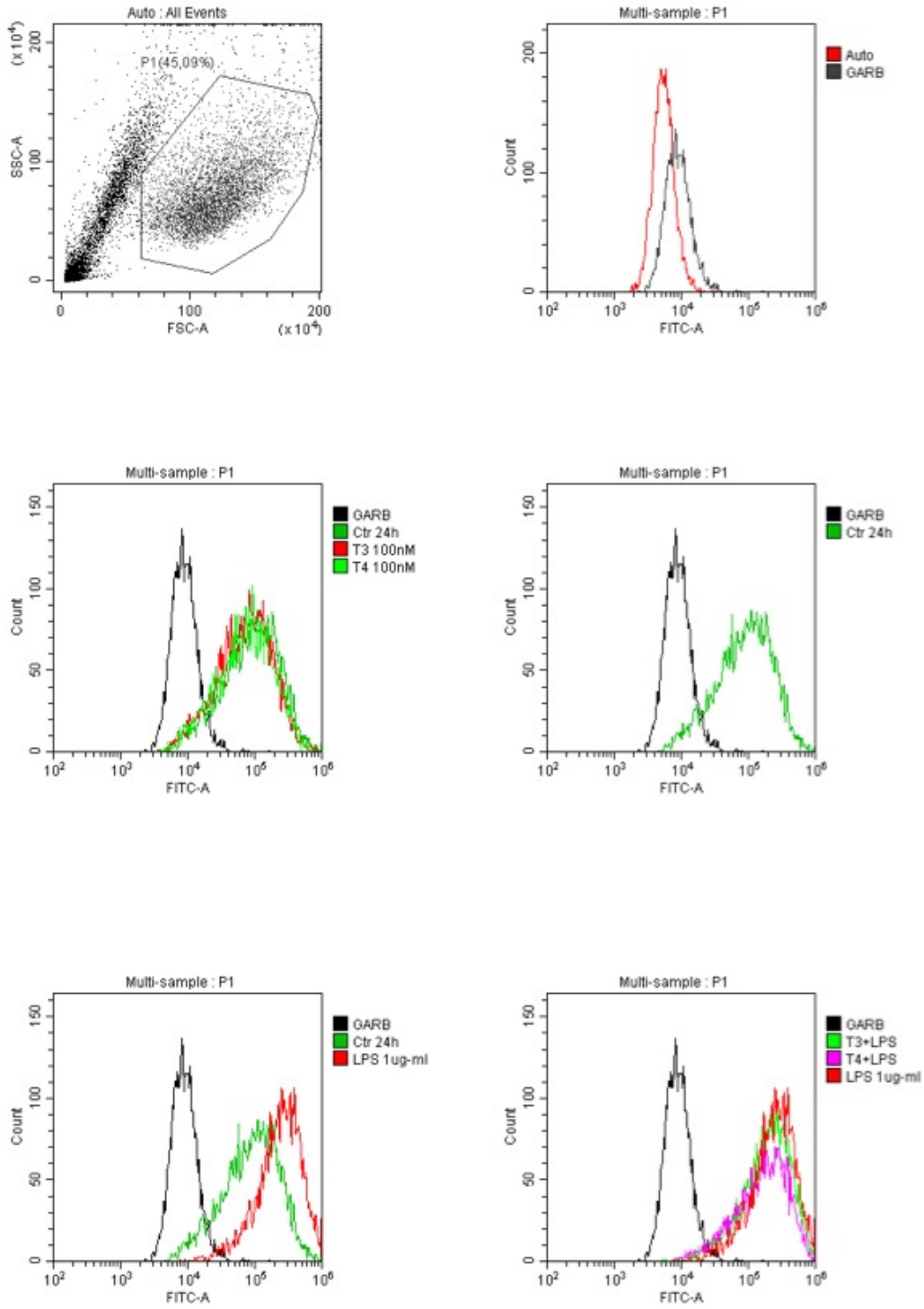

Figure 14. Flow cytometry analysis of the expression of αVβ3 integrin in BV-2 murine microglial cells, and its modulation by T_4 (10^{-7} M), T_3 (10^{-7} M) and LPS (1 μg/ml) at 24 hours.

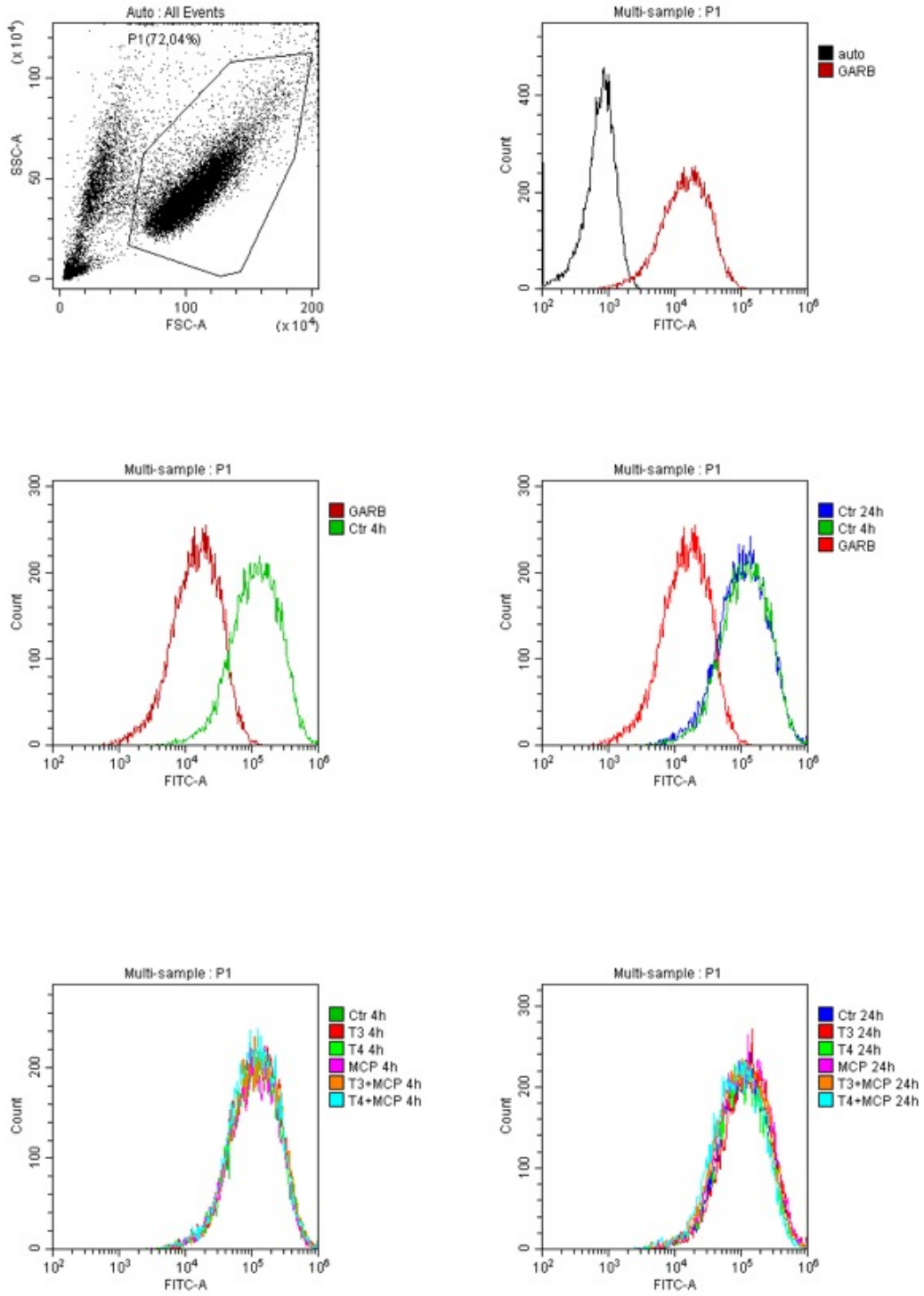

Figure 15. Flow cytometry analysis of the expression of αVβ3 integrin in THP-1 human leukemic monocytes and its modulation by T_4 (10^{-7} M), T_3 (10^{-7} M) and MCP-1 (100 ng/ml) at 4 and 24 hours.

The confocal microscopy experiment is in good agreement with the results of the FACS analysis, as far as integrin expression is concerned.

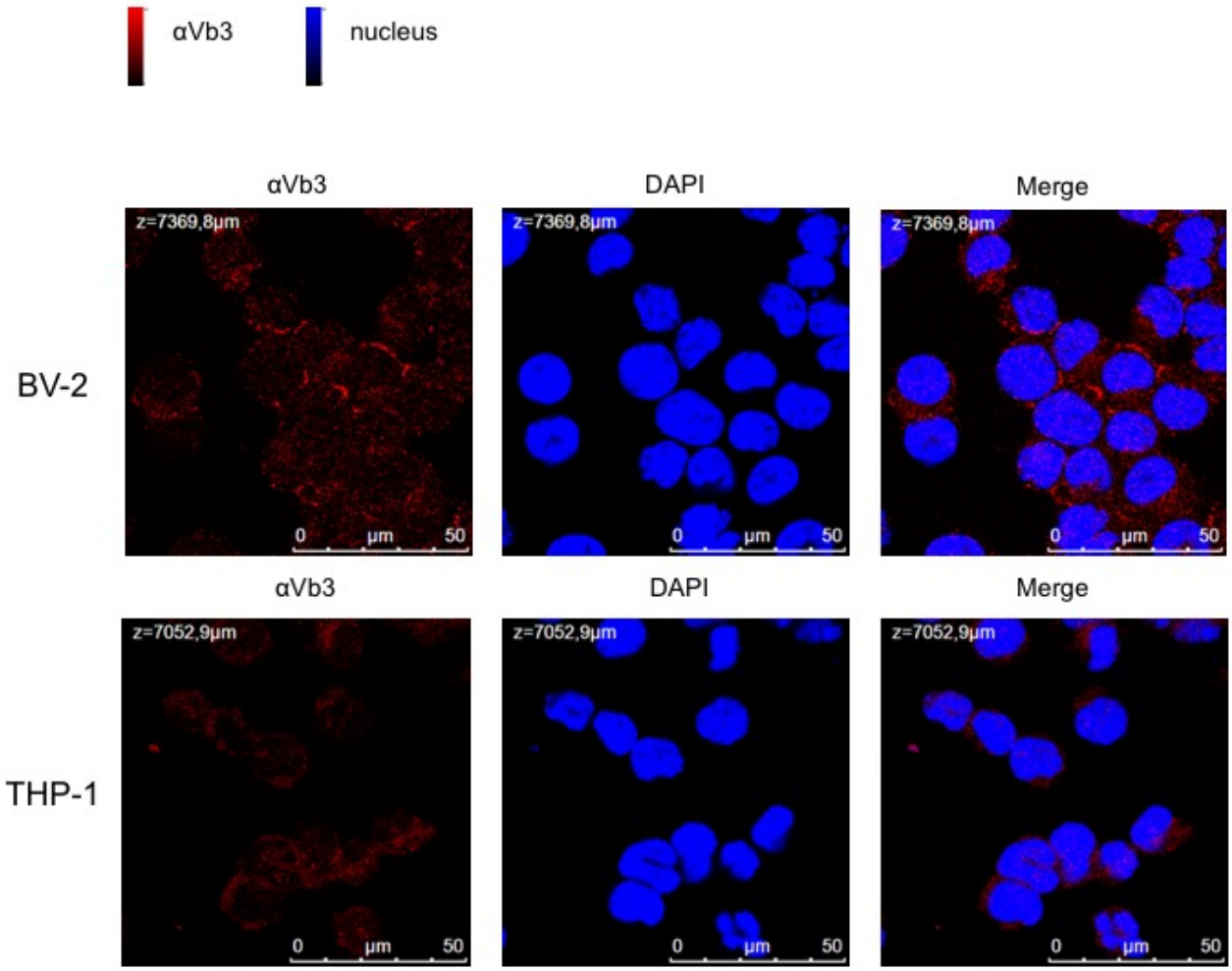

Figure 16. Expression of αVβ3 integrin in murine microglia BV-2 and human acute monocytic leukemia THP-1 cell lines measured by confocal microscopy. The red fluorophore indicates the presence of integrin αVβ3, the blue color given by DAPI instead indicates the nuclei. The third couple of panels in the right indicates the merge of the integrin signal with the nuclei.

4.3 Role of integrin αVβ3 in cell proliferation induced by IGF-1

IGF-1 is a growth factor therefore it enhances cell proliferation and survival (Laron, 2001), but also has been shown to behave as a chemokine after skeletal muscle injury (Pillon *et al*., 2013). Integrin αVβ3 is a co-receptor of IGF-1 in addition to the typical tyrosin kinase receptor IGF1R, and "ligand occupancy" of αVβ3 enhances signaling induced by IGF1 binding to IGF1R (Jones *et al*., 1996; Zheng *et al*., 1998; Clemmons, 2007; Soung *et al*., 2010; Takada *et al*., 2017). It has been proposed that ECM ligands bind to integrins and IGF1 binds to IGF1R, and two separate signals merge inside the cells (Maile *et al*., 2006). Blocking of αVβ3 integrin interaction using an anti-αVβ3 mAb and echistatin, a snake venom disintegrin that specifically inhibits αVβ3, inhibits IGF1-induced IGF1R phosphorylation, DNA synthesis and cell migration (Jones *et al*., 1995, 1996; Zheng and Clemmons, 1998).

We previously published a modulation by THs of the IGF-1 mediated short-term (glucose uptake) and long-term (proliferation) responses through integrin αVβ3 in L6 myoblasts from rat skeletal muscle (Incerpi *et al*., 2014). So once assessed the expression and the modulation by THs of integrin αVβ3, we determined whether THs modulated the stimulatory effect of IGF-I on cell proliferation in microglial cells. We first confirmed with a proliferation curve that IGF-I increased BV-2 cells number, as expected, both THs alone have nothing or small modulatory effect on cell proliferation but THs significantly inhibited the stimulatory effect of IGF-1 on proliferation (Figure 17).

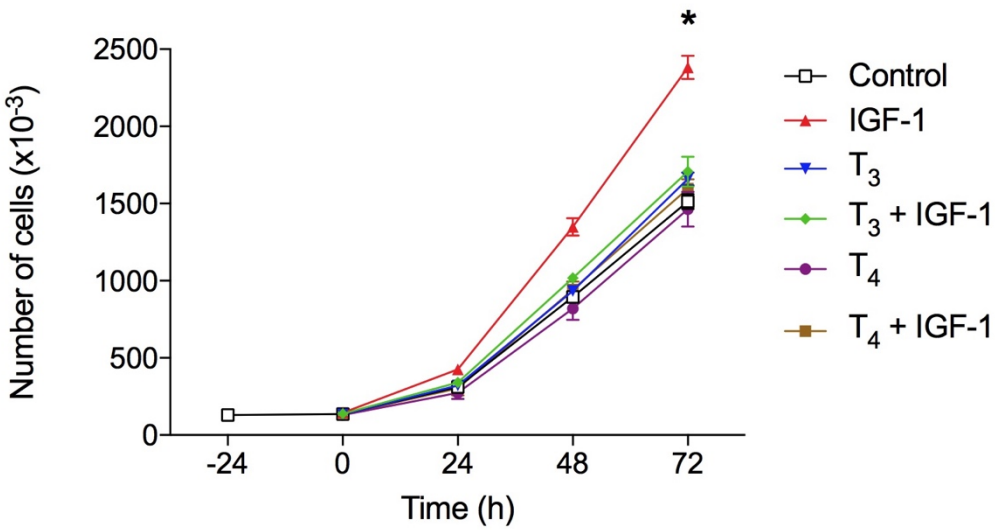

Figure 17. Effects of T_3 (10^{-7} M), T_4 (10^{-7} M) and IGF-1 (10 nM) on cell proliferation in BV-2 microglial cells. Results are reported as mean ± SD of 3 different experiments carried out in duplicate. * $p<0.001$ *vs* all.

Then addition of either RGD, tetrac or echistatin to the combination of T_4 and IGF-I completely reverted the inhibition by THs of the proliferative IGF-I effect and confirmed that integrin αVβ3 is a membrane receptor for THs and a co-receptor for IGF-1, as shown above. In particular the cell proliferation was a good example of a long-term effect that starts from the plasma membrane (Incerpi *et al*., 2014) (Figure 17).

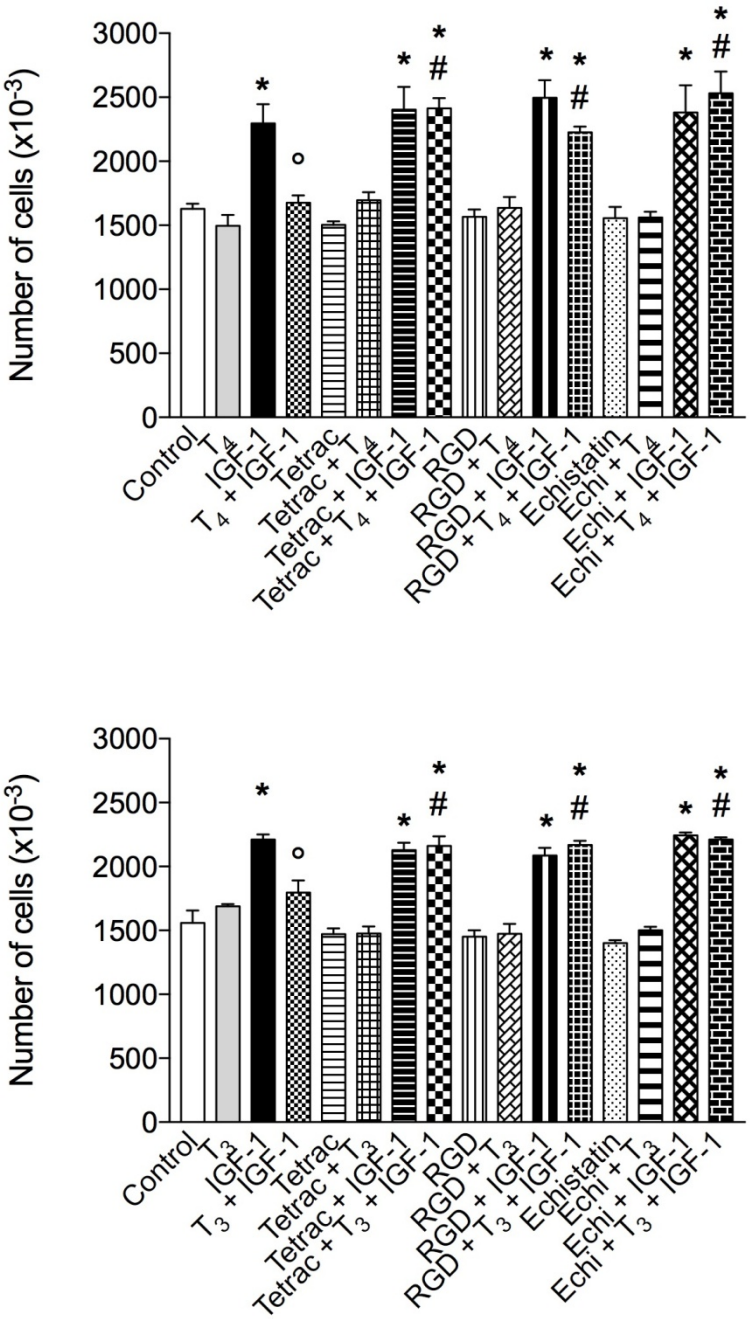

Figure 18. Effects of T_4 (10^{-7} M) in the upper panel, T_3 (10^{-7} M) in the lower panel, RGD peptide (10 μM), tetrac (10 μM) and echistatin (100 nM) on cell proliferation stimulated by IGF-1 (10 nM) in BV-2 microglia cells after 72h. Being the effect of THs on IGF-1 is more evident at confluency, after 72h, we carried out the following experiments at this fixed time. Results are reported as mean ± SD of 3 different experiments carried out in duplicate. * $p<0.01$ at least *vs* all; ◦ $p<0.001$ *vs* IGF-1: # $p<0.001$ *vs* T_4+IGF-1.

4.4 Signal transduction on BV-2 proliferation: MAPK and PI3-K pathways

In light of the above results, after evaluating the inhibitory effect of T_4 and the role of integrin αVβ3 in cell proliferation, we determined whether MAPK and PI3-K pathways are involved in this process by a pharmacological approach, because PI3-K/Akt mediates both short- and long-term cellular responses to IGF-1 (Clemmons, 2009). The results obtained are similar to L6 myoblasts: IGF-1 stimulated cell proliferation, through PI3-K, as already reported in the literature (Baserga *et al.*, 1997; Riedemann and Macaulay, 2006; Incerpi *et al.*, 2014). Figure 19 shows that ERK inhibition with PD98059 had no effect on the action of IGF-1 or on cell counts but eliminated the inhibitory effect of T_4 on cell proliferation of IGF-1. In contrast, wortmannin (a selective inhibitor for PI3-Ks) completely prevented induction of cell proliferation by IGF-I so that the cell counts remained at control level. These data confirm that the inhibitory effect of T_4 in cell proliferation through integrin αVβ3 was mediated by MAPK as reported also for L6 cells (Incerpi *et al.*, 2014).

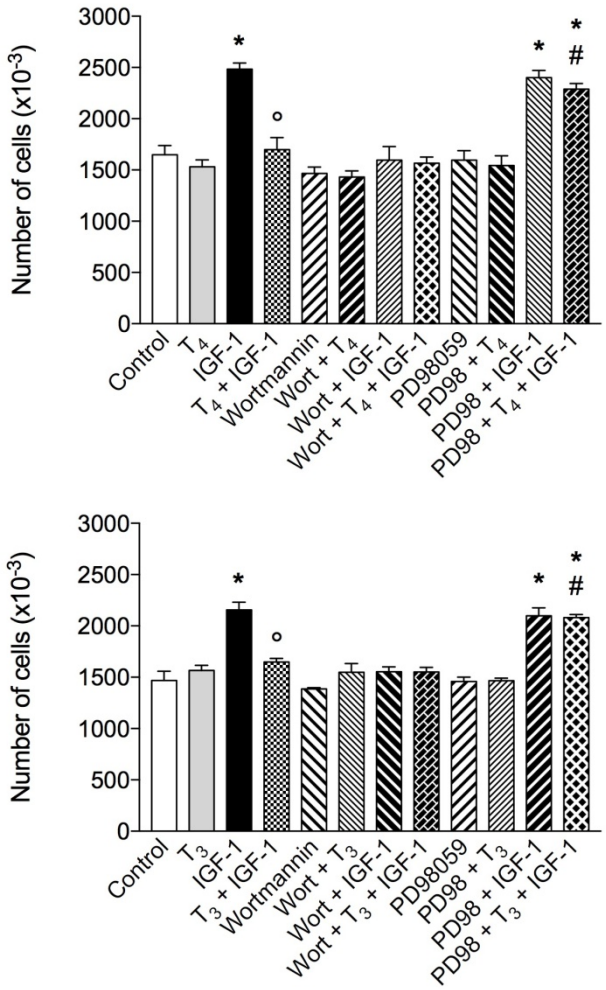

Figure 19. Effects of T_4 (10^{-7} M) in the upper panel, T_3 (10^{-7} M) in the lower panel, inhibitors of MAPK (PD98059) (10 μM) and PI3-K (wortmannin) (100 nM) pathways on cell proliferation induced by IGF-1 (10 nM) after 72h. Results are reported as mean ± SD of 3 different experiments carried out in duplicate. * $p<0.001$ *vs* all; ◦ $p<0.001$ *vs* IGF-1: # $p<0.001$ *vs* T_4+IGF-1.

4.5 Role of integrin αVβ3 in the ROS production induced by thyroid hormones

Many effects of THs are directly linked to ROS (reactive oxygen species) production and oxidative stress in various ways. ROS produced by cellular metabolism play an important role as signaling messengers in immune system cells, and their increase also occurs during the process of tissue repair (San Martin and Griendling, 2010; Chen *et al*., 2016).

Previous reports indicate that exists a crosstalk between THs and immune system (De Vito *et al*., 2012). Furthermore preliminary data from our laboratory showed that in THP-1 monocytes T_4 through integrin αVβ3 was able to increase ROS production as measured through formation of the intracellular fluorescent probe dichlorofluorescein (DCF) (De Vito *et al*., 2012).

The results showed that also in BV-2 cells, T_4 induce ROS formation and the effect was significantly inhibited by RGD peptide, tetrac and echistatin suggesting the involvement of integrin αVβ3. It was also interesting that when T_4 was given together with diphenylene iodonium (DPI) there was a further inhibition of ROS production, suggesting the involvement of a membrane NADPH oxidase system and a possibly crosstalk between integrin and NADPH oxidase (Figure 20A).

In addition, also the signal transduction pathway was studied by a pharmacological approach: both ERK1/2 and PI3-K/Akt pathways are involved, since ROS production was inhibited by wortmannin and PD98059, inhibitors of PI3-K and ERK1/2, respectively (Figure 20B).

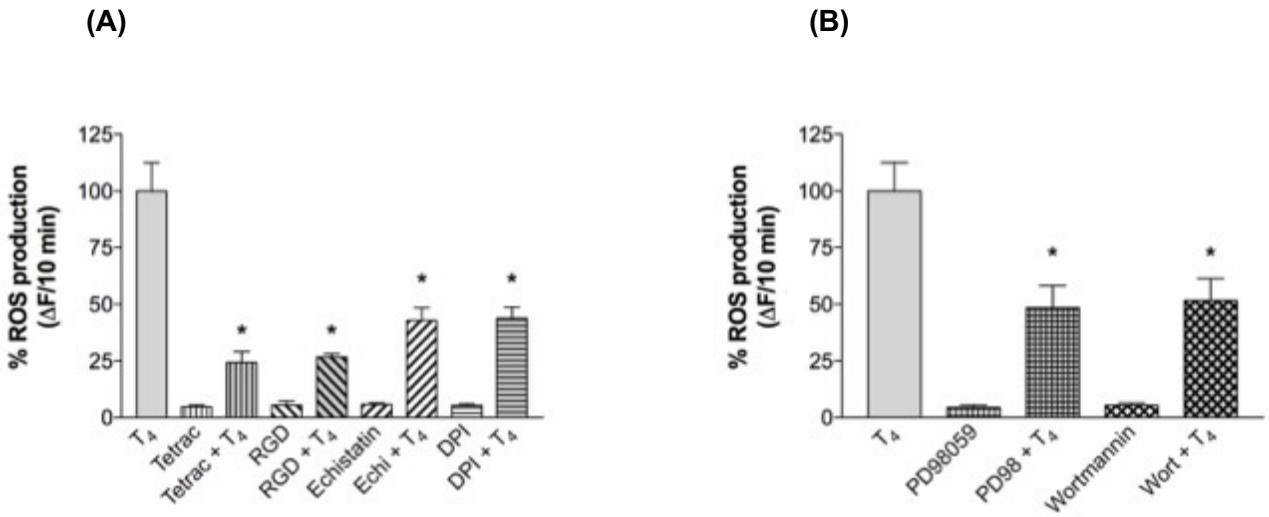

Figure 20. Effect of T_4 (10^{-6} M) on the ROS production in BV-2 microglial cells; they were pre-incubated with the inhibitors of integrin αVβ3 for 30 min at 37°C before the addition of hormone. (A) Effects of RGD (10 μM), tetrac (10 μM), echistatin (100 nM) and diphenylene iodonium (20 μM). (B) Effects of wortmannin (100 nM) and PD98059 (30 μM). Results are reported as mean ± S.D. of 3 different experiments. * $p<0.001$ *vs* T_4.

4.6 The role of thyroid hormones in wound healing

Accumulating evidence suggests that immune cells perform crucial inflammation-related functions including clearing dead tissue and promoting wound healing (Graeber and Streit, 2010). As such microglia, the brain-resident macrophages, are essential for maintaining the health and normal function of the CNS and are highly mobile after activation in response to damage or inflammation, in order to reach their target sites (Lively and Schlichter, 2013). Various stimuli have been demonstrated to induce the classical activation of microglia both *in vitro* and *in vivo*, such as lipopolysaccharide (LPS), which is a major component of the outer membrane of Gram-negative bacteria (Hoshino *et al*., 1999; Lehnardt *et al*., 2003).

It has been reported that αVβ3 integrin and LPS are involved in the production of TNF-α in THP-1 leukemic monocytes stimulated by *Coxiella burnetii* (Dellacasagrande *et al*., 2000), and data from our lab show that THs are able to inhibit the migration of THP-1 induced by MCP-1 through integrin αVβ3 after 4 hours (Candelotti, PhD thesis). Therefore, we studied the capability of THs to modulate cell migration in BV-2 cells with a wound healing assay, because this mechanism is a hallmark of wound repair involved in CNS repair after injury.

Figure 21 shows the effects of THs in the migration induced by LPS. Previous experiments using a wide range of concentrations of LPS showed that 1 μg/ml was the best concentration to stimulate BV-2 cells (data not shown). The data showed that LPS alone was able to increase the percentage of wound closure with respect to control, as expected, but both THs were able to inhibit it and bring back to basal value, aging as anti-inflammatory agents. In particular T_3 (10^{-9} M) was able to inhibit cell migration by about 20% with respect to basal value (Figure 21B).

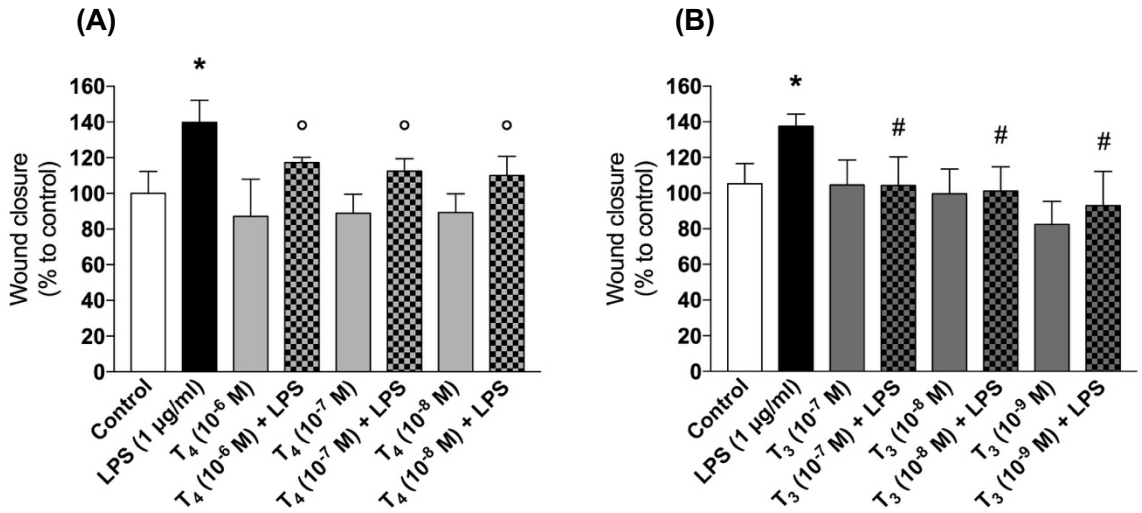

Figure 21. Effects of (A) T_4 and (B) T_3 in the modulation of BV-2 cell migration induced by LPS. Results are reported as mean ± SD of 3 different experiments carried out in triplicate. * $p<0.001$ *vs* all; ° $p<0.01$ at least *vs* LPS as from a Student's t test; # $p<0.001$ *vs* LPS as from a Student's t test.

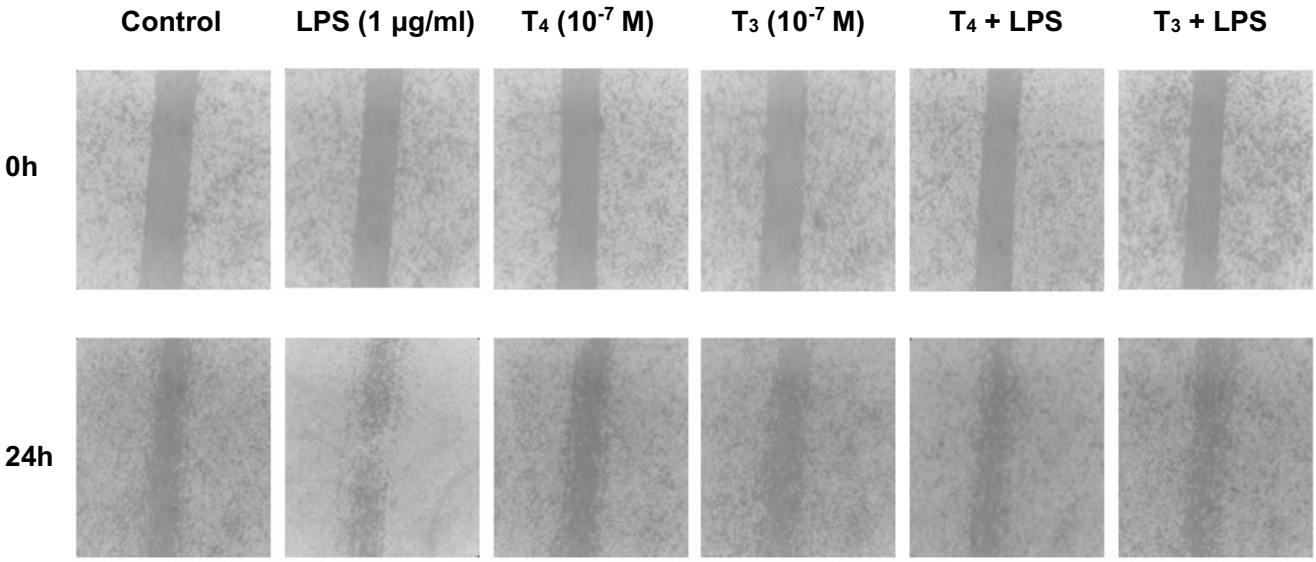

Figure 23. Images from a representative experiment of wound healing assay at 0 and 24 hours of BV-2 cell monolayer.

We then studied the signal transduction of the effect of T_4 on the migration induced by LPS using inhibitors of integrin αVβ3: RGD (Figure 22A) and tetrac (Figure 22B). Both inhibitors were unable to revert the effect of T_4, but the combination of both RGD and tetrac with T_4 potentiate the inhibition.

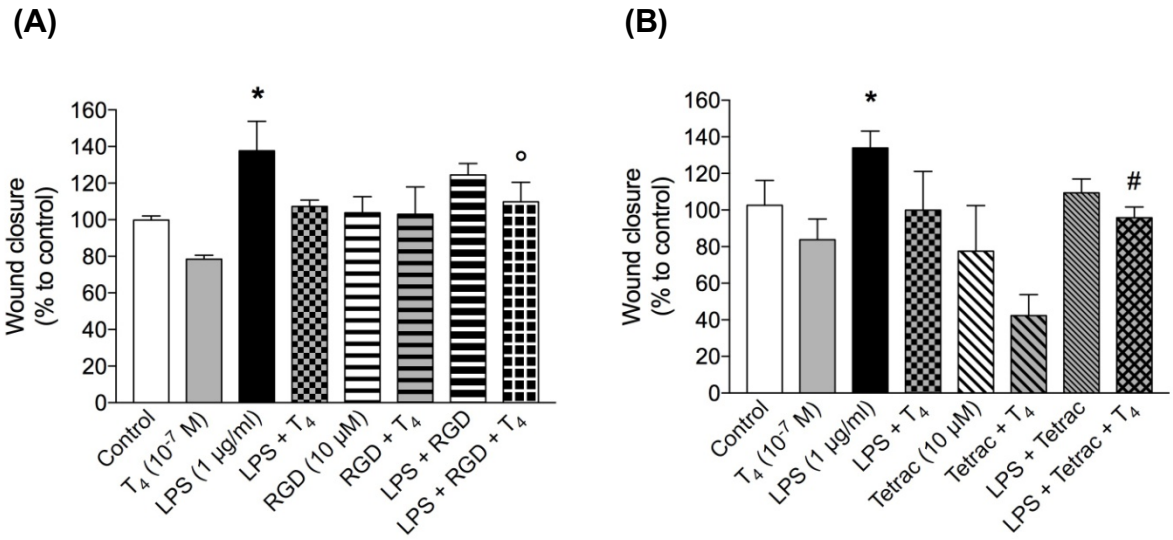

Figure 22. Effect of (A) RGD and (B) tetrac on the migration of BV-2 cells, induced by LPS. Results are reported as mean ± SD of 3 different experiments carried out in triplicate. * $p<0.001$ *vs* all; ° $p<0.05$ *vs* LPS + RGD as from a Student's t test; # $p<0.05$ vs LPS + Tetrac as from a Student's t test.

4.7 Effects of BPA on cytotoxicity and cell migration

Investigating immunomodulation effects of BPA on immune system cells, necessitates the exclusion of secondary genotoxic effects of BPA which may occur subsequent to its cytotoxic effects such as apoptosis or necrosis. We carried out a MTT assay in both BV-2 and macrophages (THP-1 monocytes differentiated with PMA for 24 hours at the final concentration of 100 nM (Lombardo *et al*., 2013)) and results show that in both cell line, BPA did not affect significantly cell viability but not at 1 mM (Figure 24).

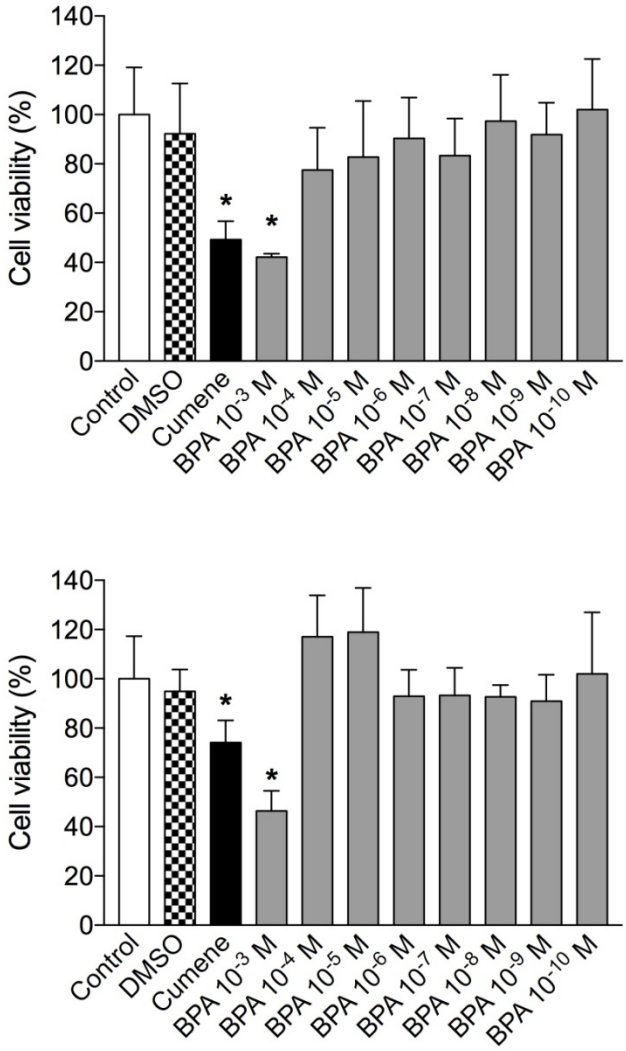

Figure 24. Cytotoxicity evaluation of BPA on BV-2 (upper panel) and macrophages (THP-1 differentiated with 100 nM PMA for 24 hours) (lower panel). Cumene hydroperoxide (200 μM), a radical generator, was used as a positive control. DMSO was used as a solvent control. Results are reported as mean ± SD of 3 different experiments carried out in triplicate. * $p<0.05$ at least *vs* all.

In the scientific literature, there are numerous research results that demonstrate a specific connection between the endocrine and immune systems. In particular Sheng and collaborators have shown that BPA can interfere with the action of thyroid hormones through integrin αVβ3 (Sheng *et al*., 2013).

We evaluated, in a wide range of concentrations, the immunomodulatory effects of BPA in THP-1 leukemic monocytes in the migration induced by MCP-1. Data shown are in line with previously experiments carried out in our laboratory with thyroid hormone, T_4 (Candelotti, PhD thesis), but at present we are not able to confirm that this inhibition is mediated by interaction between BPA and αVβ3 integrin (Figure 25).

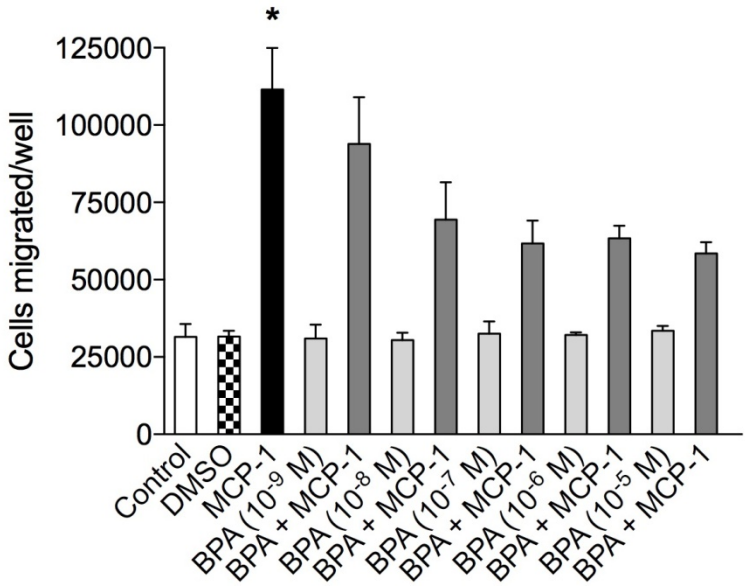

Figure 25. Dose-response of BPA (10^{-5} M - 10^{-9} M) in the transwell migration of THP-1 induced by MCP-1 (100 ng/ml). DMSO was used as a solvent control. Results are reported as mean ± SD of 3-6 different experiments carried out in duplicate. * $p<0.05$ at least *vs* all.

5. DISCUSSION

To the best of our knowledge, the present thesis was the first to demonstrate that thyroid hormones (THs), through integrin αVβ3, seems to behave as anti-inflammatory agents in activated microglia. Microglia, the resident macrophages of the CNS are generally considered the primary immune cells of the brain (Kim and de Vellis, 2005). In healthy CNS, ramified microglia are widely distributed to detect any environmental changes by their motile processes (Streit *et al.*, 1988; Kettenmann *et al.*, 2011). Pathological insults trigger microglial activation, they change into highly active phagocytic cells that proliferate and migrate to the inflammatory focus (Becher *et al.*, 2000; Hanisch and Kettenmann, 2007; Tanaka *et al.*, 2009).

The connection between THs and immune system is complex and not well-understood, however the identification of αVβ3 as a membrane receptor for TH permits speculation about clinical significance of the interaction between integrin αVβ3 and the TH, and the downstream consequence of immune responses. In fact, our group showed that THs, mainly T_4, prevented cell migration in THP-1 monocytes induced by different chemokines and this mechanism is regulated by integrin αVβ3 (Candelotti, PhD thesis). All this body of evidences prompted us to study the possible crosstalk between THs and immune system using as a model murine microglial cells BV-2. While the primary function of microglia is to protect cells of the CNS from invading microorganisms, accumulating evidence suggests that under certain conditions microglia may become overly stimulated, leading to excessive destruction of host tissue (Gonzalez-Scarano and Baltuch, 1999; Carson, 2002). In view of these findings, we focused our attention to define the possible role of TH in the regulation of activation of microglial BV-2 cells through integrin αVβ3, because in literature few information is available.

THs are important for the development and function not only in periphery but also in the CNS, supporting the development of microglia (Lima *et al.*, 2001) With the exception of direct hormonal action on mitochondrial respiration, the effects of TH have been viewed as largely mediated by genomic mechanisms (Cheng *et al.*, 2010). However, data presented in the past decade have supported the existence of a number of non-genomic mechanisms of action for the hormone initiated at a specific receptor on a cell surface protein: integrin αVβ3 (Cheng *et al.*, 2010; Davis *et al.*, 2011). Intracellular signals through integrin αVβ3 include regulation of trafficking of specific proteins from cytoplasm into the nuclear compartment, or activation of signal transducing kinases (MAPK and PI3-K) but recent evidence supports the existence of crosstalk between non-genomic and genomic effects of the hormone (Bergh *et al.*, 2005; Incerpi *et al.*, 2014; Davis *et al.*, 2016;

Kalyanaraman *et al*., 2016). Since the traditional ligands of integrins are proteins, finding that a small molecule like TH is also a ligand of an integrin is a novel finding.

The immune system provides other examples of crosstalk between genomic and non-genomic mechanisms of THs. Growing evidence compiled over recent decades has revealed a bidirectional crosstalk between THs and the immune system. This interplay has been demonstrated for several pathophysiological conditions of the thyroid functioning and the innate and adaptive immunity (De Vito *et al*., 2012). In general hyperthyroidism enhances the immune response. This is indicated in terms of antibody production, immune cell migration, lymphocyte proliferation and ROS production, and it is associated with decreased pro-inflammatory marker release, antioxidant enzyme production, and increased immune functions (De Vito *et al*., 2011, 2012). Hypothyroidism often gives rise to the opposite effects for some of these parameters, decreasing the immune response, antibody production, cell migration, and lymphocyte proliferation (Klecha *et al*., 2008; Barreiro Arcos *et al*., 2011). Though non-genomic effect of TH, its plasma membrane-bound receptor, and its signaling has been identified, precise function in each cell type of the CNS remained to be investigated. The novel homeostatic link between THs and the pathophysiological role of microglia opens new perspectives on the interactions between the endocrine and immune systems.

Integrins are transmembrane adhesion receptors that mediate cell-cell and cell-extracellular matrix adhesion and also induce bidirectional signalling across the cell membrane to regulate cell proliferation, activation, migration and homeostasis (Hynes, 2002). Integrin αVβ3 is a ubiquitous receptor that is expressed on a wide variety of cell types including macrophages (Felding-Habermann and Cheresh, 1996; Cai and Chen, 2006). Previous studies have showed that integrin αVβ3 was highly expressed on activated cells under pathological conditions and is linked to inflammation (Monick *et al*., 2002; Antonov *et al*., 2011; Kurihara *et al*., 2011; Gianni *et al*., 2012). In fact expression of integrin αVβ3 can be up-regulated by stressful signals in monocytes (Antonov *et al*., 2004), and modulated by cytokines and ECM proteins in microglial cells (Milner, 2009). We compared the integrin αVβ3 expression on THP-1 and BV-2 under different pro-inflammatory stimuli and our data show that only in BV-2 cell, LPS increases significantly the expression of the receptor partially reverted in combination with T_3 and T_4. These preliminary data together indicate that integrin αVβ3 is up-regulated in BV-2 under LPS stimulation and is required for microglial activation, but the inhibition by TH suggests an anti-inflammatory role of the hormone in the modulation of the response.

THs are also able to modulate transduction of several cytokine and growth factor signals in human cell lines such as such as EGF and TGF-α (Lin *et al*., 1999b; Shih *et al*., 2004). Previously

data from our lab demonstrated a crosstalk between THs and IGF-1 through integrin αVβ3 in L6 myoblasts from rat skeletal muscle (Incerpi *et al.*, 2014). We wanted to assess whether this interaction might be found also in BV-2 cells while IGF-1, a growth factor that has integrin αVβ3 as a co-receptor, has been recently reported to behave also as a chemokine after a skeletal muscle injury (Pillon *et al.*, 2013). In particular we focused our attention in the study of cell proliferation induced by IGF-1, a good example of a long-term effect that starts from the plasma membrane (Cheng *et al.*, 2010; Incerpi *et al.*, 2014). Our results show that in BV-2 microglial cells both THs, mainly T_4, are able to inhibit cell proliferation induced by IGF-1 and this effect is mediated by integrin αVβ3 since RGD, tetrac and echistatin completely reverted the inhibitory effects induced by TH. Then signal transduction studies of TH and IGF-I were conducted by a pharmacological approach using inhibitors of PI3-K and MAPK, since both signalling pathways mediates both short- and long-term cellular responses to IGF-1 and are co-activated by integrin αVβ3 and IGF1R. Our data show that wortmannin (an inhibitor of PI3-K) inhibited IGF-I stimulation of cell proliferation, as expected; on the other hand PD98059 (an inhibitor of MAPK) did not affects IGF-I action but blocks the action of T_4 and confirm that the modulation of cell proliferation through integrin αVβ3 was mediated by MAPK pathway.

The innate immune system is involved also in every aspect of the wound healing process and is especially significant during the inflammatory stage (Julier *et al.*, 2017), but the involvement of TH is not well understood (Safer *et al.*, 2004). The reported importance of TH in wound healing *in vivo*, both in human and in animals, is contradictory. Although some authors report improved rates and quality of wound healing in response to TH (Zamick and Mehregan, 1973; Mehregan and Zamick, 1974; Herndon *et al.*, 1979; Talmi *et al.*, 1979; Alexander *et al.*, 1982; Erdogan *et al.*, 1999), others found no apparent TH-mediated changes in wound healing (Pirk *et al.*, 1974; Ladenson *et al.*, 1984; Cannon, 1994). To gain insight, we evaluated the impact of THs on wound healing in BV-2 microglial cells. Microglia rapidly become activated in response to CNS damage or in the presence of modulator of inflammation like LPS, because among other cells in the brain microglia are the major LPS responsive cells (Lehnardt *et al.*, 2003). The principal finding in the present thesis is that both T_4 and T_3 in the physiological range are able to modulate wound closure and cell migration responses to LPS and these effects seem to be mediated by integrin αVβ3. The involvement of αVβ3 integrin in the effects of TH on wound healing was studied by the use of two different inhibitors of the binding to αVβ3 integrin: Arg-Gly-Asp peptide (RGD), tetraiodothyroacetic acid (tetrac) a metabolite of TH and a probe for integrin αVβ3. Surprisingly, T_4 was still able to reduce the migration induced by LPS, but the combination of TH and integrin αVβ3 inhibitors results in a significant potentiation of the inhibitory effect of TH on LPS-induced cell

migration. Mori and collaborators demonstrated that T_3 induces microglial migration both *in vitro* and *in vivo* and the enhancement is mediated by T_3 uptake by TH transporters and binding to TR (Mori *et al*., 2015). Our results suggest that TH is an important signaling factor that affects microglial migration via various complex mechanisms, but the difference in the response probably depends from the signal transduction pathway activated. However these results are in agreement with previous data from our lab that reported the involvement of αVβ3 integrin in the crosstalk between T_4 and MCP-1 in THP-1 monocytes cell migration (Candelotti, PhD thesis) but the unexpected results on the potentiation of the inhibitory effect of the TH needs to be elucidated.

In silico molecular docking simulations have been carried out in order to better understand the mechanism of interaction between T_4 and αVβ3 integrin, in its inactive form, both in the presence and in the absence of the peptide RGD. Data showed that T_4 mainly binds at the interface between two αVβ3 integrin subunits in the basal part of the macromolecule next to the cell membrane, a site that is different from the RGD binding site. Interestingly, T_4 was able to bind to this site both in the presence and in the absence of the RGD peptide. In particular, the putative T_4 binding site is located in the extracellular space, but very close to the plasma membrane and, interacting with both integrin subunits may stabilize the inactive conformation of the αVβ3 integrin. These results are in agreement with those obtained by the wound healing assays which show a significant potentiation in the inhibition of the migration induced by LPS, when T_4 was in the presence of the peptide RGD. As mentioned above, this effect could be due to the presence on the integrin of a binding site for the hormone, different from the RGD site.

Cell migration is a complex, dynamic and integrated process involved in several physio-pathological functions such as morpho-genesis during development. In adults, cell migration plays a pivotal role in immune response and tissue-repair (San Martin & Griendling, 2010). ROS are very important for cell migration as shown in different cell types including THP-1 monocytes and ROS production is important in different aspects of the migratory process (De Vito *et al*., 2012). Formation of ROS can be obtained by activation of plasma membrane NADPH oxidases that give rise to superoxide production, but there are other types of ROS in the cells, such as H_2O_2 which is not a free radical and is quite stable, even though it has strong oxidizing capacity, in particular as a substrate of peroxidases. The reports concerning the action of THs in cells of the immune system, mainly mononuclear and polymorphonuclear leukocytes, indicate that TH stimulates their metabolic activity and oxygen consumption, thus TH may contribute to oxidative stress both in the short- and in the long-term range (Magsino *et al*., 2010). In light of the above results, after evaluating the inhibitory effect of T_4 and the role of integrin αVβ3 in cell proliferation and migration, we evaluated the cellular and molecular mechanisms involved in THs effects on ROS production in

innate immune cells. Our results showed that in BV-2 cells T_4 is able to increase ROS production and this leads to a stimulation of cell migration. The process is mediated by integrin αVβ3, since the effect is inhibited by RGD, tetrac and echistatin whereas the ROS production seems to come from the crosstalk of integrin αVβ3 and NADPH oxidases (De Vito *et al*., 2012).

In the scientific literature, there are numerous research results that demonstrate a specific connection between the endocrine and immune systems. The structures of some compounds resemble those of natural hormones, and this enables them to act directly on the endocrine system similarly as an endogenous hormone. This finding, as well as further observations, leads to the constitution of a novel group-endocrine disrupting chemicals (EDCs), exogenous and natural and synthetic chemicals which disrupt endocrine function through mimicking or blocking endogenous hormones (Vaiserman, 2014; Nowak *et al*., 2019). Bisphenol A (BPA), a monomer of polycarbonate plastics, has been shown to possess estrogenic properties (Acconcia *et al*., 2015) and is reported that can act also as an agonist for TH and integrin αVβ3 (Moriyama *et al*., 2002; Sheng *et al*., 2019). Based on literature that demonstrates the relationship between BPA, THs and immune systems, we reported the effects of BPA exposure *in vitro* on immune system cell. Preliminary data showed that BPA at high concentrations suppress cell viability of THP-1 monocytes and BV-2 microglial cells. Segura and collaborators observed the inhibitory influence of BPA on macrophage functions (Segura *et al*., 1999), so we compared the effect of BPA in THP-1 cell migration induced by MCP-1. Results showed that BPA at concentration used seems to mimic the effects of THs in the inhibition of migration but at the moment we can't confirm the effect is mediated by αVβ3.

In conclusion, our data show for the first time that THs, T_3 and T_4, inhibit ROS production, cell proliferation and cell the migration in wound healing assay in BV-2 microglial cells. The effect is mediated by integrin αVβ3, the plasma membrane receptor for THs, as observed by the use of pharmacological inhibitors of αVβ3: RGD and tetrac. THs seem to behave as anti-inflammatory agents in several physio-pathological functions and play a pivotal role in immune response but since the response in the presence of different modulators of inflammation is different, for this reason we can hypothesize that maybe the role of THs may be different depending on the physiopathological situation.

6. REFERENCES

Acconcia F, Pallottini V, Marino M. (2015) Molecular Mechanisms of Action of BPA. *Dose Response*. 13(4):1559325815610582.

Al-Abed Y, Metz CN, Cheng KF, Aljabari B, VanPatten S, Blau S, Lee H, Ochani M, Pavlov VA, Coleman T, Meurice N, Tracey KJ, Miller EJ. (2011) Thyroxine is a potential endogenous antagonist of macrophage migration inhibitory factor (MIF) activity. *Proc Natl Acad Sci U S A*. 108(20):8224-8227.

Alamino VA, Montesinos MDM, Soler MF, Giusiano L, Gigena N, Fozzatti L, Maller SM, Mendez-Huergo SP, Rabinovich GA, Pellizas CG. (2019) Dendritic Cells Exposed to Triiodothyronine Deliver Pro-Inflammatory Signals and Amplify IL-17-Driven Immune Responses. *Cell Physiol Biochem*. 52:354-367.

Alexander MV, Zajtchuk JT, Henderson RL. (1982) Hypothyroidism and wound healing: occurrence after head and neck radiation and surgery. *Arch Otolaryngol*. 108(5):289-291.

Allen CM, Sharman WM, Madeleine CL, Van Lier JE, Weber JM. (2002) Attenuation of photodynamically induced apoptosis by an RGD containing peptide. *Photochem Photobiol Sci*. 1:246-254.

ANSES (French Agency for Food, Environmental and Occupational Health Safety) (2013) Risk assessment of bisphenol A (BPA) on human health (March 2013).

ANSES (French Agency for Food, Environmental and Occupational Health Safety) (2018) Concerns related to ED-mediated effects of Bisphenol A and their regulatory consideration. *Mol Cell Endocrinol*. (18):30055-30058 (ISSN 0303-7207).

Antonov AS, Antonova GN, Munn DH, Mivechi N, Lucas R, Catravas JD, Verin AD. (2011) αVβ3 integrin regulates macrophage inflammatory responses via PI3 kinase/Akt-dependent NF-κB activation. *J Cell Physiol*. 226(2):469-476.

Arjonen A. (Unpublished doctoral dissertation) Integrins on the move. VTT Technical Research Centre of Finland, University of Turku, Turku, Finland.

Arnaout MA, Mahalingam B, Xiong JP. (2005) Integrin structure, allostery, and bidirectional signaling. *Annu Rev Cell Dev Biol*. 21:381-410.

Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. (2010) Vitamin D: modulator of the immune system. *Curr Opin Pharmacol*. 10:482-496.

Bagriacik EU, Zhou Q, Wang HC, Klein JR. (2001) Rapid and transient reduction in circulating thyroid hormones following systemic antigen priming: implications for functional collaboration between dendritic cells and thyroid. *Cell Immunol*. 212:92-100.

Barczyk M, Carracedo S, Gullberg D. (2010) Integrins. *Cell Tissue Res*. 339(1):269-280.

Barillari G, Sgadari C, Fiorelli V, Samaniego F, Colombini S, Manzari V, Modesti A, Nair BC, Cafaro A, Stürzl M, Ensoli B. (1999) The Tat protein of human immunodeficiency virus type-1 promotes vascular cell growth and locomotion by engaging the alpha5beta1 and alphavbeta3 integrins and by mobilizing sequestered basic fibroblast growth factor. *Blood*. 94(2):663-672.

Barreiro Arcos ML, Gorelik G, Klecha A, Genaro AM, Cremaschi GA. (2006) Thyroid hormones increase inducible nitric oxide synthase gene expression downstream from PKC-zeta in murine tumor T lymphocytes. *Am J Physiol Cell Physiol*. 291:C327-336.

Baserga R, Hongo A, Rubini M, Prisco M, Valentinis B. (1997) The IGF-I receptor in cell growth, transformation and apoptosis. *Biochim Biophys Acta*. 1332(3):F105-126.

Beausoleil C, Emond C, Cravedi JP, Antignac JP, Applanat M, Appenzeller BR, Beaudouin R, Belzunces LP, Canivenc-Lavier MC, Chevalier N, Chevrier C, Elefant E, Eustache F, Habert R, Kolf-Clauw M, Le Magueresse-Battistoni B, Mhaouty-Kodja S, Minier C, Multigner L, Schroeder H, Thonneau P, Viguié C, Pouzaud F, Ormsby JN, Rousselle C, Verines-Jouin L, Pasquier E, Michel C. (2018) Regulatory identification of BPA as an endocrine disruptor: Context and methodology. *Mol Cell Endocrinol*. 475:4-9.

Becher B, Prat A, Antel JP. (2000) Brain-immune connection: immuno-regulatory properties of CNS-resident cells. *Glia*. 29(4):293-304.

Bergh JJ, Lin HY, Lansing L, Mohamed SN, Davis FB, Mousa S, Davis PJ. (2005) Integrin alphaVbeta3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis. *Endocrinology*. 146(7):2864-2871.

Bhargava M, Lei J, Mariash CN, Ingbar DH. (2007) Thyroid hormone rapidly stimulates alveolar Na, K-ATPase by activation of phosphatidylinositol 3-kinase. *Curr Opin Endocrinol Diabetes Obes*. 14:416-420.

Blalock JE, Johnson HM, Smith EM, Torres BA. (1984) Enhancement of the in vitro antibody response by thyrotropin. *Biochem Biophys Res Commun*. 125:30-34.

Blasi E, Barluzzi R, Bocchini V, Mazzolla R, Bistoni F. (1990) Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. *J Neuroimmunol*. 27(2-3):229-237.

Bozza FA, Gomes RN, Japiassú AM, Soares M, Castro-Faria-Neto HC, Bozza PT, Bozza MT. (2004) Macrophage migration inhibitory factor levels correlate with fatal outcome in sepsis. *Shock*. 22(4):309-313.

Brent GA. (2012) Mechanisms of thyroid hormone action. *J Clin Invest*. 122:3035-3043.

Brooks PC, Clark RA, Cheresh DA. (1994) Requirement of vascularintegrin alpha v beta 3 for angiogenesis. *Science*. 264:569-571.

Buckley CD, Doyonnas R, Newton JP, Blystone SD, Brown EJ, Watt SM, Simmons DL. (1996) Identification of alpha v beta 3 as a heterotypic ligand for CD31/PECAM-1. *J Cell Sci*. 109 (Pt 2):437-445.

Byun JA, Heo Y, Kim YO, Pyo MY. (2005) Bisphenol A-induced downregulation of murine macrophage activities in vitro and ex vivo. *Environmental toxicology and pharmacology*. 19:19-24.

Cai W, Chen X. (2006) Anti-angiogenic cancer therapy based on integrin alphavbeta3 antagonism. *Anticancer Agents Med Chem*. 6(5):407-428.

Candelotti E. (Unpublished doctoral dissertation) Crosstalk between thyroid hormones and IGF-1. Department of Sciences, University Roma Tre, Rome, Italy.

Cannon CR. (1994) Hypothyroidism in head and neck cancer patients: experimental and clinical observations. *Laryngoscope*. 104(11 Pt 2 Suppl 66):1-21.

Cao HJ, Lin HY, Luidens MK, Davis FB, Davis PJ. (2009) Cytoplasm-to-nucleus shuttling of thyroid hormone receptor-beta1 (TRbeta1) is directed from a plasma membrane integrin receptor by thyroid hormone. *Endocr Res*. 34:31-42.

Carr R, Bertasi F, Betancourt A, Bowers S, Gandy BS, Ryan P, Willard S. (2003) Effect of neonatal rat bisphenol a exposure on performance in the Morris watermaze. *J Toxicol Environ Health A*. 66:2077-2088.

Chen C, Sheppard D. (2007) Identification and molecular characterization of multiple phenotypes in integrin knockout mice. *Methods Enzymol*. 426:291-305.

Chen X, Song M, Zhang B, Zhang Y. (2016) Reactive Oxygen Species Regulate T Cell Immune Response in the Tumor Microenvironment. *Oxid Med Cell Longev*. 2016:1580967.

Chen Y, Chen PL, Chen CF, Sharp ZD, Lee WH. (1999) Thyroid hormone, T3-dependent phosphorylation and translocation of Trip230 from the Golgi complex to the nucleus. *Proc Natl Acad Sci U S A*. 96:4443-4448.

Chen Y, Sjölinder M, Wang X, Altenbacher G, Hagner M, Berglund P, Gao Y, Lu T, Jonsson AB, Sjölinder H. (2012) Thyroid hormone enhances nitric oxide-mediated bacterial clearance and promotes survival after meningococcal infection. *PLoS One*. 7(7):e41445.

Cheng SY, Leonard JL, Davis PJ. (2010) Molecular aspects of thyroid hormone actions. *Endocr Rev*. 31:139-170.

Clemmons DR. (2007) Modifying IGF1 activity: an approach to treat endocrine disorders, atherosclerosis and cancer. *Nat Rev Drug Discov*. 6(10):821-833.

Clemmons DR. (2009) Role of IGF-I in skeletal muscle mass maintenance. *Trends Endocrinol Metab*. 20(7):349-356.

Cody V, Davis PJ, Davis FB. (2007a) Molecular modeling of the thyroid hormone interactions with alpha v beta 3 integrin. *Steroids*. 72(2):165-170.

Cody V, Freindorf M, Furlani T, Davis PJ, Davis FB. (2007b) Computational studies of thyroid and steroid hormone interactions with alphavbeta3 integrin. *Thyroid*. 17:S130.

Danzi S, Klein I. (2014) Thyroid disease and the cardiovascular system. *Endocrinol Metab Clin North Am*. 43:517-528.

Davies AG. (1972) Thyroid physiology. *Br Med J*. 2:206-209.

Davis FB, Cody V, Davis PJ, Borzynski LJ, Blas SD. (1973) Stimulation by thyroid hormone analogues of red blood cell Ca2+-ATPase activity in vitro. Correlations between hormone structure and biological activity in a human cell system. *J Biol Chem*. 258:12373-12377.

Davis FB, Mousa SA, O'Connor L, Mohamed S, Lin HY, Cao HJ, Davis PJ. (2004) Proangiogenic action of thyroid hormone is fibroblast growth factor-dependent and is initiated at the cell surface. *Circ Res*. 94(11):1500-1506.

Davis FB, Tang HY, Shih A, Keating T, Lansing L, Hercbergs A, Fenstermaker RA, Mousa A, Mousa SA, Davis PJ, Lin HY. (2006) Acting via a cell surface receptor, thyroid hormone is a growth factor for glioma cells. *Cancer Res*. 66(14):7270-7275.

Davis PJ, Shih A, Lin HY, Martino LJ, Davis FB. (2000) Thyroxine promotes association of mitogen-activated protein kinase and nuclear thyroid hormone receptor (TR) and causes serine phosphorylation of TR. *J Biol Chem*. 275:38032-38039.

Davis PJ, Davis FB, Cody V. (2005) Membrane receptors mediating thyroid hormone action. *Trends Endocrinol Metab*. 16(9):429-435.

Davis PJ, Davis FB, Bergh JJ, Lin HY. (2006) Defining the roles of the cell surface receptor for thyroid hormone. *Hot Thyroidol*. [http://www.hotthyroidology.com/editorial_157.html].

Davis PJ, Davis FB, Lin HY. (2008) Promotion by thyroid hormone of cytoplasm-to-nucleus shuttling of thyroid hormone receptors. *Steroids*. 73:1013-1017.

Davis PJ, Davis FB, Mousa SA, Luidens MK, Lin HY. (2011) Membrane receptor for thyroid hormone: physiologic and pharmacologic implications. *Annu Rev Pharmacol Toxicol*. 51:99-115.

Davis PJ, Glinsky GV, Lin HY, Incerpi S, Davis FB, Mousa SA, Tang HY, Hercbergs A, Luidens MK. (2013) Molecular mechanisms of actions of formulations of the thyroid hormone analogue, tetrac, on the inflammatory response. *Endocr Res*. 38(2):112-118.

Davis PJ, Goglia F, Leonard JL. (2016) Nongenomic actions of thyroid hormone. *Nat Rev Endocrinol*. 12:111-121.

Davis PJ, Leonard JL, Lin HY, Leinung M, Mousa SA. (2018) Molecular Basis of Nongenomic Actions of Thyroid Hormone. *Vitam Horm*. 106:67-96.

de Mendoza D, Moreno H, Farias RN. (1978) Membrane cooperative enzymes. High molecular specificity for blocking action of thyroxine on triiodothyronine effect in rat erythrocyte and Escherichia coli systems. *J Biol Chem*. 253:6255-6259.

De Vito P, Incerpi S, Pedersen JZ, Luly P. (2010) Atrial natriuretic peptide and oxidative stress. *Peptides*. 31:1412-1419.

De Vito P, Incerpi S, Pedersen JZ, Luly P, Davis FB, Davis PJ. (2011) Thyroid hormones as modulators of immune activities at the cellular level. *Thyroid*. 21:879-890.

De Vito P, Balducci V, Leone S, Percario Z, Mangino G, Davis PJ, Davis FB, Affabris E, Luly P, Pedersen JZ, Incerpi S. (2012) Nongenomic effects of thyroid hormones on the immune system cells: New targets, old players. *Steroids*. 77:988-995.

De Vito P, Incerpi S, Affabris E, Percario Z, Borgatti M, Gambari R. Pedersen JZ, Luly P. (2013) Effect of atrial natriuretic peptide on reactive oxygen species- induced by hydrogen peroxide in THP-1 monocytes: role in cell growth, migration and cytokine release. *Peptides*. 50:100-108.

Dellacasagrande J, Ghigo E, Hammami SM, Toman R, Raoult D, Capo C, Mege JL. (2000) alpha(v)beta(3) integrin and bacterial lipopolysaccharide are involved in Coxiella burnetii-stimulated production of tumor necrosis factor by human monocytes. *Infect Immun*. 68(10):5673-5678.

Denis C, Williams JA, Lu X, Meyer D, Baruch D. (1993) Solid-phase von Willebrand factor contains a conformationally active RGD motif that mediates endothelial cell adhesion through the alpha v beta 3 receptor. *Blood*. 82(12):3622-3630.

Dentice M, Salvatore D. (2011) Deiodinases: the balance of thyroid hormone: localimpact of thyroid hormone inactivation. *J Endocrinol*. 209:273-282.

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. (2009) Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev*. 30(4):293-342.

Ebeling O, Duczmal A, Aigner S, Geiger C, Schöllhammer S, Kemshead JT, Möller P, Schwartz-Albiez R, Altevogt P. (1996) L1 adhesion molecule on human lymphocytes and monocytes: expression and involvement in binding to alpha v beta 3 integrin. *Eur J Immunol*. 26(10):2508-2516.

ECHA (European Chemicals Agency) (2015) Opinion on an annex XV dossier proposing restrictions on bisphenol A. Compiled version prepared by the ECHA Secretariat of RAC's opinion (adopted 5 June 2015) and SEAC's opinion (adopted 4 December 2015).

EFSA (European Food Safety Authority) (2015) Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in food stuffs. *EFSA J.* 13(1):3978.

Erdoğan M, Ilhan YS, Akkuş MA, Caboğlu SA, Ozercan I, Ilhan N, Yaman M. (1999) Effects of L-thyroxine and zinc therapy on wound healing in hypothyroid rats. *Acta Chir Belg*. 99(2):72-77.

Eslami A. (2005) The role of integrins in wound healing. *SQC.* [https://www.scq.ubc.ca/the-role-of-integrins-in-wound-healing].

Fabris N, Mocchegiani E, Provinciali M. (1995) Pituitary-thyroid axis and immune system: a reciprocal neuroendocrine-immune interaction. *Horm Res*. 43(1-3):29-38.

Fekete C, Lechan RM. (2014) Central regulation of hypothalamic-pituitary-thyroidaxis under physiological and pathophysiological conditions. *Endocr Rev*. 35:159-194.

Felding-Habermann B, Cheresh DA. (1993) Vitronectin and its receptors. *Curr Opin Cell Biol*. 5(5):864-868.

Foster MP, Jensen ER, Montecino-Rodriguez E, Leathers H, Horseman N, Dorshkind K. (2000) Humoral and cell-mediated immunity in mice with genetic deficiencies of prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormone. *Clin Immunol*. 96(2):140-149.

Gagnon A, Langille ML, Chaker S., Antunes TT, Durand J, Sorisky A. (2014) TSH signaling pathways that regulate MCP-1 in human differentiated adipocytes. *Metabolism*. 63:812-821.

Galo MG, Unates LE, Farias RN. (1981) Effect of membrane fatty acid composition on the action of thyroid hormones on (Ca2+ + Mg2+)-adenosine triphosphatase from rat erythrocyte. *J Biol Chem*. 256:7113-7114.

Geiger B, Yamada KM. (2011) Molecular architecture and function of matrix adhesions. *Cold Spring Harb Perspect Biol*. 3(5).

Gereben B, Zeold A, Dentice M, Salvatore D, Bianco AC. (2008a) Activation and inactivation of thyroid hormone by deiodinases: local actionwith general consequences. *Cell Mol Life Sci*. 65:570-590.

Gereben B, Zavacki AM, Ribich S, Kim BW, Huang SA, Simonides WS, Zeöld A, Bianco AC. (2008b) Cellular and molecular basis of deiodinase-regulated thyroid hormone signaling. *Endocr Rev*. 29(7):898-938.

Gianni T, Leoni V, Chesnokova LS, Hutt-Fletcher LM, Campadelli-Fiume G. (2012) αvβ3-integrin is a major sensor and activator of innate immunity to herpes simplex virus-1. *Proc Natl Acad Sci U S A*. 109(48):19792-19797.

Gionfra F, De Vito P, Pallottini V, Lin HY, Davis PJ, Pedersen JZ, Incerpi S. (2019) The Role of Thyroid Hormones in Hepatocyte Proliferation and Liver Cancer. *Front Endocrinol (Lausanne)*. 10:532.

Gnocchi D, Steffensen KR, Bruscalupi G, Parini P. (2016) Emerging role of thyroid hormone metabolites. *Acta Physiol*. 17:184-216.

Goglia F, Silvestri E, Lanni A. (2002) Thyroid hormones and mitochondria. *Biosci Rep*. 22(1):17-32.

Goto M, Ono H, Takano-Ishikawa Y, Shinmoto H, Yoshida M. (2004) Mac1 positive cells are required for enhancement of splenocytes proliferation caused by bisphenol A. *Biosci Biotechnol Biochem*. 68:263-265.

Graeber MB, Streit WJ. (1999) Microglia: biology and pathology. *Acta Neuropathol*. 119(1):89-105.

Gyftaki R, Liacos C, Politi E, Liontos M, Saltiki K, Papageorgiou T, Thomakos N, Haidopoulos D, Rodolakis A, Alevizaki M, Bamias A, Dimopoulos A. (2014) Differential transcriptional and protein expression of thyroid-stimulating hormone receptor in ovarian carcinomas. *Int J Gynecol Cancer*. 24:851-856.

Han D, Denison MS, Tachibana H, Yamada K. (2002) Effects of estrogenic compounds on immunoglobulin production by mouse splenocytes. *Biol Pharm Bull*. 25:1263-1267.

Hanisch UK, Kettenmann H. (2007) Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci*. 10(11):1387-1394.

Herndon DN, Wilmore DW, Mason AD Jr, Curreri PW. (1979) Increased rates of wound healing in burned guinea pigs treated with L-thyroxine. *Surg Forum*. 30:95-97.

Hiller-Sturmhöfel S, Bartke A. (1998) The endocrine system: an overview. *Alcohol Health Res World*. 22(3):153-164.

Hodkinson CF, Simpson EE, Beattie JH, O'Connor JM, Campbell DJ, Strain JJ, Wallace JM. (2009) Preliminary evidence of immune function modulation by thyroid hormones in healthy men and women aged 55-70 years. *J Endocrinol*. 202:55-63.

Hong CC, Shimomura-Shimizu M, Muroi M, Tanamoto K. (2004) Effect of endocrine disrupting chemicals on lipopolysaccharide-induced tumor necrosis factor-alpha and nitric oxide production by mouse macrophages. *Biol Pharm Bull*. 27:1136-1139.

Horton MA. (1997) The alpha v beta 3 integrin "vitronectin receptor". *Int J Biochem Cell Biol*. 29(5):721-725.

Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol*. 162(7):3749-3752.

Humphries MJ. (2000) Integrin structure. *Biochem Soc Trans*. 28(4):311-339.

Hynes RO. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*. 69(1):11-25.

Hynes RO. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell*. 110:673-687.

Illario M, Cavallo AL, Monaco S, Di Vito E, Mueller F, Marzano LA, Troncone G, Fenzi G, Rossi G, Vitale M. (2005) Fibronectin-induced proliferation in thyroid cells is mediated by alphavbeta3 integrin through Ras/Raf-1/MEK/ERK and calcium/CaMKII signals. *J Clin Endocrinol Metab*. 90(5):2865-2873.

Incerpi S, Luly P, De Vito P, Farias RN. (1999) Short-term effects of thyroid hormones on the Na/H antiport in L-6 myoblasts: high molecular specificity for 3,3',5-triiodo-L-thyronine. *Endocrinology*. 140:683-689.

Incerpi S, Fiore AM, De Vito P, Pedersen JZ. (2007) Involvement of plasma membrane redox systems in hormone action. *J Pharm Pharmacol*. 59:1711-1720.

Incerpi S, Hsieh MT, Lin HY, Cheng GY, De Vito P, Fiore AM, Ahmed RG, Salvia R, Candelotti E, Leone S, Luly P, Pedersen JZ, Davis FB, Davis PJ. (2014) Thyroid hormone inhibition in L6 myoblasts of IGF-I-mediated glucose uptake and proliferation: new roles for integrin αvβ3. *Am J Physiol Cell Physiol*. 307(2):C150-161.

Incerpi S, Davis PJ, Pedersen JZ, Lanni A. (2016) Nongenomic Actions of Thyroid Hormones. In: Belfiore A, LeRoith D, eds. Principles of Endocrinology and Hormone Action. *Cham: Springer International Publishing*. 1-26.

Jiao FR, Sun XJ, Pang ZT. (2008) Production and market analysis of bisphenol A. *Chem Ind*. 26(9):21-33.

Jones JI, Doerr ME, Clemmons DR. (1995) Cell migration: interactions among integrins, IGFs and IGFBPs. *Prog Growth Factor Res*. 6(2-4):319-327.

Jones JI, Prevette T, Gockerman A, Clemmons DR. (1996) Ligand occupancy of the alpha-V-beta3 integrin is necessary for smooth muscle cells to migrate in response to insulin-like growth factor. *Proc Natl Acad Sci U S A*. 93(6):2482-2487.

Jontell M, Hanks CT, Bratel J, Bergenholtz, G. (1995) Effects of unpolymerized resin components on the function of accessory cells derived from the rat incisor pulp. *J Dent Res*. 74:1162-1167.

Julier Z, Park AJ, Briquez PS, Martino MM. (2017) Promoting tissue regeneration by modulating the immune system. *Acta Biomater*. 53:13-28.

Kalyanaraman H, Schwappacher R, Joshua J, Zhuang S, Scott BT, Klos M, Casteel DE, Frangos JA, Dillmann W, Boss GR, Pilz RB. (2014) Nongenomic thyroid hormone signaling occurs through a plasma membrane-localized receptor. *Sci Signal*. 7(326):ra48.

Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. (2011) Physiology of microglia. *Physiol Rev*. 91(2):461-553.

Kim SU, de Vellis J. (2005) Microglia in health and disease. *J Neurosci Res*. 81(3):302-313.

Kim JY, Jeong, HG. (2003) Down-regulation of inducible nitric oxide synthase and tumor necrosis factor-alpha expression by bisphenol A via nuclear factor-kappaB inactivation in macrophages. *Cancer Lett*. 196:69-76.

Kimber I, Dearman RJ. (2010) An assessment of the ability of phthalates to influence immune and allergic responses. *Toxicology*. 271(3):73-82.

Kitamura S, Jinno N, Ohta S, Kuroki H, Fujimoto N. (2002) Thyroid hormonal activ-ity of theflame retardants tetrabromobisphenol A and tetrachlorobisphenol A. *Biochem Biophys Res Commun*. 293:554-559.

Kitamura S, Kato T, Iida M, Jinno N, Suzuki T, Ohta S, Fujimoto N, Hanada H, Kashiwagi K, Kashiwagi A. (2005) Anti-thyroid hormonal activity of tetrabromobisphenol A, a flame retardant, and related compounds: affinity to the mammalian thyroid hormone receptor, and effect on tadpole metamorphosis. *Life Sci*. 76:1589-1601.

Klecha AJ, Genaro AM, Lysionek AE, Caro RA, Coluccia AG, Cremaschi GA. (2000) Experimental evidence pointing to the bidirectional interaction between the immune system and the thyroid axis. *Int J Immunopharmacol*. 22:491-500.

Klecha AJ, Barreiro Arcos ML, Frick L, Genaro AM, Cremaschi G. (2008) Immune-endocrine interactions in autoimmune thyroid diseases. *Neuroimmunomodulation*. 15:68-75.

Klein JR. (2006) The immune system as a regulator of thyroid hormone activity. *Exp Biol Med (Maywood)*. 231:229-236.

Kloss CU, Bohatschek M, Kreutzberg GW, Raivich G. (2001) Effect of lipopolysaccharide on the morphology and integrin immunoreactivity of ramified microglia in the mouse brain and in cell culture. *Exp Neurol*. 168(1):32-46.

Kruger TE, Blalock JE. (1986) Cellular requirements for thyrotropin enhancement of in vitro antibody production. *J Immunol*. 137:197-200.

Krutzsch HC, Choe BJ, Sipes JM, Guo N-h, Roberts DD. (1999) Identification of an alpha(3)beta(1) integrin recognition sequence in thrombospondin-1. *J Biol Chem*. 274(34):24080-24086.

Kuan YH, Li YC, Huang FM, Chang YC. (2012) The upregulation of tumour necrosis factor-alpha and surface antigens expression on macrophages by bisphenol A-glycidyl- methacrylate. *International endodontic journal*. 45:619-626.

Kubo K, Arai O, Ogata R, Omura M, Hori T, Aou S. (2001) Exposure to bisphenol A during the fetal and suckling periods disrupts sexual differentiation of the locus coeruleus and of behavior in the rat. *Neurosci Lett*. 304:73-76.

Kumar CC, Malkowski M, Yin Z, Tanghetti E, Yaremko B, Nechuta T, Varner J, Liu M, Smith EM, Neustadt B, Presta M, Armstrong L. (2001) Inhibition of angiogenesis and tumor growth by SCH221153, a dual alphavbeta3 and alphavbeta5 integrin receptor antagonist. *Cancer Res*. 61:2232-2238.

Kurihara Y, Nakahara T, Furue M. (2011) αVβ3-integrin expression through ERK activation mediates cell attachment and is necessary for production of tumor necrosis factor alpha in monocytic THP-1 cells stimulated by phorbol myristate acetate. *Cell Immunol*. 270(1):25-31.

Ladenson PW, Levin AA, Ridgway EC, Daniels GH. (1984) Complications of surgery in hypothyroid patients. *Am J Med*. 77(2):261-266.

Laron Z. (2001) Insulin-like growth factor 1 (IGF-1): a growth hormone. *Mol Pathol*. 54(5):311-316.

Laurenzi MA, Arcuri C, Rossi R, Marconi P, Bocchini V. (2001) Effects of microenvironment on morphology and function of the microglial cell line BV-2. *Neurochem Res*. 26(11):1209-1216.

Lehnardt S, Massillon L, Follett P, Jensen FE, Ratan R, Rosenberg PA, Volpe JJ, Vartanian T. (2003) Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. *Proc Natl Acad Sci U S A*. 100(14):8514-8519.

Liang CC, Park AY, Guan JL. (2007) In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc*. 2(2):329-333.

Lima FR, Gervais A, Colin C, Izembart M, Neto VM, Mallat M. (2001) Regulation of microglial development: a novel role for thyroid hormone. *J Neurosci*. 21(6):2028-2038.

Lin CC, Chin YT, Shih YJ, Chen YR, Chung YY, Lin CY, Hsiung CN, Whang-Peng J, Lee SY, Lin HY, Davis PJ, Wang K. (2019) Resveratrol antagonizes thyroid hormone-induced expression of checkpoint and proliferative genes in oral cancer cells. *J Dent Sci*. 4(3):255-262.

Lin HY, Martino LJ, Wilcox BD, Davis FB, Gordinier JK, Davis PJ. (1998) Potentiation by thyroid hormone of human IFN-gamma-induced HLA-DR expression. *J Immunol*. 161:843-849.

Lin HY, Davis FB, Gordinier JK, Martino LJ, Davis PJ. (1999a) Thyroid hormone induces activation of mitogen-activated protein kinase in cultured cells. *Am J Physiol*. 276:C1014-1024.

Lin HY, Shih A, Davis FB, Davis PJ. (1999b) Thyroid hormone promotes the phosphorylation of STAT3 and potentiates the action of epidermal growth factor in cultured cells. *Biochem J*. 338(Pt 2):427-32.

Lin HY, Zhang S, West BL, Tang HY, Passaretti T, Davis FB, Davis PJ. (2003) Identification of the putative MAP kinase docking site in the thyroid hormone receptor-beta1 DNA-binding domain: functional consequences of mutations at the docking site. *Biochemistry*. 42:7571-7579.

Lin HY, Lansing L, Merillon JM, Davis FB, Tang HY, Shih A, Vitrac X, Krisa S, Keating T, Cao HJ, Bergh J, Quackenbush S., Davis PJ. (2006) Integrin alphavbeta3 contains a receptor site for resveratrol. *FASEB J*. 20(10):1742-1744.

Lin HY, Sun M, Lin C, Tang HY, London D, Shih A, Davis FB, Davis PJ. (2009a) Androgen-induced human breast cancer cell proliferation is mediated by discrete mechanisms in estrogen receptor-alpha-positive and -negative breast cancer cells. *J Steroid Biochem Mol Biol*. 113(3-5):182-188.

Lin HY, Sun M, Tang HY, Lin C, Luidens MK, Mousa SA, Incerpi S, Drusano GL, Davis FB, Davis PJ. (2009b) L-Thyroxine vs. 3, 5, 3′-triiodo-L-thyronine and cell proliferation: activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. *Am J Physiol Cell Physiol*. 296:C980-C991.

Lin HY, Tang HY, Davis FB, Davis PJ. (2011) Resveratrol and apoptosis. *Ann N Y Acad Sci*. 1215:79-88.

Lin HY, Chin YT, Yang YC, Lai HY, Wang-Peng J, Liu LF, Tang HY, Davis PJ. (2016) Thyroid Hormone, Cancer, and Apoptosis. *Compr Physiol*. 6(3):1221-1237.

Liu Z, Wang F, Chen X. (2008) Integrin alpha(v)beta(3)-Targeted Cancer Therapy. *Drug Dev Res*. 69(6):329-339.

Lively S, Schlichter LC. (2013) The microglial activation state regulates migration and roles of matrix-dissolving enzymes for invasion. *J Neuroinflammation*. 10:75.

Lombardo E, Sabellico C, Hájek J, Staňková V, Filipský T, Balducci V, De Vito P, Leone S, Bavavea EI, Silvestri IP, Righi G, Luly P, Saso L, Bovicelli P, Pedersen JZ, Incerpi S. (2013) Protection of cells against oxidative stress by nanomolar levels of hydroxyflavones indicates a new type of intracellular antioxidant mechanism. *PLoS One*. 8(4):e60796.

Luo BH, Carman CV, Springer TA. (2007) Structural basis of integrin regulation and signaling. *Annu Rev Immunol*. 25:619-647.

Luongo C, Dentice M, Salvatore D. (2019) Deiodinases and their intricate role in thyroid hormone homeostasis. *Nat Rev Endocrinol*. 15(8):479-488.

Mackawy AM, Al-Ayed BM, Al-Rashidi BM. (2013) Vitamin d deficiency and its association with thyroid disease. *Int J Health Sci (Qassim)*. 7:267-275.

Magsino CH Jr, Hamouda W, Ghanim H, Browne R, Aljada A, Dandona P. (2000) Effect of triiodothyronine on reactive oxygen species generation by leukocytes, indices of oxidative damage, and antioxidant reserve. *Metabolism*. 49(6):799-803.

Maile LA, Busby WH, Sitko K, Capps BE, Sergent T, Badley-Clarke J, Clemmons DR. (2006) Insulin-like growth factor-I signaling in smooth muscle cells is regulated by ligand binding to the 177CYDMKTTC184 sequence of the beta3-subunit of alphaVbeta3. *Mol Endocrinol*. 20(2):405-413.

Marino F, Guasti L, Cosentino M, De Piazza D, Simoni C, Piantanida E, Cimpanelli M, Klersy C, Bartalena L, Venco A, Lecchini S. (2006) Thyroid hormone regulation of cell migration and oxidative metabolism in polymorphonuclear leukocytes: clinical evidence in thyroidectomized subjects on thyroxine replacement therapy. *Life Sci*. 78(10):1071-1077.

Meerovitch K, Bergeron F, Leblond L, Grouix B, Poirier C, BubenikM, Chan L, Gourdeau H, Bowlin T, Attardo G. (2003) A novel RGD antagonist that targets both alphavbeta3 and alpha5beta induces apoptosis of angiogenic endothelial cells on type I collagen. *Vascul Pharmacol*. 40:77-89.

Mehregan AH, Zamick P. (1974) The effect of triiodothyronine in healing of deep dermal burns and marginal scars of skin grafts. A histologic study. *J Cutan Pathol*. 1(3):113-116.

Monick MM, Powers L, Butler N, Yarovinsky T, Hunninghake GW. (2002) Interaction of matrix with integrin receptors is required for optimal LPS-induced MAP kinase activation. *Am J Physiol Lung Cell Mol Physiol*. 283(2):L390-402.

Moon MK, Kang GH, Kim HH, Han SK, Koo YD, Cho SW, Kim YA, Oh BC, Park do J, Chung SS, Park KS, Park YJ. (2016) Thyroid-stimulating hormone improves insulin sensitivity in skeletal

muscle cells via cAMP/PKA/CREB pathway-dependent upregulation of insulin receptor substrate-1 expression. *Mol Cell Endocrinol*. 436:50-58.

Moreno M, de Lange P, Lombardi A, Silvestri E, Lanni A, Goglia F. (2008) Metabolic effects of thyroid hormone derivatives. *Thyroid*. 18:239-253.

Mori Y, Tomonaga D, Kalashnikova A, Furuya F, Akimoto N, Ifuku M, Okuno Y, Beppu K, Fujita K, Katafuchi T, Shimura H, Churilov LP, Noda M. (2015) Effects of 3,3',5-triiodothyronine on microglial functions. *Glia*. 63(5):906-920.

Moriyama K, Tagami T, Akamizu T, Usui T, Saijo M, Kanamoto N, Hataya Y, Shimatsu A, Kuzuya H, Nakao K. (2002) Thyroid hormone action is disrupted by bisphenol A as an antagonist. *J Clin Endocrinol Metab*. 87:5185-5190.

Mosser DM. (2003) The many faces of macrophage activation. *J Leukoc Biol*. 73(2):209-212.

Mousa SA, O'Connor LJ, Bergh JJ, Davis FB, Scanlan TS, Davis PJ. (2005) The proangiogenic action of thyroid hormone analogue GC-1 is initiated at an integrin. *J Cardiovasc Pharmacol*. 46(3):356-360.

Mousa SA, O'Connor L, Davis FB, Davis PJ. (2006a) Proangiogenesis action of the thyroid hormone analog 3,5-diiodothyropropionic acid (DITPA) is initiated at the cell surface and is integrin mediated. *Endocrinology*. 147(4):1602-1607.

Mousa SA, O'Connor LJ, Davis FB, Bergh JJ, Davis PJ. (2006b) Tetraiodothyroacetic acid (tetrac) inhibits angiogenesis. In: Program of the 77th annual meeting of the american thyroid association. Phoenix, AZ. [Abstract 108].

Mousa SA, Davis FB, Mohamed S, Davis PJ, Feng X. (2006c) Pro-angiogenesis action of thyroid hormone and analogs in a three-dimensional in vitro microvascular endothelial sprouting model. *Int Angiol*. 25(4):407-413.

Mousa SA, Yalcin M, Bharali DJ, Meng R, Tang HY, Lin HY, Davis FB, Davis PJ. (2012) Tetraiodothyroacetic acid and its nanoformulation inhibit thyroid hormone stimulation of non-small cell lung cancer cells in vitro and its growth in xenografts. *Lung Cancer*. 76:39-45.

Milner R. (2009) Microglial expression of alphavbeta3 and alphavbeta5 integrins is regulated by cytokines and the extracellular matrix: beta5 integrin null microglia show no defects in adhesion or MMP-9 expression on vitronectin. *Glia*. 57(7):714-723.

Murray PJ, Wynn TA. (2011) Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol*. 11(11):723-737.

Nath D, Slocombe PM, Stephens PE, Warn A, Hutchinson GR, Yamada KM, Docherty A, Murphy G. (1999) Interaction of metargidin (ADAM-15) with alphavbeta3 and alpha5beta1 integrins on different haemopoietic cells. *J Cell Sci*. 112(Pt 4):579-587.

Neff S, Sá-Carvalho D, Rieder E, Mason PW, Blystone SD, Brown EJ, Baxt B. (1998) Foot-and-mouth disease virus virulent for cattle utilizes the integrin alpha(v)beta3 as its receptor. *J Virol*. 72(5):3587-3594.

Nettore IC, Albano L, Ungaro P, Colao A, Macchia PE. (2017) Sunshine vitamin and thyroid. *Rev Endocr Metab Disord*. 18:347-354.

Noda M. (2018) Thyroid Hormone in the CNS: Contribution of Neuron-Glia Interaction. *Vitam Horm*. 106:313-331.

Nowak K, Jabłońska E, Ratajczak-Wrona W. (2019) Immunomodulatory effects of synthetic endocrine disrupting chemicals on the development and functions of human immune cells. *Environ Int*. 125:350-364.

Ohnishi T, Yoshida T, Igarashi A, Muroi M, Tanamoto K. (2008) Effects of possible endocrine disruptors on MyD88-independent TLR4 signaling. *FEMS Immunol Med Microbiol*. 52(2):293-295.

Ortiga-Carvalho TM, Chiamolera MI, Pazos-Moura CC, Wondisford FE. (2016) Hypothalamus-pituitary-thyroid axis. *Compr Physiol*. 6:1387-1428.

Pedersen JZ, Oliveira C, Incerpi S, Kumar V, Fiore AM, De Vito P, Prasad AK, Malhotra SV, Parmar VS, Saso L. (2007) Antioxidant activity of 4-methylcoumarins. *J Pharm Pharmacol*. 59(12):1721-1728.

Perrotta C, Buldorini M, Assi E, Cazzato D, De Palma C, Clementi E, Cervia D. (2014) The thyroid hormone triiodothyronine controls macrophage maturation and functions: protective role during inflammation. *Am J Pathol*. 184(1):230-247.

Perrotta C, De Palma C, Clementi E, Cervia D. (2015) Hormones and immunity in cancer: are thyroid hormones endocrine players in the microglia/glioma cross-talk? *Front Cell Neurosci*. 9:236.

Pillon NJ, Bilan PJ, Fink LN, Klip A. (2013) Cross-talk between skeletal muscle and immune cells: muscle-derived mediators and metabolic implications. *Am J Physiol Endocrinol Metab*. 304(5):E453-465.

Pirk FW, ElAttar TM, Roth GD. (1974) Effect of analogues of steroid and thyroxine hormones on wound healing in hamsters. *J Periodontal Res*. 9(5):290-297.

Plow EF, Haas TA, Zhang L, Loftus J, Smith JW. (2000) Ligand binding to integrins. *J Biol Chem*. 275(29):21785-21788.

Pytela R, Pierschbacher MD, Ruoslahti E. (1985) A 125/115-kDa cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic acid adhesion sequence derived from fibronectin. *Proc Natl Acad Sci U S A*. 82(17):5766-5770.

Ramakrishnan S, Wayne NL. (2008) Impact of bisphenol A on early embryonic development and reproductive maturation. *Reprod Toxicol*. 25:177-183.

Riedemann J, Macaulay VM. (2006) IGF1R signalling and its inhibition. *Endocr Relat Cancer*. 13(Suppl 1):S33-43.

Rochester JR. (2013) Bisphenol A and human health: a review of the literature. *Reprod Toxicol*. 42:132-155.

Rogers JA, Metz L, Yong VW. (2013) Review: Endocrine disrupting chemicals and immune responses: a focus on bisphenol-A and its potential mechanisms. *Molecular immunology*. 53:421-430.

Roy A, Bauer SM, Lawrence BP. (2012) Developmental exposure to bisphenol A modulates innate but not adaptive immune responses to influenza A virus infection. *PloS One*. 7(6):e38448.

Rucci N, Di Giacinto C, Orrù L, Millimaggi D, Baron R, Teti A. (2005) A novel protein kinase C alpha-dependent signal to ERK1/2 activated by alphaVbeta3 integrin in osteoclasts and in Chinese hamster ovary (CHO) cells. *J Cell Sci*. 118(Pt 15):3263-3275.

Ruoslahti E. (1999) Fibronectin and its integrin receptors in cancer. *Adv Cancer Res*. 76:1-20.

Rusnati M, Tanghetti E, Dell'Era P, Gualandris A, Presta M. (1997) alphavbeta3 integrin mediates the cell-adhesive capacity and biological activity of basic fibroblast growth factor (FGF-2) in cultured endothelial cells. *Mol Biol Cell*. 8(12):2449-2461.

Safer JD, Crawford TM, Holick MF. (2004) A role for thyroid hormone in wound healing through keratin gene expression. *Endocrinology*. 145(5):2357-2361

Sakazaki H, Ueno H, Nakamuro K. (2002) Estrogen receptor alpha in mouse splenic lymphocytes: possible involvement in immunity. *Toxicol Lett*. 133:221-229.

Salvatore D, Simonides WS, Dentice M, Zavacki AM, Larsen PR. (2014) Thyroid hormones and skeletal muscle-new insights and potential implications. *Nat Rev Endocrinol*. 10:206-214.

San Martin A, Griendling KK. (2010) Redox control of vascular smooth muscle migration. *Antiox Redox Signal*. 12:625-640.

Scapin S, Leoni S, Spagnuolo S, Fiore AM, Incerpi S. (2009) Short-term effects of thyroid hormones on Na+-K+-ATPase activity of chick embryo hepatocytes during development: focus on signal transduction. *Am J Physiol Cell Physiol*. 296:C4-12.

Schmohl KA, Nelson PJ, Spitzweg C. (2019) Tetrac as an anti-angiogenic agent in cancer. *Endocr Relat Cancer*. 26(6):R287-R304.

Schwartz MA, Schaller MD, Ginsberg MH. (1995) Integrins: emerging paradigms of signal transduction. *Annu Rev Cell Dev Biol*. 11:549-599.

Segal J, Ingbar SH. (1979) Stimulation by triiodothyronine of the in vitro uptake of sugars by rat thymocytes. *J Clin Invest*. 63:507-515.

Segal J, Ingbar SH. (1989) Evidence that an increase in cytoplasmic calcium is the initiating event in certain plasma membrane-mediated responses to 3,5,3'-triiodothyronine in rat thymocytes. *Endocrinology*. 124:1949-1955.

Segura JJ, Jiménez-Rubio A, Pulgar R, Olea N, Guerrero JM, Calvo JR. (1999) In vitro effect of the resin component bisphenol A on substrate adherence capacity of macrophages. *J Endod*. 25(5):341-344.

Senese R, Cioffi F, de Lange P, Goglia F, Lanni A. (2014a) Thyroid: biological actions of 'nonclassical' thyroid hormones. *J Endocrinol*. 221:R1-12.

Senese R, Lasala P, Leanza C, de Lange P. (2014b) New avenues for regulation of lipid metabolism by thyroid hormones and analogs. *Front Physiol*. 5:475.

Shattil SJ. (1999) Signaling through platelet integrin aIIbb3: Inside-out, outside-in, and sideways. *Thrombosis and haemostasis*. 82:318-325.

Shattil SJ, Kim C, Ginsberg MH. (2010) The final steps of integrin activation: the end game. *Nat Rev Mol Cell Biol*. 11(4):288-300.

Shen B, Delaney MK, Du X. (2012) Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. *Current opinion in cell biology*. 24:600-606.

Sheng ZG, Tang Y, Liu YX, Yuan Y, Zhao BQ, Chao XJ, Zhu BZ. (2012) Low concentrations of bisphenol a suppress thyroid hormone receptor transcription through a nongenomic mechanism. *Toxicol Appl Pharmacol*. 259(1):133-142.

Shih A, Lin HY, Davis FB, Davis PJ. (2001) Thyroid hormone promotes serine phosphorylation of p53 by mitogen-activated protein kinase. *Biochemistry*. 40:2870-2878.

Shih A, Zhang S, Cao HJ, Tang HY, Davis FB, Davis PJ, Lin HY. (2004) Disparate effects of thyroid hormone on actions of epidermal growth factor and transforming growth factor-alpha are mediated by 3',5'-cyclic adenosine 5'-monophosphate-dependent protein kinase II. *Endocrinology*. 145(4):1708-1717.

Siegrist-Kaiser CA, Juge-Aubry C, Tranter MP, Ekenbarger DM, Leonard JL. (1990) Thyroxine-dependent modulation of actin polymerization in cultured astrocytes. A novel, extranuclear action of thyroid hormone. *J Biol Chem*. 265:5296-5302.

Smith EM, Phan M, Kruger TE, Coppenhaver DH, Blalock JE. (1983) Human lymphocyte production of immunoreactive thyrotropin. *Proc Natl Acad Sci U S A*. 80:6010-6013.

Sonnenberg A. (1995) Integrins and their ligands. *Curr Top Microbiol Immunol*. 184:7-35.

Sorice A, Guerriero E, Capone F, Colonna G, Castello G, Costantini S. (2014) Ascorbic acid: its role in immune system and chronic inflammation diseases. *Mini Rev Med Chem*. 4:444-452.

Soung YH, Clifford JL, Chung J. (2014) Crosstalk between integrin and receptor tyrosine kinase signaling in breast carcinoma progression. *BMB Rep*. 43(5):311-318.

Sterling K, Lazarus JH, Milch PO, Sakurada T, Brenner MA. (1978) Mitochondrial thyroid hormone receptor: localization and physiological significance. *Science*. 201:1126-1129.

Sterling K, Brenner MA, Sakurada T. (1980) Rapid effect of triiodothyronine on the mitochondrial pathway in rat liver in vivo. *Science*. 210:340-342.

Streit WJ, Graeber MB, Kreutzberg GW. (1988) Functional plasticity of microglia: a review. *Glia*. 1(5):301-307.

Su Y, Xia W, Li J, Walz T, Humphries MJ, Vestweber D, Cabañas C, Lu C, Springer TA. (2016) Relating conformation to function in integrin α5β1. *Proceedings of the National Academy of Sciences*. 113:E3872-E3881.

Suzuki S, Argraves WS, Pytela R, Arai H, Krusius T, Pierschbacher MD, Ruoslahti E. (1986) cDNA and amino acid sequences of the cell adhesion protein receptor recognizing vitronectin reveal a transmembrane domain and homologies with other adhesion protein receptors. *Proc Natl Acad Sci U S A*. 83(22):8614-8618.

Tadokoro S, Shattil SJ, Eto K, Tai V, Liddington RC, De Pereda JM, Ginsberg MH, Calderwood DA. (2003) Talin binding to integrin ß tails: a final common step in integrin activation. *Science*. 302:103-106.

Takada Y, Takada YK, Fujita M. (2017) Crosstalk between insulin-like growth factor (IGF) receptor and integrins through direct integrin binding to IGF1. *Cytokine Growth Factor Rev*. 34:67-72.

Takagi J, Petre BM, Walz T, Springer TA. (2002) Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell*. 110(5):599-611.

Takahashi K, Ohsawa M, Utsumi H. (2002) A simple bioassay for evaluating immunotoxic properties of chemicals by use of in vitro antibody production system. *J Health Sci*. 48:161-167.

Talmi YP, Finkelstein Y, Zohar Y. (1989) Pharyngeal fistulas in postoperative hypothyroid patients. *Ann Otol Rhinol Laryngol*. 98(4 Pt1):267-268.

Tanaka T, Ueno M, Yamashita T. (2009) Engulfment of axon debris by microglia requires p38 MAPK activity. *J Biol Chem*. 284(32):21626-21636.

Tang HY, Lin HY, Zhang S, Davis FB, Davis PJ. (2004) Thyroid hormone causes mitogen-activated protein kinase-dependent phosphorylation of the nuclear estrogen receptor. *Endocrinology*. 145:3265-3272.

Triantafilou K, Triantafilou M, Takada Y, Fernandez N. (2000) Human parechovirus 1 utilizes integrins alphavbeta3 and alphavbeta1 as receptors. *J Virol*. 74(13):5856-5862.

US Food and Drug Administration. (2017) Bisphenol A (BPA): Use in Food Contact Application.

Vaiserman A. (2014) Early-life Exposure to Endocrine Disrupting Chemicals and Later-life Health Outcomes: An Epigenetic Bridge? *Aging Dis*. 5(6):419-429.

Wang H, Wang F, Tao X, Cheng H. (2012) Ammonia-containing dimethyl sulfoxide: an improved solvent for the dissolution of formazan crystals in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. *Anal Biochem*. 421(1):324-326.

Watanabe H, Adachi R, Kusui K, Hirayama A, Kasahara T, Suzuki K. (2003) Bisphenol A significantly enhances the neutrophilic differentiation of promyelocytic HL-60 cells. *Int Immunopharmacol*. 3(12):1601-1608.

Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. (1993) Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell*. 73(2):309-319.

Wilder RL. (2002) Integrin alpha V beta 3 as a target for treatment of rheumatoid arthritis and related rheumatic diseases. *Ann Rheum Dis*. 61(Suppl 2):96-99.

Wu SY, Green WL, Huang WS, Hays MT, Chopra IJ. (2005) Alternate pathways of thyroid hormone metabolism. *Thyroid*. 15(8):943-958.

Xiong JP, Stehle T, Zhang R, Joachimiak A, Frech M, Goodman SL. Arnaout MA. (2002) Crystal structure of the extracellular segment of integrin alphavbeta3 in complex with an Arg-Gly-Asp ligand. *Science*. 296(5565):151-155.

Xiong JP, Stehle T, Goodman SL, Arnaout MA. (2003) New insights into the structural basis of integrin activation. *Blood.* 102(4):1155-1159.

Yalcin M, Dyskin E, Lansing L, Bharali DJ, Mousa SS, Bridoux A, Hercbergs AH, Lin HY, Davis FB, Glinsky GV, Glinskii A, Ma J, Davis PJ, Mousa SA. (2010) Tetraiodothyroacetic acid (tetrac) and nanoparticulate tetrac arrest growth of medullary carcinoma of the thyroid. *J Clin Endocrinol Metab*. 95(4):1972-1980.

Yalcin M, Lin HY, Sudha T, Bharali DJ, Meng R, Tang HY, DavisFB, Stain SC, Davis PJ, Mousa SA. (2013) Response of human pancreaticcancer cell xenografts to tetraiodothyroacetic acid nanoparticles. *HormCancer*. 4:176-185.

Yamazaki T, Okada Y, Hisamatsu Y. (2000) Effect of endocrine disrupting chemicals on lymphocyte responses. *Organohalogen Compounds*. 49:394-396.

Yang J, Zhang L, Yu C, Yang XF, Wang H. (2014) Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomark Res*. 2(1):1.

Yoshida T, Gong J, Xu Z, Wei Y, Duh EJ. (2012) Inhibition of pathological retinal angiogenesis by the integrin alphavbeta3 antagonist tetraiodothyroacetic acid (tetrac). *Exp Eye Res*. 94:41-48.

Yoshino S, Yamaki K, Yanagisawa R, Takano H, Hayashi H, Mori Y. (2003) Effects of bisphenol A on antigen-specific antibody production, proliferative responses of lymphoid cells, and TH1 and TH2 immune responses in mice. *Br J Pharmacol*. 138:1271-1276.

Youn JY, Park HY, Lee JW, Jung IO, Choi KH, Kim K, Cho KH. (2002) Evaluation of the immune response following exposure of mice to bisphenol A: induction of Th1 cytokine and prolactin by BPA exposure in the mouse spleen cells. *Arch Pharm Res*. 25:946-953.

Yurino H, Ishikawa S, Sato T, Akadegawa K, Ito T, Ueha S, Inadera H, Matsushima K. (2004) Endocrine Disruptors (Environmental Estrogens) Enhance Autoantibody Production by B1 Cells. *Toxicol Sci.* 81(1):139-147.

Zamick P, Mehregan AH. (1973) Effect of 1-tri-iodothyronine on marginal scars of skin grafted burns in rats. *Plast Reconstr Surg*. 51(1):71-75.

Zheng B, Clemmons DR. (1998) Blocking ligand occupancy of the alphaVbeta3 integrin inhibits insulin-like growth factor I signaling in vascular smooth muscle cells. *Proc Natl Acad Sci U S A*. 95(19):11217-11222.

Zheng B, Duan C, Clemmons DR. (1998) The effect of extracellular matrix proteins on porcine smooth muscle cell insulin-like growth factor (IGF) binding protein-5 synthesis and responsiveness to IGF-I. *J Biol Chem*. 273(15):8994-9000.

Zoeller RT, Dowling ALS, Herzig CTA, Iannacone EA, Gauger KJ, Bansal R. (2002) Thyroid hormone, brain development, and the environment. *Environ Health Perspect*. 110:355-361.